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Metabonomics and Gut Microbiota in Nutrition and Disease

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Preface

Being the most densely populated microbial ecosystem on earth, gut microbiota coevolved as a key component of human biology and physiology. Human beings share an intimate and mutualistic relationship with their gut microbiota, and recent findings have demonstrated that the role of our gut microbial symbionts into health and disease has been so far underestimated. Increasingly, scientific evidence identifies gut microbiota as a key biological interface between human genetics and environmental conditions encompassing nutrition. Microbiota dysbiosis or variation in metabolic activity has been associated to metabolic deregulation (e.g., obesity, inflammatory bowel disease), disease risk factor (e.g., coronary heart disease), or even in the etiology of various pathologies (e.g., autism), although causal role into impaired metabolism still needs to be established.

The rise in multifactorial disorders, the lack of understanding of the molecular processes at play, and the needs for disease prediction in asymptomatic conditions are some of the many questions that systems biology approaches are well suited to address. Achieving this goal lies in our ability to model and understand the complex web of interactions between genetics, metabolism, environmental factors, and gut microbiota. The adaptation of systems biology to translational and clinical sciences has been termed network medicine and is changing the way we think about preventing, predicting, diagnosing, and treating complex human diseases. Through consolidating knowledge across intermediate organizational levels of life such as the epigenome, transcriptome, proteome, metabolome or microbiome, and its integration with gene-disease traits, systems biology approaches are becoming highly relevant for assessing the clinical characteristics of human health and disease.

The advent of metabonomics as a powerful systems biology approach opens new opportunities to deepen and model the complex web of molecular interactions between nutrition and health encompassing the understanding on how to modulate gut microbiota. While the study of the gut microbiota required the development of modern molecular biology techniques (many of the organisms present cannot be cultured and are only known as a result of their detection via ribosomal 16S DNA), we now know that these microbial populations are highly complex with many hundreds of different species cohabiting in the gut and forming a complex ecology.

Its composition and activity coevolve with the host from birth and is subject to a complex interplay that depends on the host genome, nutrition, and lifestyle.

Undoubtedly, the interplay between gut microbiome and host and its modulation by nutrition will benefit from the integration of information on a systems biology-wide approach. Integration of gene sequence of the microbiome and metabolomics is currently envisioned to pave the way towards a better molecular understanding of the complex mammalian superorganism, which is a prerequisite for optimizing therapeutic strategies to manipulate the gut microbiota to combat disease and improve health.

The field of endeavor is expanding rapidly, but we believe that now is a good time to review the achievements in the area of metabonomics and gut microbiota research. Therefore, this book will aim at providing state-of-the-art information on the key findings and methodologies and point future directions towards understanding how to beneficially modulate our gut functional ecology for health and nutritional benefits.

This book provides a comprehensive overview of metabonomics and gut microbiota research from molecular analysis to population-based global health considerations. The topics include the discussion of the applications in relation to metabonomics and gut microbiota in nutritional research and in health and disease and a review of future therapeutical, nutraceutical, and clinical applications. It also examines the translatability of systems biology approaches into applied clinical research and to patient health and nutrition.

The book begins with a general introduction on major concepts and research directions with emphasis on core indicators of health and functions in infant, adult, and elderly populations (Chap. 1). The second and third chapters will provide some background information on the metabonomics technology and its implementation in clinical research and data modeling.

Chapters 4, 5, 6, 7, 8, 9, and 10 describe extensively some key applications in nutritional research. These sections provide examples and expectations in the field of personalized medicine and nutrition, including the study of infant-nutrition and healthy aging paradigms and studies of microbial and human metabolism of macronutrients (protein, fat, carbohydrates, and fiber) and specific non-nutrient food components (polyphenols, flavonoids) and herbal medicine.

Chapters 11, 12, 13, and 14 describe the applications and perspectives of combining metabonomics and gut microbiota approaches in health and disease research. Chapter 11 covers the introduction of the field of microbio-immuno-metabolism, based on the role of the environment, genetic background, and individual diversity in relation to the onset and development of metabolic diseases. Chapter 12 gives emphasis on the role of microbiota in modulating lipid metabolism and related disease risks, while Chap. 13 comprehensively describes the intimate relationship between gut microbiota metabolism and gastrointestinal disorders with emphasis on inflammatory bowel diseases. Chapter 14 extends the discussion to the field of the gut brain axis and its role in the etiology of several disorders with emphasis on autism.

Chapters 15 and 16 review the applications towards future therapeutical, nutraceutical, and clinical applications, with emphasis on two main examples, bariatric surgery and drug efficacy and toxicity.

Metabonomics and gut microbiota in nutrition and disease serves as a handbook for postgraduate students, researchers in life sciences or health sciences, and scientists in academic and industrial environments working in application areas as diverse as health, disease, nutrition, microbial research, and human clinical medicine.

We would like to acknowledge the chapter authors for their efforts in creating this book. Even though they all have many demands on their time, they generously contributed towards this effort to publicize Metabonomics and gut microbiota research efforts towards deciphering human health and disease.

Lausanne, Switzerland

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François-Pierre Martin, Ph.D., Eng., is Senior Scientist and Research Program Manager in the Department of Molecular Biomarkers, at the Nestlé Institute of Health Sciences, Lausanne, Switzerland. He received his Ph.D. in Biochemistry and Metabonomics from Imperial College London, UK, in 2006, and has a background in biotechnology and microbiology from the engineering school Polytech-Marseille, France. From 2006 to 2012, François-Pierre has developed and expanded metabonomics applications to nutrition and gut microbiota research for health and disease management, at the Nestlé Research Center, Switzerland. Emphasis was given to investigate transgenomic interactions between the mammalian and the gut microbial metabolic space using systems biology. Dr. Martin has developed a strong expertise in investigating gut microbial effects on diverse mammalian processes and their impact upon the etiology of several conditions (gastrointestinal health, diabetes, obesity). In July 2012, Dr. Martin joined the Nestlé Institute of Health Sciences, Switzerland, to further develop systems biology approaches in the frame of health, disease, and nutrition research. His main research interests include gastrointestinal health (e.g., inflammatory bowel disease, irritable bowel syndrome), metabolic health (metabolic syndrome and diabetes), and the study of the underlying mechanisms of foods and bioactives in pediatric and adult populations. This research helps identify new targets for nutraceutical and/or therapeutical applications. Dr. Martin has authored over 60 research papers published in internationally peer-reviewed journals, 6 book chapters, and 20 patents.

Sunil Kochhar obtained his Ph.D. from the Indian Institute of Technology, New Delhi (1986), and after postdoctorate at the University of Delaware, DE USA, and University of Zurich, Zurich Switzerland, he joined Nestlé Research Center, Lausanne, Switzerland. Following different lead scientist and line management functions, he became Head of the BioAnalytical Sciences Department in 2002. He has authored over 100 scientific articles in the internationally peer-reviewed journals, 20 book chapters, and 14 patents. He is invited lecturer in many international conferences. His research interests are in enzyme and protein chemistry, functional genomics, glycobiology, and taste receptors. In early 2000, he initiated the concept

of “nutritional metabonomics” to assess metabolic health and its early deviations in healthy humans with a notion to provide appropriate nutritional solutions to maintain and/or prolong the healthier status. Metabonomics research leads to the concept of “metabotype” that included full metabolite signature of an individual including metabolites of the gut microbiota. Over the past 15 years, he has intensively studied the influence of dietary intake and mammalian gut microbiota on the overall health status in healthy individuals. Currently, Dr. Kochhar is the head of Analytical Sciences department and holds an honorary professorship at Imperial College, London UK.

Chapter 1
Introduction to Metabonomics in Systems Biology Research

François-Pierre Martin and Sunil Kochhar

Abstract There is a growing interest to understand the paradigm of healthy aging since the aging population and longevity increases worldwide. However, chronic diseases cover a broad range of organ and biological functions, ranging from metabolic to digestive health, as well as from mental to physical functions. In the meantime, rising evidence also point towards the critical and long-term involvement of prenatal and early nutrition on later health and disease risk predisposition. Dietary preferences and nutrient composition have been shown to influence human and gut microbial metabolism, which ultimately have specific effects on health and disease risk. Increasingly, results from molecular biology and microbiology demonstrate the key role of the gut microbiota metabolic interface to the overall mammalian host's nutritional and disease status.

There is therefore raising interest in nutrition and disease research to characterize the molecular foundations of the gut microbial mammalian cross talk at both physiological and biochemical pathway levels. Tackling these challenges can be achieved through systems biology approaches, such as metabonomics, to underpin the highly complex metabolic exchanges between diverse biological compartments, including organs, systemic biofluids, and microbial symbionts. The generalization of such approaches has opened new research areas to deepen our current understanding on many physiological processes as well as food functionalities in general and targeted populations. Such novel insights are envisioned for aiding strategies for a tailored therapeutic and nutritional program. By the development of specific biomarkers for prediction of health and disease, metabonomics is increasingly used in clinical applications as regard to disease etiology, diagnostic stratification, and potentially mechanism of action for therapeutical and nutraceutical solutions.

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This chapter focuses on introducing major concepts and research directions with emphasis on core indicators of health and functions in infant, adult, and elderly populations. The different themes will be developed further under the related book sections.

Keywords Disease • Gut microbiota • Health • Mass spectrometry • Metabonomics • Metagenomics • Nuclear magnetic resonance spectroscopy • Nutrition • Systems biology

1.1 Introduction

There is a growing interest to understand the paradigm of healthy aging since the aging population and longevity increases worldwide. Indeed, it has been estimated that by 2020 chronic disease in developing countries will account for almost three-quarters of all deaths worldwide with 75 % of death due to stroke and 70 % of death due to diabetes [1, 2]. Awareness about the role of nutrition and lifestyle for health and disease risk management has increased, with key emphasis on the prevention of metabolic disorders, including cardiometabolic diseases and type 2 diabetes [1–3]. In parallel, rising evidence pointed towards the critical and long-term involvement of early nutrition and lifestyle on later health and disease risk predisposition [4]. Thus, it becomes pertinent to look at metabolism throughout life, disease development, and nutritional requirements to understand the onset of certain child and adult physiological conditions [3, 4].

With the advent of the post-genomic era, nutrition research benefits from all state-of-the-art analytical strategies that could be used for understanding the complex relationship between nutrition and health [5]. In the meantime, modern nutrition has shifted its focus towards molecular biology, genetics, and metabolic pathways with the goal of preventing disease and enhancing the health and well-being of individuals [6]. Since population and individual physiological features are encoded at the different levels of biological compartments and organization, there is growing interest in modeling gene expression, protein or metabolite concentrations, and their dynamic pathways in cells, tissues, and organs to generate biological system models. Recent revolution in omics technologies has generated various promising concepts aiming to generate a global systems view of physiological and pathological processes [6, 7]. The concept of systems biology has then been developed and related to the integration of information at the different levels of genomic expression (mRNA, protein, metabolite). Thus, systems biology generates pathway information and provides the capacity to measure subtle perturbations of metabolic pathways resulting from various intrinsic and extrinsic effects, including disease or nutrition. Dietary preferences and nutrient composition have been shown to influence human and gut microbial metabolism, which ultimately impact health and disease risk. It is therefore envisioned that the integration of systems biology

into nutritional and health research will provide novel perspectives [8, 9]. This has led to the development of nutrigenomics that tackles how diet influences gene transcription, protein expression, and metabolism [6, 7, 10].

Systems biology applications are rising and are envisioned to drive a change in clinical practice through generalizing large population-based studies, aiming at enhancing our understanding of the role of genetics, environmental factors, and their interactions on individual susceptibility to disease and health [11, 12]. Therefore, one of the most powerful strategies for deciphering gene-environment interactions and their role in individual variability, disease etiology, and nutritional outcomes lies in the ability to combine data from different omics technologies, establishing the way for systems biology approaches [13, 14]. In this, metabonomics is able to generate multivariate information on a wide range of molecules and provide the ability to measure subtle changes in biological processes as a result of different nutritional effects [15–17]. Metabolic profiling of biological fluids by nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS) ensures a simultaneous analysis of a wide range of metabolites that are the endpoints of molecular regulatory processes, diet, and gut microbiota metabolism and influenced by other environmental factors (Fig. 1.1). Metabonomics therefore enables monitoring metabolite concentrations and dynamics in cells, tissues, and multi-compartmental biological systems [18–21]. By opening a direct biochemical window into the metabolome, metabonomics is uniquely suited to develop new generations of biomarkers that are capable of providing a better understanding of complex metabolic phenomena as well as assessing intra- and interindividual differences. This feature makes metabonomics very efficient for the generation of biomarker patterns for the comprehensive characterization of metabolic health, the prognostics and the diagnostics of diseases, and the generation of new insights in the understanding of the interactions of diet and metabolism and/or medical conditions. Therefore, the identification of specific metabolic fingerprints vows strong potentials for nutraceutical and therapeutical surveillance.

To date, numerous successful applications of metabonomics are reported in toxicity screening, drug metabolism, and functional genomics, a major part of them involving animal models rather than human subjects due to the greater control of conditions to reduce extrinsic variability [22–27]. Metabonomics' introduction in the field of nutrition research, i.e., nutritional metabonomics, has already delivered interesting insights for the understanding of metabolic responses of human or animals in response to dietary interventions and for the definition of metabolic phenotypes [5, 28–31]. Defining the metabolic phenotype or "metabotype" of human populations will offer a great opportunity to evaluate the metabolic response and the degree of this response to specific dietary modulations at the individual level, similarly to the "pharmaco-metabonomic" concept [32].

One particular feature that such an approach enables is rediscovering and revisiting gut functional ecology and its role in the etiology of many metabolic disorders. Metabonomics has indeed offered an unprecedentedly new way to capture the complex metabolic interactions of the host with its commensal microbial partners providing a new way to define individual and population phenotypes [18, 33, 34]. This

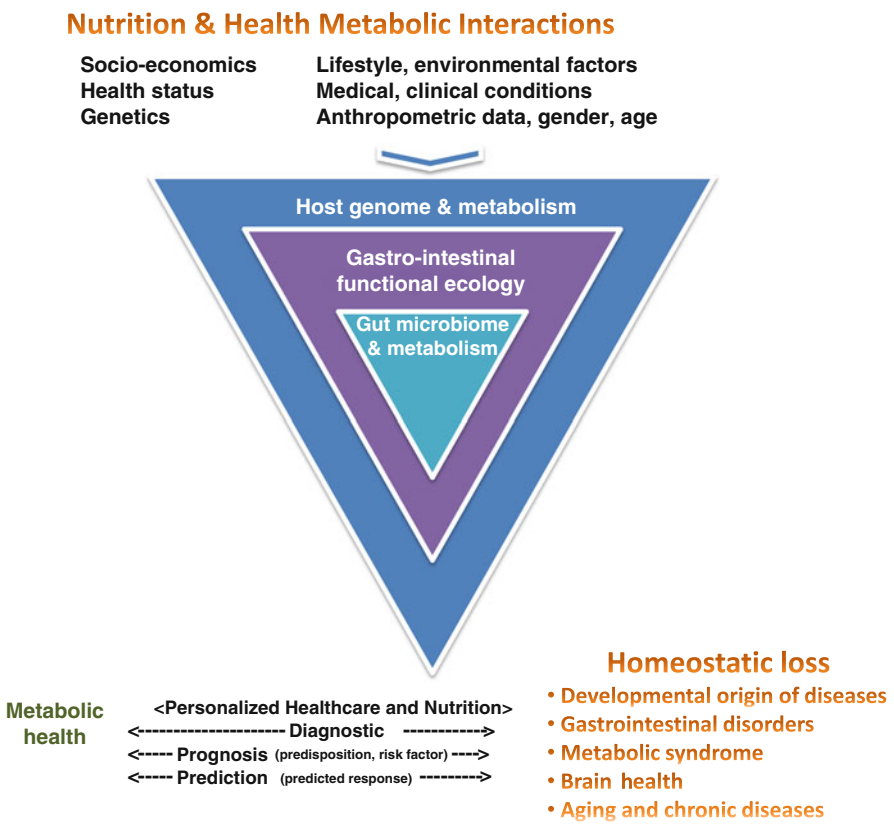

Fig. 1.1 Conceptualization of systems biology approaches for gastrointestinal health and risk management. The metabolic status of individuals results from gene, environment, lifestyle, food, and gut microbiota interactions. The relationship between host and gut microbiota, with the metabolic influence of gut symbionts at different level of biological organization, underpins the depth of controls over multiple host cell metabolic functions

feature offers a new perspective to study the role of mammalian gut microbial metabolic interactions in individual susceptibility to health and disease outcomes, which is of increasing importance in many health research fields ranging from metabolic to gastrointestinal health and even brain health [35–42]. The mammalian gut microbial relationship is a key determinant by performing multiple digestive, immune, and metabolic functions [43–45]. The gastrointestinal tract of adult humans contains around 1.5 k of biomass composed by gut microbial symbiotic and commensal organisms that are in intimate communication with the host, which is the result of a long period of coadaptation between the host genotype and the gut microbiome [46]. The gut microbiota provides to the host specific capacities ranging from dietary energy recovery from nutrients, generating digestible carbohydrates and short-chain fatty acids (SCFAs) from otherwise nondigestible fibers, amino acids, and vitamins to protect against infectious diseases [47–50]. Gut

microbial activities were shown to be extremely specific, as exemplified via its essential role in the development and maintenance of the mucosal innate and adaptive immune system [51]. In parallel, there is increasing awareness that many health disorders involve a significant perturbation of immune and energy metabolism which are intrinsically linked to the gut functional ecology [45, 52, 53]. The consortium of symbiotic gut microorganisms can be viewed as a metabolically adaptable, rapidly renewable, and metabolically flexible ecosystem varying in addition with the host's age, diet, and health status. Consequently, the microbiome is a nutritional target today and might also become the foundation of future drug targeting and interventions [54].

Undoubtedly, the interplay between gut microbiome and host and its modulation by nutrition will benefit from the integration of information on a system's biology-wide approach. Integration of gene sequence of the microbiome and metabolomics is currently envisioned to pave the way towards a better molecular understanding of the complex mammalian superorganism, which is a prerequisite for optimizing therapeutic strategies to manipulate the gut microbiota to combat disease and improve health. The field of endeavor is expanding rapidly, but we believe that now is a good time to review the achievements in the area of metabonomics and gut microbiota research. Therefore, this book will aim at providing state-of-the-art information on the key findings and methodologies and point future directions towards understanding how to beneficially modulate our gut functional ecology for health and nutritional benefits.

1.2 Metabonomics-Based Systems Approaches: Lessons Learnt and Highlights

Over the last three decades, the majority of metabonomic-based investigations in human populations have applied metabonomic analysis of biofluids to screen and diagnose certain diseases and monitor physiological changes caused by toxic insults [23–25, 32, 55–58]. Metabonomics is being widely employed in the area of food research to investigate the mutual link among the fields of metabonomic and nutrition research [5, 31]. Systems biology approaches employing systemic and compartmental metabonomics are therefore applied to assess the molecular processes reflecting metabolic adaptation to disease etiology, development, or management with drugs. One key feature of such an approach lies in its ability to capture the individual status prior to intervention which encapsulates both intrinsic and extrinsic determinants of the individual. Increasingly, results from molecular biology and microbiology demonstrate the key role of the gut microbiota metabolic interface to the overall mammalian host's health status. There is therefore raising interest in nutrition and disease research to characterize the molecular foundations of the gut microbial mammalian cross talk at both physiological and biochemical pathway levels. Tackling these challenges can be achieved through systems biology approaches, such as metabonomics, to underpin the highly complex metabolic

exchanges between diverse biological compartments, including organs, systemic biofluids, and microbial symbionts. To explore how the changes in environmental conditions and lifestyle influence human physiology, a large-scale metabonomic study was conducted to investigate metabolic phenotype variation across and within four human population groups. Holmes et al. used metabonomics in the context of a large-scale epidemiological study to identify metabolic signatures across and within selected human populations in relation to geography, diet-related major risk factors, and cardiovascular diseases [59]. In this study, urinary metabolite excretion patterns differ between East Asian and Western populations, Japanese individuals living in Japan or in the USA, and Chinese participants living in the northern and southern parts of China. For example, urinary excretion of formate, a mammalian gut microbial co-metabolite, was shown for the first time to be inversely correlated with blood pressure.

Overall, the increasing rate of metabolic syndrome is a worldwide challenge for both health and nutritional research [60, 61]. By 2015, the world health organization (WHO) projections predict 2.3 billion overweight adults and more than 700 million obesity cases [62]. This overweight and obesity pandemic continues to rise particularly among children [63, 64]. This rapid pandemic, which cannot be explained on the sole basis of genetic heritability and susceptibility, has raised awareness on the critical role of changes in dietary habits and lifestyle in the metabolic etiology of the diseases. It is nowadays well admitted that a main obesity determinant is the energy imbalance between calorie intakes and expenditures which can be ascribed to a global dietary shift towards high energy density foods and sedentary behaviors. Existing therapeutic strategies to obesity lies in changing dietary habits and lifestyle or pharmacotherapies, yet, with only marginally beneficial effects for morbidly obese patients. Nowadays, much interest is therefore given for a tailor-made weight management program and more recently to body composition rather than BMI-driven approaches. Body fat distribution, and visceral fat in particular, was demonstrated as a key determinant of increased risk of cardiovascular disease [65–67], diabetes [68, 69], hypertension [70], nonalcoholic fatty liver disease [71], and a higher risk of mortality [72]. Over the last decades, genetic and environmental promoters for obesity-related metabolic disorders were investigated, including genes and transcription factors associated with fat storage and obesity [73, 74], genetic inheritability [75], or even gut microbiota influence [76]. Nevertheless, the generated insights face the challenges of explaining why similar obesogenic and diabetogenic conditions do not necessarily lead to a universal response to insulin and adiposity-associated cardiometabolic risks [77, 78]. Individuals with normal weight (body mass index, BMI <25) are increasingly shown to also express cardiometabolic abnormalities [77]. Increasing evidence pinpoints the key role of region-specific body composition and metabolism underpinning individual susceptibility to metabolic disease risks. In this search for expanding our knowledge on the etiology of metabolic disorders, systems biology models may lead to new working hypotheses underlying one's predisposition to develop specific disorders. For instance, in an elegant series of studies integrating metabolic, endocrine, inflammatory, and physiologic differences between obese and lean subjects, a branched-chain

amino acid (BCAA)-related metabolic signature was highlighted as a robust metabolic readout of insulin resistance (IR) [79], even present under subclinical conditions [80]. The catabolism of BCAAs was tightly intertwined with the IR levels, while greater levels of BCAAs were detected in the obese and IR phenotype [81–83]. Further studies confirmed the predictive values of these markers for the development of diabetes in prospective human studies [84, 85]. Five branched-chain and aromatic amino acids were indeed associated with IR, namely, isoleucine, leucine, valine, tyrosine, and phenylalanine, and a combination of three amino acids (isoleucine, phenylalanine, tyrosine) could predict future diabetes (>5-fold higher risk for individuals in the top quartile) [85]. However, supplementation of BCAAs in a diet-induced obesity rat model did not result in any improvement of the metabolic status and IR and only led to reduced food intake and weight gain. Together these key findings demonstrate a critical role of BCAA metabolism in the early onset of IR and diabetes type 2 developments, but the understanding of the biological specificities in certain organ still remains to be understood in order to better decipher how the modulation of BCAA and amino acid metabolism could help disease management. Recent report on obese Japanese subjects further demonstrated a physiological inference between IR; plasma levels of alanine, glycine, glutamate, tryptophan, tyrosine, and BCAA; and visceral fat metabolism [86]. Complementary findings further described a complex relationship between dyslipidemia and IR development [84], while there is growing interest towards understanding the specificities of fatty liver and visceral adiposity in the development of metabolic disorders. For instance, a complex remodeling of triglycerides [84] and phospholipids [87] species were highlighted in relation to the distribution of visceral and subcutaneous fat within the body. These observations may result from a multifactorial origin, including dietary factors, gut functional ecology, intestinal absorption, as well as platelet-activating factor metabolic pathways [88, 89], which are modulated by obesity and insulin resistance [90, 91]. Another physiological peculiarity of the metabolic syndrome encompasses nonalcoholic fatty liver disease, ranging from steatosis to nonalcoholic steatohepatitis [92, 93]. For instance, using a parallel animal model/human design, biochemical perturbations linked to liver dysfunction through increased concentrations in bile acids and eicosanoids were highlighted. Such findings corroborate metabolic steatosis markers in liver tissues, including altered levels of bile acids, glutathione, and lipids. All together, these metabolic findings provided further insight into specific metabolic contribution of liver dysfunction in an overall metabolic syndrome context, as per specific cholesterol, lipid, and oxidative stress readouts. More recently, metabonomics was successfully employed to demonstrate a relationship between gut microbial metabolism of dietary phosphatidylcholine and cardiovascular pathogenesis in humans [94].

Over the last two decades, gastrointestinal surgery has been revealed as a potential "holy grail" solution to treat diabetes in several obese populations, and its applications is envisioned to be increasingly applied worldwide [95]. Effective weight loss was achieved in morbidly obese patients after undergoing bariatric surgery. A substantial majority of patients with diabetes, hyperlipidemia, and hypertension generally experience complete resolution or improvement [95]. However, major

gaps still obscure proper understanding on the mechanisms at play for improving diabetes. In the meantime, clinicians and patients are also facing the challenge of understanding why a significant percentage of the subjects do not respond post-surgery and reacquire insulin-dependent disorders and how to tackle all the subsequent adverse events, including micronutrient deficiencies. Metabolic insights are envisioned to help generate reference data that will help further the understanding of the underlying biological deregulations, for instance, through better monitoring of the metabolic and nutritional requirements of patients who underwent various interventions (e.g., gastric banding, gastric bypass, gastroplasty, biliopancreatic diversion, or duodenal switch). Recent findings in animal models reported how urinary phenotyping may indicate weight loss-independent metabolic effects of Roux-en-Y gastric bypass, which sets a first step towards promising novel insights for human applications [96].

Furthermore, the gastrointestinal tract (GIT) is one of the most essential interfaces of mammalian organisms interacting with nutrients, exogenous compounds, and gut microbiota, and its condition is influenced by the complex interplay between these environmental factors and host genetic elements. Along the GIT, the gut microbiota is a key determinant of the gut functional ecology and regulatory processes involved in the absorption, digestion, metabolism, and excretion of dietary nutrients as well as barrier integrity, motility, and mucosal immunity [35, 97]. The evolution of nutrition, sanitary, and medical care conditions has led to rediscovering host-gut microbial metabolic interactions in health and disease [98]. Gut microbial activities can be extremely complex, such as in the etiology and development of several chronic inflammatory disorders, including inflammatory bowel disease (IBD) or irritable bowel syndrome (IBS) [99, 100]. Since normal aging is associated with a number of significant changes in GIT function and with the development and progression of chronic disease, such insights will be key for tailoring personal nutritional intervention and monitoring patient response on an individual basis [101]. Advancing knowledge regarding the mechanisms of IBD has led, for instance, to the development of different therapeutic solutions based on surgery [102], cannabinoids [103], or immunosuppression [104]. Although prognostic and monitoring tools are currently lacking, the use of metabolic readouts in combination with state-of-the-art clinical and medical readouts is to be a valuable tool to differentiate and follow-up disease evolution and respond to disease-modifying interventions. This is, for instance, exemplified through the discovery of metabolic indicators of different IBD determinants, including mucosal healing, gut permeability, absorption, digestion, or inflammatory states. As an example, Winterkamp et al. reported previously how N-methylhistamine, a key metabolite in mast cell metabolism involved in the pathogenesis of IBD, could be used as an indicator of disease activity in patients [105]. In this study, the urinary excretion of N-methylhistamine was associated with elevated histamine production and metabolism in IBD and could be used as a reliable diagnostic tool to monitor clinical and endoscopic disease activity in IBD. Additional animal studies may further substantiate proofs of concept on the feasibility to identify blood-related metabolic indicators of early onsets of chronic inflammatory development for patient monitoring [106]. Finally, noninvasive stool

analysis was shown to be very promising for monitoring the remodeling of the gut functional ecology, concomitant to malabsorption and element of protein losing enteropathy [107, 108]. Other noninvasive approaches to monitor gut permeability functions may also be promising prospects for future translation to patient monitoring [109].

It can be forecasted that such integrative systems biology approaches would help to delineate different behavioral and response phenotypes, with which to personalize the disease and nutritional management programs. For instance, beneficial effects of exercise in patients with type 1 diabetes were assessed using such a metabonomic approach [110], a concept that can be extended to a larger range of individuals under different age, nutritional, and health conditions. Nevertheless, the translation of this unprecedented source of metabolic knowledge which is now at hand into actionable clinical practices for healthcare requires addressing properly the dynamics of physiological and biological processes when defining metabolic phenotypes. This implies the development of analytical strategies based on the measurement of metabolites to assess the effects of nutrition at both the organ-specific compartment and system levels [25, 26, 111]. Therefore, the understanding of regulatory metabolic processes of a complex living organism at the system level involves the assessment of spatiotemporal interorgan metabolic cross talks through the analysis of biofluids. Recently, Montoliu et al. applied unsupervised chemometric methods for integrating ^{1}H NMR metabolic profiles from mouse plasma, liver, pancreas, adrenal gland, and kidney cortex matrices in order to infer inter-compartment functional links [112]. The authors showed that integration of metabolic profiles using advanced mathematical modeling approaches provided an overview of functional relationships across matrices and enabled the characterization of compartment-specific metabolite signatures, the spectrotypes. In particular, the methodology enables the modeling of biochemical signatures common to different biological matrices, which may highlight specific metabolic processes or cross talks shared by different organs or specific to a given biological tissue or biofluid. In parallel, developments on computational modeling have established a global systems view of human metabolism. A global genome- and bibliome-wide reconstruction of the human metabolism was built using 3,311 reactions [113]. Metabolic activities at the tissue level were predicted by a constraint-based computational model, from integration a genome-scale model with tissue-specific gene and protein data. Results were obtained for ten human tissues (the brain, heart, kidney, ling, pancreas, prostate, spleen, thymus, skeletal muscle, and liver) [114]. A genome-scale stoichiometric model of hepatic metabolism was obtained by combining literature-based knowledge, transcriptomic, proteomic, metabolomic, and phenotypic data [115]. Likewise, modeling of the gut ecosystem could be applied as a combinatory analysis of individual genome-scale metabolic models of gut bacteria, taking into account their interactions [116]. System-wide computational approaches can be then useful in nutritional applications to test hypothesis in silico on whole systems, in order to study potential effects of diet or modulation of metabolic diseases. Such a combinatorial approach could provide new research avenues to assess the role of gut microbiota and nutritional modulation of bacterial metabolic activities on host metabolism.

In the meantime, it is expected that analytical technologic developments will enable sustainable deployment of such tests into clinical routine setups thanks to improved automation, miniaturization, time to result, and cost per analysis.

The assessment of the inherent interindividual variability especially as regards variable environmental conditions and genetic background will have to be properly considered through longitudinal studies of a large cohort of subjects. The existence of unique individual metabolic phenotypes has been hypothesized, but the experimental evidence has been only recently collected [117, 118]. This was exemplified by Bertini et al. through the analysis of individual phenotypes over the timescale of years, which shows that the metabolic phenotypes are largely invariant. Such reports also support the idea that the individual metabolic phenotype can also be considered a metagenomic entity that is strongly affected by both gut microbiome and host metabolic phenotype, the latter defined by both genetic and environmental contributions [117, 118]. Longitudinal metabolic profiling (e.g., generating individual metabolic trajectories) and metagenomics are therefore foreseen as phenotypic variables to stratify genetic backgrounds, offering an alternative to the averaging of genetic variability within study groups defined using clinical criteria currently available. One can foresee that such phenotypic-determined groups will aid in exploring the underlined causes of inherent individual response to clinical therapeutic and nutrition schemes (e.g., responders and nonresponders). This is also a feature of key importance in the early life period for gastrointestinal and immune system maturations and understanding metabolic and nutritional requirements for optimal growth and development and their inferred effects with later-life diseases. The applications of metabonomics have been so far very promising in the field of neonatology, including intrauterine growth restriction, perinatal transition, asphyxia, brain injury and hypothermia, metabolic diseases, perinatal programming, as well as inborn error metabolism screening [3, 4, 119]. Such data will serve as reference to provide understanding at the cellular and molecular level of the relationships between early life nutritional status and the later disease risk predisposition and formulate future nutritional concepts.

1.3 Gut Microbiota Metabolism in Nutrition, Health, and Disease

Recently, an exhaustive gene catalogue containing virtually all of the prevalent gut microbial genes in large human cohort and reported to which extent many bacterial species are shared by different individuals [120]. Such an approach could be used for global characterization of the genetic potential of ecologically complex environments [120] but also to help understand how gut microbiota specificities could be exploited to develop new therapeutic and nutritional strategies. In particular, demographics have made aging and age-related chronic disease an enormous and growing biomedical and societal challenge [38]. The immune system undergoes profound

and multifaceted changes with aging. In particular, the homeostatic balance between the proinflammatory and the antiinflammatory arms of the immune system is skewed, resulting in a state of persistent low-grade systemic inflammation. In this the origins and drivers of the "inflammaging" process are still poorly understood [121], for instance, but intestinal dysbiosis appears a cornerstone in growth, development, and aging.

The gastrointestinal tract is a complex ecosystem host to a diverse and highly evolved microbial community composed of hundreds of different microbial species. The human body contains circa 10 trillion parenchymal cells, but the gut contains approximately 100 trillion of microbial organisms, which vary in community composition through life according to lifestyle and nutrition [48, 122–124]. On average, each individual harbors a unique combination of about 500–1,000 different bacterial species [48, 122–124]. In mammals, microbial communities differ in composition from the stomach to the colon, where the competition for space and nutrients in the large bowel contributes to the microbial composition of this internal ecosystem. The main human intestinal bacteria coexist in a dynamic ecological equilibrium together with various yeasts and other microorganisms [125]. The members of the gut microbiota consortium are diverse and impart to the host-specific capacities ranging from dietary energy recovery from nutrient load, generating digestible carbohydrates, short-chain fatty acids, amino acids, and vitamins, to protection against infectious diseases [47–50]. Activities of the diverse gut microbiota can be highly specific, and it has been reported that the establishment of *Bifidobacteria* is important for the development of the immune system and management of gut functions [126–128]. As the microbiome interacts strongly with the host to determine the metabolic phenotype [129, 130], and the metabolic phenotype influences the outcomes of drug interventions [18, 27], understanding these interactions as part of personalized healthcare solutions is clearly an important role [26]. Advances in metabonomic applications are providing novel insights into the molecular foundations of these host-microbial relationships and their influence onto health and disease risks [131, 132]. In particular, a series of investigations in humans [133], rats [134], and gnotobiotic mice [131, 135] have provided a set of reference metabolic profiles of gut intestinal biopsies that can be used to assess not only the compartment structure and function but also the gut microbial impact at the tissue level [135]. These studies therefore indicated that the type of gut microbiota may be a key factor in the determination of the intestinal homeostasis, osmo-protection, motility, and calorie recovery from the diet. For instance, the ileum is regarded as the major site for absorption of luminal bile acids and emulsified dietary lipids, and the report of higher concentrations of glycerophospholipids, glutathione, taurine, and betaine in this tissue was consistent with its role in lipid and bile acid metabolism. The jejunum of the mice harboring a nonadapted microbiota showed metabolic similarities to the ileal profile, marked by higher levels of glutathione and lower concentrations of its precursors in the γ-glutamyl cycle when compared to conventional animals. Such an observation illustrates the essential role of the gut microbiota to influence the surrounding tissue metabolic pathways, ultimately to shape a host environment that fosters their implantation and persistence. The

investigations illustrated how microbial-dependent variations along the upper intestine, an element often underestimated due to low bacterial populations, may affect utilization efficiency of dietary proteins and amino acids and their subsequent availability to extra-intestinal tissues. Moreover, some reference data were generated to investigate changes in gut functionality, such as gut permeability, using metabolic profiling of biofluids [109, 136]. Since growth and aging are associated with a number of significant changes in GI function, which may impact the daily energy intake [101], such insights will be key for tailoring personal nutritional intervention and monitoring patient response on an individual basis.

1.4 System Biology Approaches: Applications with Specific Reference to Gut Microbial Metabolic Interactions

Recent findings are describing the deep and fundamental role of gut microbiota in both positive and negative triggers of specific metabolic states of individuals and populations [37, 98]. In this the generation of novel and more specific metabolic readouts will help the deciphering of gut microbial influence on human health and nutritional status [31, 36, 119]. In particular, both system-wide and organ-specific metabolism may have components driven by gut microbial activities [137, 138], which suggests that the dynamics of the gut microbiome could help maintain or restore host metabolic homeostasis in disease and early onsets of metabolic deregulations.

Today, direct metabolic insights into gut microbiota metabolism remain limited due to the inaccessibility of the intestinal habitat and by the sheer complexity of the gut microbiota [139]. The measurement of the gut microbial metabolism is generally confined to fecal samples, which is generally limited due to the elevated colonic absorption of bacterial metabolites [36, 140]. However, such measurable outcomes provide some essential insights – yet limited – into a small range of microbial activities within the colon. In particular, fecal analysis provides essential information on the impact of microbial activities on specific biological processes, including the metabolism of bile acids, SCFAs, or heterocyclic amines [141–143]. The metabolic composition of fecal extracts provides therefore a window for elucidating the complex metabolic interplay between mammals and their intestinal ecosystems, and these metabolic profiles can yield information on a range of gut diseases. Saric et al. employed ^{1}H NMR-based metabolic analysis of fecal water extracts to assess inherent similarities and dissimilarities across different mammals, namely, humans, mice, and rats [144]. This study provided an important milestone as per characterization of many fecal metabolites common to the three species, such as SCFAs and branched-chain amino acids. The authors also described how not only the presence but also the proportion of the different biochemical compounds resulted in a species-specific profile, encapsulating interindividual variations (i.e., the natural genetic and environmental diversity in human populations). Furthermore, NMR spectroscopy,

using high-resolution magic angle spinning NMR (HR-MAS) [145], offers a unique prospect by the holistic and simultaneous profile of hundreds of metabolites in intact tissue biopsies. Therefore, HR-MAS presents the unique feature of ensuring the integrity and organizational compartmentation of the biological samples. Advanced data modeling strategies enabled the visualization of metabolic similarities and differences along the gut tract that were induced by different gut microbial ecologies, namely, mice with a conventional mouse microbiota or inoculated with a simplified model of human-derived microbiota [131, 132]. These studies indicated that the type of gut microbiota may be a key factor in the determination of intestinal homeostasis, osmo-protection, motility, and calorie recovery from the diet. Indeed, the symbiotic gut microbiome exerts a strong influence on the metabolic phenotype of the mammalian host and participates in extensive microbial-mammalian co-metabolism [26, 112, 146, 147]. The integrated metabolism of the bile acid pools in mammals is a good example of the complex transgenomic biochemical interactions between host and microbiome symbionts [33], which are crucial for the absorption of dietary fats and lipid-soluble vitamins in the intestine [148].

Additional applications of top-down systems biology approaches revealed the depth and width of the long-range effects of gut microbiota modulation in complex organisms, resulting in modulation of host lipid, carbohydrate, and amino acid metabolism at a panorganismal scale [44, 137, 149–151]. Wikoff et al. provided additional evidence that the specific metabolic activities of a single gut bacterial species can provide the host with new biochemical compounds in sufficient amount to be detected in the general blood circulation [152]. Martin et al. exemplified how the gut microbial modulation of the gastrointestinal system [131] and extensive microbial-mammalian co-metabolism may fine-tune host metabolic processes and may induce metabolic deregulations [33]. In this case, gut bacteria exert modulation over the host metabolism via reprocessing of signaling molecules, i.e., bile acids. As such, bile acids may be an example of transgenomic mechanism of quorum sensing [44] whereby microbial cells communicate with each other and disperse their metabolic functions, thus behaving like a multicellular organism [33]. Martin et al. evaluated the effects of the induction of a nonadapted microbiota in a murine model (human-derived microbiota) on the host metabolism by comparison with animals colonized with a natural gut microbiota (conventional), the result of a long period of coevolution [33]. The simplified human microbiota was not adapted to mice, which modified the physiology of the murine host towards a pre-pathologic state. While conventionalized mice evolve to normal gut flora from an ecological point of view, the simplified human microbiota maintained the gut tract and the liver out of a sustainable mouse ecological equilibrium, as denoted by increased lipid accumulation in the liver and lower concentrations of glutathione, which together are associated with a higher lipoperoxidation risk. In another study, Claus et al. described the determinant role of the gut microbiota on the metabolism of bile acids through the enterohepatic cycle, as noted by higher levels of phospholipids in the liver of germ-free mice and higher levels of bile acids in gut tissues [153]. The presence of the gut microbiota was shown as a key regulator of the bile acids metabolism and some CYP family enzymes.

In addition to the building awareness that specific environmental exposure (e.g., stress perception) may induce specific and chronic gut functional activities [109, 154], increasing evidence tends to show that long-term dietary habits may also have a significant impact in determining chronically the gut functional ecology, which may have a long-term health consequence [24, 155, 156]. Therefore, the gut microbiota functionality remains to be further explored and understood, in order to assess how its nutritional modulation at individual and population level may be used for future nutritional management solutions. It is therefore crucial to decipher the foundations of the reciprocal metabolic influences between host and microbiota to better define the role of gut microbes in determining gastrointestinal functional ecology. The gut microbiota does not only determine absorption, digestion, metabolism, and excretion of dietary nutrients but may also determine the metabolic fate of both ingested nutrients and host cell molecular machinery, which nowadays become fundamental for developing individual disease and nutritional management solutions.

Recent evidence has shown that the influence of the gut microorganisms might be more important in the progression of human diseases than was previously suspected [157] and is of main concern in the etiology and/or maintenance of gut dysfunctions, such as IBS [158] or IBD [159]. Metabonomics was successfully applied to characterize the metabolic response to the induction of intestinal ischemia reperfusion injury by portal vein outflow occlusion in rats [160]. This combinatorial approach provides novel insights into metabolic signatures associated with oxidative stress, as noted by increased glycolysis and fatty acid and amino acid accumulation. Metabonomics was proven to be a valuable diagnostic tool to differentiate active and quiescent ulcerative colitis, as per the analysis of intact gut biopsies and colonocytes [161]. This approach was also successfully employed to provide insights into the molecular processes associated with the development of ulcerative colitis, using blood plasma [162] or urine [163] analysis, the latter revealing a possible contribution of gut microbiota via methylamine metabolism. Bertini et al. also demonstrated that combinatorial metabonomic analysis of blood sera and urine could help further the understanding of Celiac disease [164]. The authors highlighted major urinary changes in gut microbial co-metabolites phenylacetylglycine, indoxylsulfate, and meta-hydroxy-phenylpropionate, which may be associated with aberrant microbiota previously characterized in the small bowel of subjects suffering from Celiac disease [165].

More recently, metabonomics was successfully employed to demonstrate a relationship between gut microbial metabolism of dietary phosphatidylcholine and cardiovascular pathogenesis in humans [94]. Intestinal microbiota metabolism of choline and phosphatidylcholine produces trimethylamine (TMA), which is further metabolized to a proatherogenic species, trimethylamine-N-oxide (TMAO). Wang et al. described how the circulating levels of choline, trimethylamine-N-oxide, and betaine were shown to predict the risk for cardiovascular events, which offer novel perspectives for therapeutical and nutraceutical approaches, as demonstrated by their follow-up [166, 167]. They further demonstrated how metabolism by intestinal microbiota of dietary l-carnitine, a trimethylamine abundant in red meat, also produces TMAO and accelerates atherosclerosis in mice. In humans they reported how

the presence of specific bacterial taxa in human feces was associated with both plasma TMAO concentration and dietary status. More interestingly, they described that high plasma TMAO levels combined with plasma l-carnitine levels in subjects undergoing cardiac evaluation predicted increased risks for both prevalent cardiovascular disease and incident major adverse cardiac events (myocardial infarction, stroke, or death) [166]. In parallel, significant scientific efforts were put in identifying specific disease risk genotypes. But it is only recently that the incorporation of metabonomics in genome-wide association studies has offered novel opportunities for exploring disease-related metabolic deregulations and interactions between environmental exposure, lifestyle, genetic predisposition, and actual metabolic phenotype at individual and population scale [28]. Such an approach has recently described an association between urinary trimethylamine and plasma dimethylamine – two gut microbial metabolites – and the pyridine nucleotide-disulfide oxidoreductase gene PYROXD2 [168]. The present association is of importance and suggests how the conversion rate TMAO/TMA can be related with key hepatic functions that are under genetic determinant and may predispose individuals to specific disease risks. Such approaches offer novel avenues to screen individuals with specific predisposition and determine candidate metabolic targets to be used to develop tailor-made nutritional management program.

1.5 Novel Directions and Perspectives

Metabonomics is increasingly becoming popular in clinical research due to its unique attractiveness to generate functional and system readouts of individuals, building the elementary steps for future personalized nutraceutical and therapeutical care. Yet, the clinical translation of this unprecedented source of metabolic knowledge, which is now at hand of clinical practices, requires properly addressing the dynamics of physiological and biological adaptations throughout lifetime, including the various intrinsic and extrinsic influential factors. In addition, the development of systems biology approaches and the new generation of biomarker patterns will provide the opportunity to associate complex metabolic regulations with key mammalian and gut microbial biological processes. The link between nutrition, host-microbe interactions, and metabolism is so tightly interlinked in mammalian systems that it is still difficult to understand and unravel causality and molecular mechanisms of action. In this the lessons learnt and to be learnt from epidemiological metabonomic studies, including twin cohorts, will help in delineating the interaction of nutrients, gene expression, and metabolism from environmental pressures which ultimately determine the physiological and the disease risk status of individuals. Furthermore, metabolic phenotypes will need to capture these dynamics at the molecular and system levels, requiring longitudinal sampling and proper assessment of inherent interindividual variability. Therefore, advances in linking metabolite data to known and validated clinically relevant indices will have to be seriously considered in addition to consolidate metabonomics and metabolic markers in clinical translation/applications.

The generalization of systems biology approaches will subsequently lead to the development of system mechanistic hypotheses that could be targeted with new nutritional and therapeutic personalized concepts. As displayed above, both system-wide (i.e., whole organism) and organ-specific changes in metabolic profiles may have components driven by gut microbial activities which are of relevance in nutrition, health, and disease research as exemplified hereafter in the book. In particular, high-resolution metabonomics data combined with high-resolution microbial profiling are envisioned as a way forward to demonstrate direct and indirect functional relationships of gut microbiota metabolic activities on host cell metabolic pathways in key organs and biofluids. But we are far from the complete elucidation of the molecular processes linking bacteria, metabolic enzymes, and metabolites with their corresponding biological functions. There are indeed several diverting issues to be taken into account, such as the fact that abundant concentrations of either proteins or metabolites may actually be mediated by a low-abundance microbe. Because all studies so far have relied on fecal samples as a mirror of the whole gut microbiome, these might not accommodate local functionalities, as environments between distal, transverse, and proximal colon are known to be divergent [169]. Undoubtedly, the interplay between gut microbiome and host and its impact in nutrition will benefit from the integration of information on a systems biology-wide approach. Gene sequencing of the microbiome, metaproteomics, and metabolomics are starting points to understand the complex mammalian superorganism.

Such future scientific developments are foreseen as a promising step forward to study the spatiotemporal changes in interorgan metabolic cross talks and quantify the metabolic contribution of the gut microbiota to host cell machinery and physiological states. Moreover, such novel concepts have great potential for advancing our mechanistic knowledge of how different environmental triggers, including nutrition, can impact metabolic health of complex mammalian organisms.

References

1. Abegunde DO, Mathers CD, Adam T, Ortegon M, Strong K. The burden and costs of chronic diseases in low-income and middle-income countries. Lancet. 2007;370(9603):1929–38.
2. Strong K, Mathers C, Leeder S, Beaglehole R. Preventing chronic diseases: how many lives can we save? Lancet. 2005;366(9496):1578–82.
3. Antonucci R, Atzori L, Barberini L, Fanos V. Metabolomics: the “new clinical chemistry” for personalized neonatal medicine. Minerva Pediatr. 2010;62(3 Suppl 1):145–8.
4. Atzori L, Antonucci R, Barberini L, Griffin JL, Fanos V. Metabolomics: a new tool for the neonatologist. J Matern Fetal Neonatal Med. 2009;22 Suppl 3:50–3.
5. Rezzi S, Ramadan Z, Fay LB, Kochhar S. Nutritional metabonomics: applications and perspectives. J Proteome Res. 2007;6(2):513–25.
6. Van Ommen B, Stierum R. Nutrigenomics: exploiting systems biology in the nutrition and health arena. Curr Opin Biotechnol. 2002;13(5):517–21.
7. Trujillo E, Davis C, Milner J. Nutrigenomics, proteomics, metabolomics, and the practice of dietetics. J Am Diet Assoc. 2006;106(3):403–13.

8. Van Ommen B. Nutrigenomics: exploiting systems biology in the nutrition and health arenas. Nutrition. 2004;20:4–8.
9. Muller M, Kersten S. Nutrigenomics: goals and strategies. Nat Genet. 2003;4:315–22.
10. Corthesy-Theulaz IE, Dunnen J-T, Ferre P, Geurts J-MW, Mueller M, Belzen N, et al. Nutrigenomics: the impact of biomics technology on nutrition research. Ann Nutr Metab. 2005;49(6):355–65.
11. Ordovas JM. Integrating environment and disease into "omic" analysis. Rev Esp Cardiol. 2009;62 Suppl 2:17–22.
12. Ordovas JM, Shen J. Gene-environment interactions and susceptibility to metabolic syndrome and other chronic diseases. J Periodontol. 2008;79(8 Suppl):1508–13.
13. van der Greef J, Hankemeier T, McBurney RN. Metabolomics-based systems biology and personalized medicine: moving towards n = 1 clinical trials? Pharmacogenomics. 2006;7(7):1087–94.
14. van der GJ. Systems biology, connectivity and the future of medicine. Syst Biol (Stevenage). 2005;152(4):174–8.
15. Nicholson JK, Lindon JC. Systems biology: metabonomics. Nature. 2008;455(7216): 1054–6.
16. Holmes E, Wilson ID, Nicholson JK. Metabolic phenotyping in health and disease. Cell. 2008;134(5):714–7.
17. Nicholson JK, Wilson ID. Opinion: understanding 'global' systems biology: metabonomics and the continuum of metabolism. Nat Rev Drug Discov. 2003;2(8):668–76.
18. Nicholson JK, Holmes E, Lindon JC, Wilson ID. The challenges of modeling mammalian biocomplexity. Nat Biotechnol. 2004;22(10):1268–74.
19. Keurentjes JJ. Genetical metabolomics: closing in on phenotypes. Curr Opin Plant Biol. 2009;12(2):223–30.
20. Saito K, Matsuda F. Metabolomics for functional genomics, systems biology, and biotechnology. Annu Rev Plant Biol. 2008.
21. Weckwerth W. Metabolomics in systems biology. Annu Rev Plant Biol. 2003;54:669–89.
22. Lindon JC, Keun HC, Ebbels TM, Pearce JM, Holmes E, Nicholson JK. The Consortium for Metabonomic Toxicology (COMET): aims, activities and achievements. Pharmacogenomics. 2005;6(7):691–9.
23. Nicholson JK, Connelly J, Lindon JC, Holmes E. Metabonomics: a platform for studying drug toxicity and gene function. Nat Rev Drug Discov. 2002;1(2):153–61.
24. Nicholson JK, Wilson ID, Lindon JC. Pharmacometabonomics as an effector for personalized medicine. Pharmacogenomics. 2011;12(1):103–11.
25. Nicholson JK. Omics dreams of personalized healthcare. J Proteome Res. 2006;5(9):2067–9.
26. Nicholson JK. Global systems biology, personalized medicine and molecular epidemiology. Mol Syst Biol. 2006;2:52.
27. Clayton TA, Lindon JC, Cloarec O, Antti H, Charuel C, Hanton G, et al. Pharmacometabonomic phenotyping and personalized drug treatment. Nature. 2006;440(7087): 1073–7.
28. Montoliu I, Genick U, Ledda M, Collino S, Martin FP, le Coutre J, et al. Current status on genome-metabolome-wide associations: an opportunity in nutrition research. Genes Nutr. 2013;8(1):19–27.
29. Moco S, Martin FP, Rezzi S. A metabolomics view on gut microbiome modulation by polyphenol-rich foods. J Proteome Res. 2012;11:4781–90.
30. Kussmann M, Rezzi S, Daniel H. Profiling techniques in nutrition and health research. Curr Opin Biotechnol. 2008;19(2):83–99.
31. Claus SP, Swann JR. Nutrimetabonomics: applications for nutritional sciences, with specific reference to gut microbial interactions. Annu Rev Food Sci Technol. 2013;4:381–99.
32. Nicholson JK, Everett JR, Lindon JC. Longitudinal pharmacometabonomics for predicting patient responses to therapy: drug metabolism, toxicity and efficacy. Expert Opin Drug Metab Toxicol. 2012;8(2):135–9.

33. Martin FP, Dumas ME, Wang Y, Legido-Quigley C, Yap IK, Tang H, et al. A top-down systems biology view of microbiome-mammalian metabolic interactions in a mouse model. Mol Syst Biol. 2007;3:112.
34. Martin FP, Verdu EF, Wang Y, Dumas ME, Yap IK, Cloarec O, et al. Transgenomic metabolic interactions in a mouse disease model: interactions of *Trichinella spiralis* infection with dietary *Lactobacillus paracasei* supplementation. J Proteome Res. 2006;5(9):2185–93.
35. Sommer F, Backhed F. The gut microbiota – masters of host development and physiology. Nat Rev Microbiol. 2013;11(4):227–38.
36. Martin FP, Collino S, Rezzi S, Kochhar S. Metabolomic applications to decipher gut microbial metabolic influence in health and disease. Front Physiol. 2012;3:113.
37. Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W, et al. Host-gut microbiota metabolic interactions. Science. 2012;336(6086):1262–7.
38. Rehman T. Role of the gut microbiota in age-related chronic inflammation. Endocr Metab Immune Disord Drug Targets. 2012;12(4):361–7.
39. Zheng X, Xie G, Zhao A, Zhao L, Yao C, Chiu NH, et al. The footprints of gut microbial-mammalian co-metabolism. J Proteome Res. 2011;10(12):5512–22.
40. Tiihonen K, Ouwehand AC, Rautonen N. Human intestinal microbiota and healthy ageing. Ageing Res Rev. 2010;9(2):107–16.
41. Biagi E, Nylund L, Candela M, Ostan R, Bucci L, Pini E, et al. Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. PLoS One. 2010;5(5):e10667.
42. Cani PD, Delzenne NM. Gut microflora as a target for energy and metabolic homeostasis. Curr Opin Clin Nutr Metab Care. 2007;10(6):729–34.
43. Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, et al. Evolution of mammals and their gut microbes. Science. 2008;320(5883):1647–51.
44. Nicholson JK, Holmes E, Wilson ID. Gut microorganisms, mammalian metabolism and personalized health care. Nat Rev Microbiol. 2005;3(5):431–8.
45. Dethlefsen L, Fall-Ngai M, Relman DA. An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature. 2007;449(7164):811–8.
46. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, et al. Metagenomic analysis of the human distal gut microbiome. Science. 2006;312(5778):1355–9.
47. Gilmore MS, Ferretti JJ. The thin line between gut commensal and pathogen. Science. 2003;299:1999–2002.
48. Tannock GW. New perceptions of the gut microbiota: implications for future research. Gastroenterol Clin North Am. 2005;34(3):361–82, vii.
49. Xu J, Bjursell MK, Himrod J, Deng S, Carmichael LK, Chaing HC, et al. A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis. Science. 2003;299:2074–6.
50. Cummings JH, Pomare EW, Branch WJ, Naylor CP, Macfarlane GT. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut. 1987;28(10):1221–7.
51. Guigoz Y, Dore J, Schiffrin EJ. The inflammatory status of old age can be nurtured from the intestinal environment. Curr Opin Clin Nutr Metab Care. 2008;11(1):13–20.
52. Dunne C. Adaptation of bacteria to the intestinal niche: probiotics and gut disorder. Inflamm Bowel Dis. 2001;7(2):136–45.
53. Turnbaugh P, Ley R, Mahowald M, Magrini V, Mardis E, Gordon J. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 2006;444:1027–31.
54. Jia W, Li H, Zhao L, Nicholson JK. Gut microbiota: a potential new territory for drug targeting. Nat Rev Drug Discov. 2008;7(2):123–9.
55. Lindon JC, Nicholson JK, Holmes E, Antti H, Bollard ME, Keun H, et al. Contemporary issues in toxicology the role of metabonomics in toxicology and its evaluation by the COMET project. Toxicol Appl Pharmacol. 2003;187(3):137–46.
56. Clayton TA, Baker D, Lindon JC, Everett JR, Nicholson JK. Pharmacometabonomic identification of a significant host-microbiome metabolic interaction affecting human drug metabolism. Proc Natl Acad Sci U S A. 2009;106(34):14728–33.

57. Cunningham K, Claus SP, Lindon JC, Holmes E, Everett JR, Nicholson JK, et al. Pharmacometabonomic characterization of xenobiotic and endogenous metabolic phenotypes that account for inter-individual variation in isoniazid-induced toxicological response. J Proteome Res. 2012;11:4630–42.
58. Coen M, Goldfain-Blanc F, Rolland-Valognes G, Walther B, Robertson DG, Holmes E, et al. Pharmacometabonomic investigation of dynamic metabolic phenotypes associated with variability in response to galactosamine hepatotoxicity. J Proteome Res. 2012;11(4):2427–40.
59. Holmes E, Loo RL, Stamler J, Bictash M, Yap IK, Chan Q, et al. Human metabolic phenotype diversity and its association with diet and blood pressure. Nature. 2008;453(7193): 396–400.
60. Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, et al. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute scientific statement: executive summary. Crit Pathw Cardiol. 2005;4(4):198–203.
61. Wirfalt E, Hedblad B, Gullberg B, Mattisson I, Andren C, Rosander U, et al. Food patterns and components of the metabolic syndrome in men and women: a cross-sectional study within the Malmo Diet and Cancer cohort. Am J Epidemiol. 2001;154(12):1150–9.
62. Flegal KM, Carroll MD, Kit BK, Ogden CL. Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999–2010. JAMA. 2012;307(5):491–7.
63. Lasserre AM, Chiolero A, Paccaud F, Bovet P. Worldwide trends in childhood obesity. Swiss Med Wkly. 2007;137(9–10):157–8.
64. Chiolero A, Lasserre AM, Paccaud F, Bovet P. Childhood obesity: definition, consequences, and prevalence. Rev Med Suisse. 2007;3(111):1262–9.
65. Donahue RP, Abbott RD. Central obesity and coronary heart disease in men. Lancet. 1987;2(8569):1215.
66. Lapidus L, Bengtsson C, Larsson B, Pennert K, Rybo E, Sjostrom L. Distribution of adipose tissue and risk of cardiovascular disease and death: a 12 year follow up of participants in the population study of women in Gothenburg, Sweden. Br Med J (Clin Res Ed). 1984;289(6454):1257–61.
67. Larsson B, Renstrom P, Svardsudd K, Welin L, Grimby G, Eriksson H, et al. Health and ageing characteristics of highly physically active 65-year-old men. Eur Heart J. 1984;5(Suppl E):31–5.
68. Kalkhoff RK, Hartz AH, Rupley D, Kissebah AH, Kelber S. Relationship of body fat distribution to blood pressure, carbohydrate tolerance, and plasma lipids in healthy obese women. J Lab Clin Med. 1983;102(4):621–7.
69. Hartz AJ, Rupley Jr DC, Kalkhoff RD, Rimm AA. Relationship of obesity to diabetes: influence of obesity level and body fat distribution. Prev Med. 1983;12(2):351–7.
70. Despres JP, Tremblay A, Leblanc C, Bouchard C. Effect of the amount of body fat on the age-associated increase in serum cholesterol. Prev Med. 1988;17(4):423–31.
71. Park SH, Kim BI, Kim SH, Kim HJ, Park DI, Cho YK, et al. Body fat distribution and insulin resistance: beyond obesity in nonalcoholic fatty liver disease among overweight men. J Am Coll Nutr. 2007;26(4):321–6.
72. Folsom AR, Kaye SA, Sellers TA, Hong CP, Cerhan JR, Potter JD, et al. Body fat distribution and 5-year risk of death in older women. JAMA. 1993;269(4):483–7.
73. Sorensen TI, Boutin P, Taylor MA, Larsen LH, Verdich C, Petersen L, et al. Genetic polymorphisms and weight loss in obesity: a randomised trial of hypo-energetic high- versus low-fat diets. PLoS Clin Trials. 2006;1(2):e12.
74. Klannemark M, Orho M, Langin D, Laurell H, Holm C, Reynisdottir S, et al. The putative role of the hormone-sensitive lipase gene in the pathogenesis of Type II diabetes mellitus and abdominal obesity. Diabetologia. 1998;41(12):1516–22.
75. Teran-Garcia M, Bouchard C. Genetics of the metabolic syndrome. Appl Physiol Nutr Metab. 2007;32(1):89–114.

76. Ley R, Turnbaugh P, Klein S, Gordon J. Microbial ecology: human gut microbes associated with obesity. Nature. 2006;444(7122):1022–3.
77. Wildman RP, Muntner P, Reynolds K, McGinn AP, Rajpathak S, Wylie-Rosett J, et al. The obese without cardiometabolic risk factor clustering and the normal weight with cardiometabolic risk factor clustering: prevalence and correlates of 2 phenotypes among the US population (NHANES 1999–2004). Arch Intern Med. 2008;168(15):1617–24.
78. Smith Jr SC, Haslam D. Abdominal obesity, waist circumference and cardio-metabolic risk: awareness among primary care physicians, the general population and patients at risk–the Shape of the Nations survey. Curr Med Res Opin. 2007;23(1):29–47.
79. Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF, et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab. 2009;9(4):311–26.
80. Suhre K, Meisinger C, Doring A, Altmaier E, Belcredi P, Gieger C, et al. Metabolic footprint of diabetes: a multiplatform metabolomics study in an epidemiological setting. PLoS One. 2010;5(11):e13953.
81. Huffman KM, Shah SH, Stevens RD, Bain JR, Muehlbauer M, Slentz CA, et al. Relationships between circulating metabolic intermediates and insulin action in overweight to obese, inactive men and women. Diabetes Care. 2009;32(9):1678–83.
82. Fiehn O, Garvey WT, Newman JW, Lok KH, Hoppel CL, Adams SH. Plasma metabolomic profiles reflective of glucose homeostasis in non-diabetic and type 2 diabetic obese African-American women. PLoS One. 2010;5(12):e15234.
83. Boulange CL, Claus SP, Chou CJ, Collino S, Montoliu I, Kochhar S, et al. Early metabolic adaptation in C57BL/6 mice resistant to high fat diet induced weight gain involves an activation of mitochondrial oxidative pathways. J Proteome Res. 2013;12:1956–68.
84. Rhee EP, Cheng S, Larson MG, Walford GA, Lewis GD, McCabe E, et al. Lipid profiling identifies a triacylglycerol signature of insulin resistance and improves diabetes prediction in humans. J Clin Invest. 2011;121(4):1402–11.
85. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, et al. Metabolite profiles and the risk of developing diabetes. Nat Med. 2011;17(4):448–53.
86. Yamakado M, Tanaka T, Nagao K, Ishizaka Y, Mitushima T, Tani M, et al. Plasma amino acid profile is associated with visceral fat accumulation in obese Japanese subjects. Clin Obes. 2012;2(1–2):29–40.
87. Floegel A, Stefan N, Yu Z, Muhlenbruch K, Drogan D, Joost HG. et al. Identification of serum metabolites associated with risk of type 2 diabetes using a targeted metabolomic approach. Diabetes. 2013;62(2):639–48.
88. Snyder F. Platelet-activating factor and its analogs: metabolic pathways and related intracellular processes. Biochim Biophys Acta. 1995;1254(3):231–49.
89. McIntyre TM, Prescott SM, Stafforini DM. The emerging roles of PAF acetylhydrolase. J Lipid Res. 2009;50(Suppl):S255–9.
90. Davi G, Guagnano MT, Ciabattoni G, Basili S, Falco A, Marinopiccoli M, et al. Platelet activation in obese women: role of inflammation and oxidant stress. JAMA. 2002;288(16):2008–14.
91. Basili S, Pacini G, Guagnano MT, Manigrasso MR, Santilli F, Pettinella C, et al. Insulin resistance as a determinant of platelet activation in obese women. J Am Coll Cardiol. 2006;48(12):2531–8.
92. Fabbrini E, Sullivan S, Klein S. Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. Hepatology. 2010;51(2):679–89.
93. Johnson NA, Walton DW, Sachinwalla T, Thompson CH, Smith K, Ruell PA, et al. Noninvasive assessment of hepatic lipid composition: advancing understanding and management of fatty liver disorders. Hepatology. 2008;47(5):1513–23.
94. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature. 2011;472(7341):57–63.

95. Buchwald H, Avidor Y, Braunwald E, Jensen MD, Pories W, Fahrbach K, et al. Bariatric surgery: a systematic review and meta-analysis. JAMA. 2004;292(14):1724–37.
96. Seyfried F, Li JV, Miras AD, Cluny NL, Lannoo M, Fenske WK, et al. Urinary phenotyping indicates weight loss-independent metabolic effects of Roux-en-Y gastric bypass in mice. J Proteome Res. 2013;12(3):1245–53.
97. Tremaroli V, Backhed F. Functional interactions between the gut microbiota and host metabolism. Nature. 2012;489(7415):242–9.
98. Holmes E, Kinross J, Gibson GR, Burcelin R, Jia W, Pettersson S, et al. Therapeutic modulation of microbiota-host metabolic interactions. Sci Transl Med. 2012;4(137):137rv6.
99. Collino S, Martin FP, Rezzi S. Clinical metabolomics paves the way towards future healthcare strategies. Br J Clin Pharmacol. 2013;75(3):619–29.
100. Martin FP, Collino S, Rezzi S. 1H NMR-based metabonomic applications to decipher gut microbial metabolic influence on mammalian health. Magn Reson Chem. 2011;49 Suppl 1:S47–54.
101. Parker BA, Chapman IM. Food intake and ageing–the role of the gut. Mech Ageing Dev. 2004;125(12):859–66.
102. Harper PH, Truelove SC, Lee EC, Kettlewell MG, Jewell DP. Split ileostomy and ileocolostomy for Crohn's disease of the colon and ulcerative colitis: a 20 year survey. Gut. 1983;24(2):106–13.
103. Duncan M, Davison JS, Sharkey KA. Review article: endocannabinoids and their receptors in the enteric nervous system. Aliment Pharmacol Ther. 2005;22(8):667–83.
104. Actis GC, Pazienza P, Rosina F. Mesalamine for inflammatory bowel disease: recent reappraisals. Inflamm Allergy Drug Targets. 2008;7(1):1–5.
105. Winterkamp S, Weidenhiller M, Otte P, Stolper J, Schwab D, Hahn EG, et al. Urinary excretion of N-methylhistamine as a marker of disease activity in inflammatory bowel disease. Am J Gastroenterol. 2002;97(12):3071–7.
106. Baur P, Martin FP, Gruber L, Bosco N, Brahmbhatt V, Collino S, et al. Metabolic phenotyping of the Crohn's disease-like IBD etiopathology in the TNF(DeltaARE/WT) mouse model. J Proteome Res. 2011;10(12):5523–35.
107. Marchesi JR, Holmes E, Khan F, Kochhar S, Scanlan P, Shanahan F, et al. Rapid and noninvasive metabonomic characterization of inflammatory bowel disease. J Proteome Res. 2007;6(2):546–51.
108. Le GG, Noor SO, Ridgway K, Scovell L, Jamieson C, Johnson IT, et al. Metabolomics of fecal extracts detects altered metabolic activity of gut microbiota in ulcerative colitis and irritable bowel syndrome. J Proteome Res. 2011;10(9):4208–18.
109. Rezzi S, Martin FP, Alonso C, Guilarte M, Vicario M, Ramos L, et al. Metabotyping of biofluids reveals stress-based differences in gut permeability in healthy individuals. J Proteome Res. 2009;8(10):4799–809.
110. Brugnara L, Vinaixa M, Murillo S, Samino S, Rodriguez MA, Beltran A, et al. Metabolomics approach for analyzing the effects of exercise in subjects with type 1 diabetes mellitus. PLoS One. 2012;7(7):e40600.
111. Rezzi S, Martin FP, Kochhar S. Defining personal nutrition and metabolic health through metabonomics. Ernst Schering Found Symp Proc. 2007;4:251–64.
112. Montoliu I, Martin FP, Collino S, Rezzi S, Kochhar S. Multivariate modeling strategy for intercompartmental analysis of tissue and plasma 1H NMR spectrotypes. J Proteome Res. 2009;8(5):2397–406.
113. Duarte NC, Becker SA, Jamshidi N, Thiele I, Mo ML, Vo TD, et al. Global reconstruction of the human metabolic network based on genomic and bibliomic data. Proc Natl Acad Sci U S A. 2007;104(6):1777–82.
114. Shlomi T, Cabili MN, Herrgard MJ, Palsson BO, Ruppin E. Network-based prediction of human tissue-specific metabolism. Nat Biotechnol. 2008;26(9):1003–10.
115. Jerby L, Shlomi T, Ruppin E. Computational reconstruction of tissue-specific metabolic models: application to human liver metabolism. Mol Syst Biol. 2010;6:401.

116. Karlsson FH, Nookaew I, Petranovic D, Nielsen J. Prospects for systems biology and modeling of the gut microbiome. Trends Biotechnol. 2011;29(6):251–8.
117. Bernini P, Bertini I, Luchinat C, Nepi S, Saccenti E, Schafer H, et al. Individual human phenotypes in metabolic space and time. J Proteome Res. 2009;8(9):4264–71.
118. Assfalg M, Bertini I, Colangiuli D, Luchinat C, Schafer H, Schutz B, et al. Evidence of different metabolic phenotypes in humans. Proc Natl Acad Sci U S A. 2008;105(5):1420–4.
119. Moco S, Collino S, Rezzi S, Martin FP. Metabolomics perspectives in pediatric research. Pediatr Res. 2013;73(4 Pt 2):570–6.
120. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464(7285):59–65.
121. Franceschi C, Capri M, Monti D, Giunta S, Olivieri F, Sevini F, et al. Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans. Mech Ageing Dev. 2007;128(1):92–105.
122. Xu J, Gordon JI. Inaugural article: honor thy symbionts. Proc Natl Acad Sci U S A. 2003;100(18):10452–9.
123. Xu J, Mahowald MA, Ley RE, Lozupone CA, Hamady M, Martens EC, et al. Evolution of symbiotic bacteria in the distal human intestine. PLoS Biol. 2007;5(7):e156.
124. Beale B. Probiotics: their tiny worlds are under scrutiny. Scientist. 2002;16(15):20–2.
125. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal microbial flora. Science. 2005;308(5728):1635–8.
126. Ouwehand AC. Antiallergic effects of probiotics. J Nutr. 2007;137(3 Suppl 2):794S–7.
127. Salminen SJ, Gueimonde M, Isolauri E. Probiotics that modify disease risk. J Nutr. 2005;135(5):1294–8.
128. Blum S, Schiffrin EJ. Intestinal microflora and homeostasis of the mucosal immune response: implications for probiotic bacteria? Curr Issues Intest Microbiol. 2003;4(2):53–60.
129. Gavaghan McKee CL, Wilson ID, Nicholson JK. Metabolic phenotyping of nude and normal (Alpk:ApfCD, C57BL10J) mice. J Proteome Res. 2006;5(2):378–84.
130. Holmes E, Nicholson J. Variation in gut microbiota strongly influences individual rodent phenotypes. Toxicol Sci. 2005;87(1):1–2.
131. Martin FP, Wang Y, Yap IK, Sprenger N, Lindon JC, Rezzi S, et al. Topographical variation in murine intestinal metabolic profiles in relation to microbiome speciation and functional ecological activity. J Proteome Res. 2009;8(7):3464–74.
132. Martin FP, Montoliu I, Kochhar S, Rezzi S. Chemometric strategy for modeling metabolic biological space along the gastrointestinal tract and assessing microbial influences. Anal Chem. 2010;82(23):9803–11.
133. Wang Y, Holmes E, Comelli EM, Fotopoulos G, Dorta G, Tang H, et al. Topographical variation in metabolic signatures of human gastrointestinal biopsies revealed by high-resolution magic-angle spinning (1)H NMR spectroscopy. J Proteome Res. 2007;6(10):3944–51.
134. Wang Y, Tang H, Holmes E, Lindon JC, Turini ME, Sprenger N, et al. Biochemical characterization of rat intestine development using high-resolution magic-angle-spinning ^{1}H NMR spectroscopy and multivariate data analysis. J Proteome Res. 2005;4(4):1324–9.
135. Martin FP, Wang Y, Sprenger N, Holmes E, Lindon JC, Kochhar S, et al. Effects of probiotic *Lactobacillus paracasei* treatment on the host gut tissue metabolic profiles probed via magic-angle-spinning NMR spectroscopy. J Proteome Res. 2007;6(4):1471–81.
136. Alonso C, Guilarte M, Vicario M, Ramos L, Rezzi S, Martinez C, et al. Acute experimental stress evokes a differential gender-determined increase in human intestinal macromolecular permeability. Neurogastroenterol Motil. 2012;24(8):740-e349.
137. Martin FP, Sprenger N, Yap IK, Wang Y, Bibiloni R, Rochat F, et al. Panorganismal gut microbiome-host metabolic crosstalk. J Proteome Res. 2009;8(4):2090–105.
138. Martin FP, Sprenger N, Montoliu I, Rezzi S, Kochhar S, Nicholson JK. Dietary modulation of gut functional ecology studied by fecal metabonomics. J Proteome Res. 2010;9(10): 5284–95.

139. Tuohy KM, Gougoulias C, Shen Q, Walton G, Fava F, Ramnani P. Studying the human gut microbiota in the trans-omics era–focus on metagenomics and metabonomics. Curr Pharm Des. 2009;15(13):1415–27.
140. Cummings JH, Macfarlane GT. The control and consequences of bacterial fermentation in the human colon. J Appl Bacteriol. 1991;70(6):443–59.
141. Woodmansey EJ, McMurdo ME, Macfarlane GT, Macfarlane S. Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects. Appl Environ Microbiol. 2004;70(10):6113–22.
142. Coldham NG, Darby C, Hows M, King LJ, Zhang AQ, Sauer MJ. Comparative metabolism of genistin by human and rat gut microflora: detection and identification of the end-products of metabolism. Xenobiotica. 2002;32(1):45–62.
143. Gonthier MP, Verny MA, Besson C, Remesy C, Scalbert A. Chlorogenic acid bioavailability largely depends on its metabolism by the gut microflora in rats. J Nutr. 2003;133(6): 1853–9.
144. Saric J, Wang Y, Li J, Coen M, Utzinger J, Marchesi JR, et al. Species variation in the fecal metabolome gives insight into differential gastrointestinal function. J Proteome Res. 2008;7(1):352–60.
145. Beckonert O, Coen M, Keun HC, Wang Y, Ebbels TM, Holmes E, et al. High-resolution magic-angle-spinning NMR spectroscopy for metabolic profiling of intact tissues. Nat Protoc. 2010;5(6):1019–32.
146. Wilson ID, Nicholson JK. The role of gut microbiota in drug response. Curr Pharm Des. 2009;15(13):1519–23.
147. Wei H, Dong L, Wang T, Zhang M, Hua W, Zhang C, et al. Structural shifts of gut microbiota as surrogate endpoints for monitoring host health changes induced by carcinogen exposure. FEMS Microbiol Ecol. 2010;73(3):577–86.
148. Staggers JE, Frost SC, Wells MA. Studies on fat digestion, absorption, and transport in the suckling rat. III. Composition of bile and evidence for enterohepatic circulation of bile salts. J Lipid Res. 1982;23(8):1143–51.
149. Claus SP, Ellero SL, Berger B, Krause L, Bruttin A, Molina J, et al. Colonization-induced host-gut microbial metabolic interaction. MBio. 2011;2(2):e00271–10.
150. Mestdagh R, Dumas ME, Rezzi S, Kochhar S, Holmes E, Claus SP, et al. Gut microbiota modulate the metabolism of brown adipose tissue. J Proteome Res. 2011;11(2):620–30.
151. Merrifield CA, Lewis M, Claus SP, Beckonert OP, Dumas ME, Duncker S, et al. A metabolic system-wide characterisation of the pig: a model for human physiology. Mol Biosyst. 2011;7(9):2577–88.
152. Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, Peters EC, et al. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proc Natl Acad Sci U S A. 2009;106(10):3698–703.
153. Claus SP, Tsang TM, Wang Y, Cloarec O, Skordi E, Martin FP, et al. Systemic multicompartmental effects of the gut microbiome on mouse metabolic phenotypes. Mol Syst Biol. 2008;4:219.
154. Alonso C, Guilarte M, Vicario M, Ramos L, Ramadan Z, Antolin M, et al. Maladaptive intestinal epithelial responses to life stress may predispose healthy women to gut mucosal inflammation. Gastroenterology. 2008;135(1):163–72.
155. Rezzi S, Ramadan Z, Martin FP, Fay LB, Bladeren PV, Lindon JC, et al. Human metabolic phenotypes link directly to specific dietary preferences in healthy individuals. J Proteome Res. 2007;6(11):4469–77.
156. Stella C, Beckwith-Hall B, Cloarec O, Holmes E, Lindon JC, Powell J, et al. Susceptibility of human metabolic phenotypes to dietary modulation. J Proteome Res. 2006;5(10):2780–8.
157. Guarner F, Malagelada JR. Gut flora in health and disease. Lancet. 2003;361(9356):512–9.
158. Madden JA, Hunter JO. A review of the role of the gut microflora in irritable bowel syndrome and the effects of probiotics. Br J Nutr. 2002;88 Suppl 1:S67–72.

159. Bickston SJ, Comerford LW, Cominelli F. Future therapies for inflammatory bowel disease. Curr Gastroenterol Rep. 2003;5(6):518–23.
160. Vincenti M, Behrends M, Dang K, Park YH, Hirose R, Blasi-Ibanez A, et al. Induction of intestinal ischemia reperfusion injury by portal vein outflow occlusion in rats. J Gastroenterol. 2010;45(11):1103–10.
161. Bjerrum JT, Nielsen OH, Hao F, Tang H, Nicholson JK, Wang Y, et al. Metabonomics in ulcerative colitis: diagnostics, biomarker identification, and insight into the pathophysiology. J Proteome Res. 2010;9(2):954–62.
162. Martin FP, Rezzi S, Philippe D, Tornier L, Messlik A, Holzlwimmer G, et al. Metabolic assessment of gradual development of moderate experimental colitis in IL-10 deficient mice. J Proteome Res. 2009;8(5):2376–87.
163. Murdoch TB, Fu H, Macfarlane S, Sydora BC, Fedorak RN, Slupsky CM. Urinary metabolic profiles of inflammatory bowel disease in interleukin-10 gene-deficient mice. Anal Chem. 2008;80(14):5524–31.
164. Bertini I, Calabro A, De Carli V, Luchinat C, Nepi S, Porfirio B, et al. The metabonomic signature of celiac disease. J Proteome Res. 2009;8(1):170–7.
165. Tjellstrom B, Stenhammar L, Hogberg L, Falth-Magnusson K, Magnusson KE, Midtvedt T, et al. Gut microflora associated characteristics in children with celiac disease. Am J Gastroenterol. 2005;100(12):2784–8.
166. Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, et al. Intestinal microbiota metabolism of l-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat Med. 2013;19(5):576–85.
167. Tang WH, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, et al. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med. 2013;368(17):1575–84.
168. Nicholson G, Rantalainen M, Maher AD, Li JV, Malmodin D, Ahmadi KR, et al. Human metabolic profiles are stably controlled by genetic and environmental variation. Mol Syst Biol. 2011;7:525.
169. Payne AN, Zihler A, Chassard C, Lacroix C. Advances and perspectives in in vitro human gut fermentation modeling. Trends Biotechnol. 2012;30(1):17–25.

Chapter 2
Metabonomics in Clinical Practice

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Abstract Metabonomics is recognized as a powerful top-down system biology approach to understand genetic-environment-health paradigm paving new avenues to identify clinically relevant biomarkers. It is nowadays commonly used in clinical applications shedding new light on physiological regulatory processes of complex mammalian systems as regards disease etiology, diagnostic stratification, and potentially mechanism of action of therapeutic solutions. It therefore offers opportunities to associate complex metabolic regulations to the etiology of multifactorial diseases and metabolic dysfunctions, which may subsequently lead to mechanistic hypotheses and targets for new nutritional concepts. This review aims at describing recent applications of metabonomics in clinical fields with insight into diseases diagnostics/monitoring and improvement of homeostasis metabolic regulation.

Keywords Aging • Biomarkers • Cancer • Metabonomics • Lipidomics • Metabolic syndrome • Mental health

2.1 Introduction

Nutritional research has shifted from the measurements of a few, but key, physiological descriptors to a large-scale screening of molecular processes at different levels of biological organization, from gene to mRNA, to proteins and enzymes, and to metabolic pathways [1]. Integration of this biological information is a vital factor for nutritional research, as population and individual physiological features are not only reflected in protein concentrations and gene expression but also in metabolite

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concentrations and their kinetic changes in cells, tissues, and organs. However, studies at individual and population scale are often a complex task because biological processes are under the influence of numerous intrinsic and extrinsic factors such as environmental agents, drugs, diet, lifestyle, stress, and microbiome modulations [2, 3].

Recent revolutions in omics technologies are promising today substantial capability in achieving a global systems view of physiological and pathological processes [4]. Their application is driving a rapid shift from the clinic style investigations to much more large population-based studies, aiming at enhancing our understanding of the role of genetics, environmental factors, and their interactions on individual susceptibility to disease and health [5, 6]. Therefore, one of the most powerful strategies for deciphering gene-environment interactions and their role in individual variability, to pathology and nutritional outcome, lies in the ability to combine data from different omics technologies, establishing the way for system biology approaches [7, 8].

2.2 State-of-the-Art Metabonomics and Lipidomics Technologies

Metabonomics is able to generate multivariate information on a wide range of molecules and provides the ability to measure subtle changes in biological processes as a result of different nutritional effects [2, 9, 10]. Based on the study of metabolic profiles of biofluids from a complex biological system, over the past decade, metabonomics appeared rapidly as a powerful technology to diagnose and identify biomarkers for a medical condition. Metabonomics measures and monitors metabolite concentrations and dynamics in cells, tissues, and multicompartmental biological systems, revealing not only the end products of enzyme expression and activity but as well the ultimate information contained in the genetic code [11–13]. Because specific physiological states, gene expression, and environmental stressors can cause changes in the steady state of a biological system, monitoring the resulting metabolic variations provides a unique insight into intra- and extra-cellular regulatory processes involved in maintaining homeostasis. Therefore, the identification of specific metabolic fingerprints vows strong potentials for nutriceutical and therapeutic surveillance. Nowadays, metabonomics applications have evolved towards deciphering the cellular and molecular processes in response to different individual dietary modulations, predicting health and disease outcomes. By the global study of low molecular weight compounds (<1,500 Da) in biofluids (plasma/serum and urine), complex biological matrixes, and tissues, it assures the characterization of individual metabolic phenotypes, or metabotypes. Metabonomics employs mainly two analytical techniques based on ^{1}H nuclear magnetic resonance (NMR) and mass spectrometry coupled to gas/high performance liquid chromatography (GC-MS and LC-MS) with lately the addition of ultrahigh performance liquid chromatography systems coupled to mass

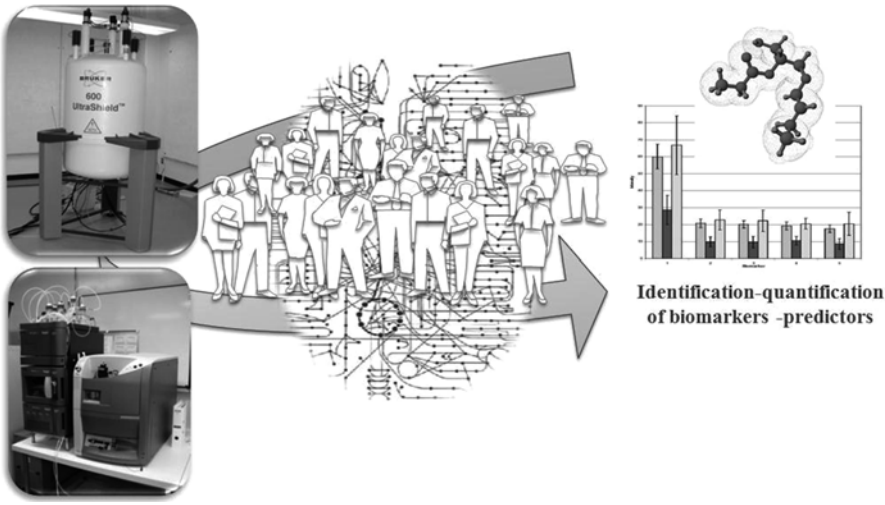

Fig. 2.1 Metabonomics as a tool to probe human metabolism and health. By using NMR- and MS-based metabonomics, biomarkers of disease, nutrition, and general development can be assessed in a qualitative and quantitative way

spectrometry (UPLC/MS) (Fig. 2.1). Both methods are then comprehensively used to generate multivariate datasets which are then employed, in coordination with advance statistical tools, to recover meaningful biological information from the complex metabolic profiles.

NMR-based metabonomics provides efficient high-throughput analysis of biological samples, making it a relatively cost-effective approach. NMR spectroscopy offers the unique prospect to holistically profile hundreds of metabolites with no a priori selection. In proton NMR spectroscopy (^{1}H NMR), all covalently attached protons from mobile molecules within a very high dynamic range of concentrations, i.e., from millimolar to nanomolar range, are simultaneously scanned, thus providing a biochemical fingerprint of biological sample. ^{1}H NMR-based metabonomics is generally preferred to other nuclei-like carbon-13 due to highest sensitivity and the relative short experimental time needed to acquire metabolic profiles. However, resonances of metabolites may be highly overlapped within the proton resonance window. In such case, ultrahigh magnetic field and/or two-dimensional (2D) NMR spectroscopy can be used to resolve overlapped resonances.

Urine and blood plasma are the most commonly used biofluids for metabonomics studies due to their intrinsic richness in metabolic information and their relatively easy and noninvasive access. Detailed procedures to collect, store, and prepare biofluids or tissue samples for NMR analysis have been provided as guidelines for metabonomics [14]. Urine, serum, and plasma usually require minimal pretreatment such as the addition of sodium azide to control bacterial growth, phosphate buffer to control pH-induced shift in resonance, and deuterated water to lock the magnetic field, TSP (3-(trimethylsilyl)-propionate, sodium salt), and DSS (2,2-dimethyl-2-silapentane-5-sulfonate, sodium salt) for chemical shift calibration. Recent introduction of

cryoprobes has strongly improved NMR sensitivity to generally a fourfold factor relatively to conventional room temperature probes [15]. Furthermore, NMR spectroscopy, using high-resolution magic angle spinning NMR (HR-MAS) [16], offers a unique prospect to generate metabolic profiles from intact tissues, ensuring thus the biological integrity of the investigated sample. One of the key features of NMR remains definitely the robustness as showed, i.e., reproducibility >98 % [17].

Along with NMR, LC-MS is probably the most widely used technique in metabonomics. Compared to NMR, LC-MS allows the detection of a wide range of metabolites, reaching higher sensitivity levels. Essentially two different approaches have been developed: the detection of all instrumentally possible metabolites and detection of specific classes of metabolites, named targeted and untargeted methods, respectively. The detection of metabolites by LC-MS is firstly obtained with optimization of the sample preparation, chromatographic separation, and ionization. The sample preparation in metabonomics is perhaps the most underestimated step and is crucial as the first selection of the aimed metabolite classes is according to their chemical properties. Typically samples are quenched and then extracted, often by liquid extraction with organic solvents or solid-phase extraction, so that eventual enzymatic activity is stopped and the conservation of the metabolite pool is insured [18, 19]. The integrity and recovery of the metabonome is an important parameter, as well as repeatability, so that comparison between samples is made possible in a robust way. Sample preparation strategies have been described for biofluids [19, 20]), tissues [21], and cells [22, 23]. Similarly to GC-MS, the use of internal and external standards, such as non-endogenous compounds or isotopically labeled species, is recommended whenever possible for better compensation of liabilities during sample preparation [24, 25]. The ionization of analytes prior to MS analysis is a prerequisite for the detection of metabolites. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), which are chemical ionization techniques, are the most used in LC-MS. The chemistry involved in ionization is complex and strongly depends on the characteristics of the solvents and additives (volatility, surface tension, viscosity, conductivity, ionic strength, dielectric constant, electrolyte concentration, pH, and gas-phase ion-molecule reactions), analyte (acid dissociation constant, hydrophobicity, surface activity, ion salvation energy, proton affinity), and operational parameters such as flow rate, temperature, and ESI voltage [26]. Nordström and co-workers [27] have applied a multiple ionization strategy, using ESI and APCI, in both positive and negative ionization modes, matrix-assisted laser desorption ionization (MALDI), and desorption ionization on silicon (DIOS) for the analysis of human serum. The combination of ionization techniques maximizes the coverage of measured metabolite classes. The variety of hardwares in mass spectrometry offers a wide-range technical solution for metabonomics. Some of these include quadrupole (Q), time of flight (TOF), orbitrap, ion trap (IT), ion cyclotron resonance (ICR), and combinations (such as QQQ, QTOF, TOF-TOF, IT-orbitrap). Typically in targeted methods, QQQ-MS or QTrap-MS is used, allowing the optimization of parameters for each

selected ion pairs (typically molecular ion and abundant fragment) or transitions called selected reaction monitoring (SRM) or multiple reactions monitoring (MRM) can be used for quantitative purposes. IT allows the isolation of ions for fragmentation up to *n*th time (with n being the number of collision events), while TOFs, ICRs, and orbitraps provide very high mass accuracy and resolution which can be useful for metabolite identification purposes. Plasma has been analyzed by untargeted GC-TOF-MS [28] leading to the detection of 46 endogenous metabolites. Using a 12T Fourier transform ICR-MS, about 570 distinct metabolite features, represented by monoisotopic masses above signal-to-noise ratio 3 within the mass range m/z 90–570, were detected in murine plasma.

The central carbon metabolism, including glycolysis, pentose phosphate pathway, tricarboxylic acid cycle (TCA cycle), and surrounding metabolic reactions, contains mostly polar compounds such as sugars, sugar phosphates, and organic and amino acids and has been covered by GC-MS after derivatization [28], ion pairing LC-MS [24], hydrophobic interaction LC-MS [29], and CE-MS [30].

In addition, a comprehensive analysis of biological lipids can be performed using MS-based lipidomics. A number of analytical approaches can be deployed to access the lipid inventory of a given biological matrix [31]. Recent advances in LC-MS technologies make this field a promising area of lipid biochemical research [32]. Although such approaches are reasonably well established for high-throughput analysis of the major lipid classes (phospholipids, sphingolipids) [33–36], they still have to be fine-tuned for the quantification of low abundant signaling lipids such as sphingosine, sohingosine-1-phosphate, or lysophosphatidic acid [37–39]. Standardization of metabonomics practices has been proposed by the Chemical Analysis Working Group from the metabonomics standards initiative where a general consensus concerning the minimum reporting standards for metabonomics experiments has been described [40]. Such a position document sets the basis for better intra- and interlaboratory reproducibility. The comparison between LC-MS urinary profiles obtained in three different labs has shown a reproducibility of over 0.95 (coefficient of determination) [41]. The compliance to quality controls, signal intensity checks, and post-analysis signal drift corrections is some of the solutions to warrant high-quality LC-MS datasets [42].

Yet, one of the bottlenecks in metabonomics remains the identification of metabolites in particular biological matrices. Traditionally, full identification of molecules is a laborious procedure, including enrichment, isolation, purification, and full characterization by several analytical strategies, to reach unambiguous identification. MS offers valuable pieces of information in the identification of metabolites: molecular mass and molecular fragmentation pattern, taken from MS and MS/MS fragmentation, respectively. In combination with LC, a relative polarity index can be obtained, according to the used stationary phase. Often, efficient identification relies with the use of a combination of analytic techniques such as NMR and MS and access to biochemical database and computational techniques (data preprocessing, statistical modeling, data mining) [43–49].

2.3 Metabonomics Flow Chart in Epidemiological Studies

Epidemiological studies aim to substantiate the causes, distribution, and control of health and diseases in populations. They integrate scientific information derived from complementary disciplines (e.g., microbiology, genetics) across a large-scale population to assess etiological hypotheses and provide the basis for developing and evaluating health management solutions [50]. Typically, epidemiological studies deal with a large number of subjects and observations, relying on statistics to establish validity levels and predict risk factors. Metabonomics has been recently used as an analytical tool in large epidemiological studies assessing several public health challenges, including cardiovascular disease [51], diabetes [52], schizophrenia [53], and aging [54]. On one hand, having access to a large number of samples is essential to validate health or disease trends, at a population level. On the other hand, the demand in organization, compliance to ethic procedures, bookkeeping, and standardized sampling procedures is imperative to carry out such a study. Therefore, this type of studies is undertaken under the control of dedicated study centers, where rigorous standardized procedures, under safety precautions, can be applied [52]. Epidemiological studies can produce thousands of samples and involve different centers, where different groups work as a consortium. In order to make sure all sample information is recorded, and traced, sample management becomes an important part of the process, dealing with sample identification, sample labeling, storage and access to analytical data, sample history, etc. Normally unique identifiers should be given to each sample (e.g., sample barcodes), and all information should be stored in established databases [55].

Preferably, samples are collected in dedicated centers under defined protocols and upon ethical approval. According to the type of sample to collect and analysis to follow, different procedures are applied [56]. Urine is obtained by noninvasive means which is an advantage, as subjects are more prone to participate in the study, and it allows the access to otherwise difficult/impossible studies. Typically, urine can be collected in random samples (any time of the day), timed samples (at a specific time in the day or at x hours after a specific intervention), and 24-h samples (pooling of urine during 24 h). The last allows compensating for large variability owed to short collection times. Obtaining blood plasma is more invasive, but remains largely applicable in the pediatric, child, and elderly fields, mainly through technological development requiring low sample volumes. For instance, successful analyses can be conducted on blood serum, plasma, or even blood spot (collected using Gutri paper or capillaries in combination to finger prick). The analysis of stool samples is also widely generalized and provides information on digestive and gut microbial metabolism, but suffers of the individual acceptance in providing samples from children and adults. In the context of disease diagnostics requiring biopsies analyses, metabonomics is increasingly envisioned as a new way to investigate the physiological integrity of tissue and personalize healthcare. Under these conditions, the collection of tissue samples will be conducted according to best clinical practice and ensuring sample biochemical integrity prior to analysis. Finally, metabonomics

analysis is also applicable to food itself to identify major nutritional constituents and/or particular metabolites showing a significant bioactivity, including milk, cereals, wine, fruits, vegetables, and meat.

Integrity of biological samples is of utmost importance when the aim of the analysis is to obtain a reflex of the metabolism taking place. Alteration of integrity of samples due to long periods at room temperature is sample dependent and should be avoided, as potentially leading to an unreal representation of physiological status. Samples should be therefore stored at −80 °C whenever possible to allow a maximum storage time [57]. In fact, the cold chain from sample collection to sample storage should be preserved, especially when home collection is involved. Samples should be collected in sterile containers and sodium azide should be added to prevent bacterial growth whenever possible. In conclusion, when dealing with a large number of samples, as in epidemiological studies, planning and automation of procedures is imperative, including study design, sample collection and storage, metabonomics analysis, data analysis, and storage and retrieval of metadata (Fig. 2.2).

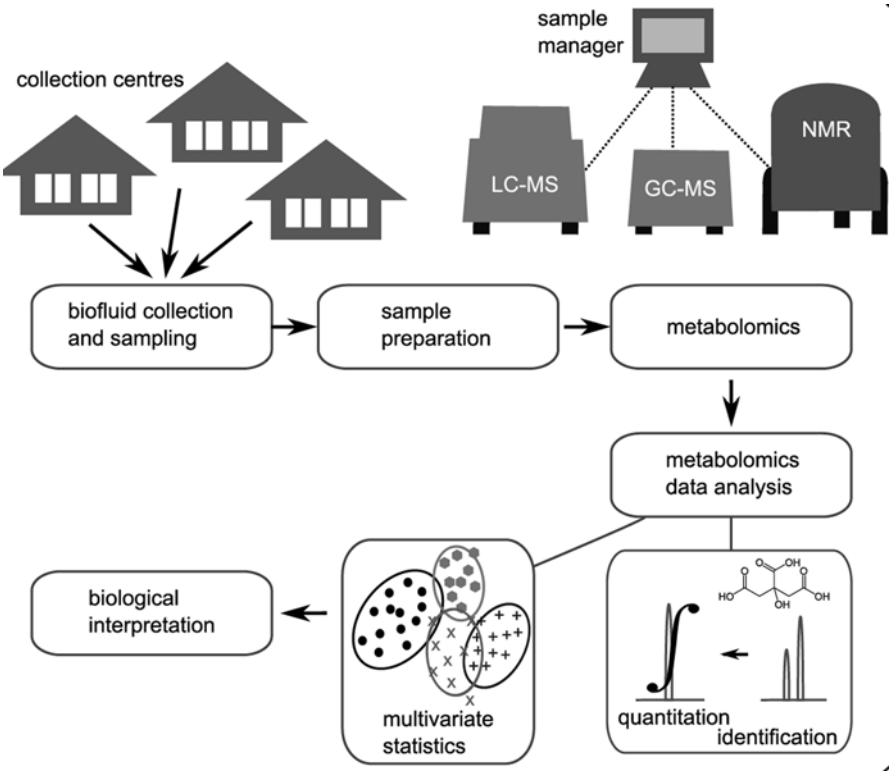

Fig. 2.2 Flowchart for epidemiological metabonomics studies. Planning and automation of procedures is crucial, including study design, as sample collection and storage, data analysis, and retrieval of metadata

2.4 Clinical Applications of Metabonomics for Aging Biomarkers

Decades of research on aging have found hundreds of genes [58, 59] and many biological processes [60–62] that are associated to the aging process, but up to date, metabonomics applications covering aging as a multifactorial event are sparse [63]. In particular, the frailty syndrome is increasingly being considered as a risk indicator of adverse health outcomes. Elderly may be also prone to be resistant to anabolic stimuli which is likely a key factor in the loss of skeletal muscle mass with aging. Vital to understand these biological processes is the development of biological markers, through system biology approaches, aiding at strategies for tailored therapeutic and personalized nutritional program. The overall aim is to prevent or attenuate decline of key physiological functions required to live an active and independent life. Therefore, it is crucial to develop core indicators (biomarkers) of health and function in older adults, where nutrition and tailored personalized programs could exhibit preventive roles and where the aid of omics technologies is increasingly displaying potential in revealing key molecular mechanisms/targets linked to specific aging and/or healthy aging processes (Fig. 2.3). In one of the first aging study, Lawton et al. analyzed the human plasma metabonome in 269 individuals (both men and women, 20–65 years old) identifying significant changes in relative concentrations of more than 100 metabolites [64]. Here changes in protein, energy, oxidative stress, and lipid metabolism were observed with increasing age. In addition, certain xenobiotics (i.e., caffeine) were higher in older subjects, displaying possibly decreased hepatic cytochrome P540 activity. Further, Nikkila and colleagues performed a metabonomics study on early childhood, following 59 children from birth to an age of 4 years old and identified that major developmental state differences between girls and boys are attributed to sphingolipids metabolism [65]. Moreover, comparison of longitudinal metabolic trajectories between boys and girls revealed

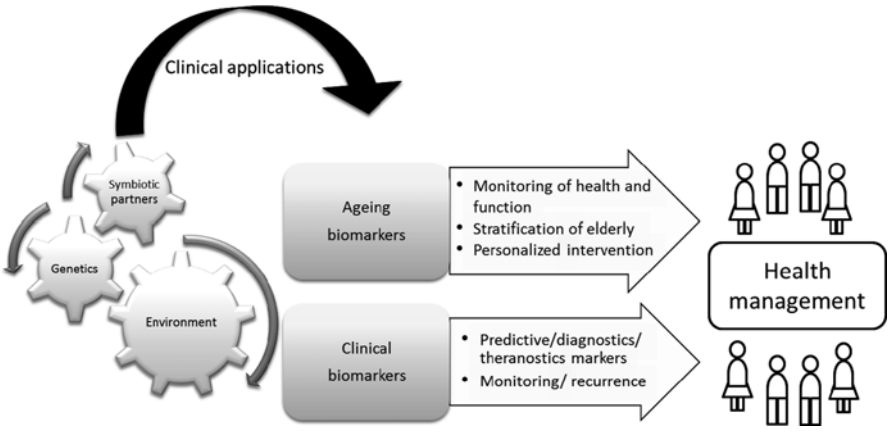

Fig. 2.3 Selected biomarker applications for aging and clinical research

higher levels of sphingomyelins in girls than in boys. More recently Zhonghao et al. characterized the metabolic profile of a large group of subjects with a wide age range (32–81 years) and identified metabolites related to chronological age, independent of BMI [54]. Here, two population-based studies were used: a German aging population as a discovery cohort, with 1,038 female and 1,124 male participants (32–81 years), and a UK study as replication, with 724 female participants. Targeted metabonomics of fasting serum samples quantified 131 metabolites. Among these, 71 out of 34 metabolites were significantly associated with age in women/men reflecting mostly incomplete mitochondrial fatty acid oxidation (elevated serum levels of acylcarnitines).

Overall, while the identification of biological markers specific of aging is still on its infancy, their characterization is crucial in providing insights into mechanisms or strategies that can prevent or reverse the decline in certain of the affected networks and as such could extend health span, preventing accelerated aging.

2.5 Clinical Application of Metabonomics for Biomarkers of Metabolic Syndrome

The continuously increasing prevalence of obesity in many countries around the world is strongly linked to the projected pandemic of type 2 diabetes (T2D) and its cardiovascular complications [66, 67]. However, there are many individuals under the same obesogenic and diabetogenic environments who remain metabolically healthy. Newgard et al. have studied metabolic, endocrine, inflammatory, and physiologic differences between obese and lean subjects and reported a branched-chain amino acids (BCAAs)-related metabolic signature contributing to insulin resistance [68]. Suhre et al. recently reported the outcomes from a multiplatform metabonomics analysis of an epidemiological study on diabetes in which diabetes-related complications could be detected already under subclinical conditions in a general German population [52]. In addition to previously reported T2D biomarkers, including sugar metabolites, ketone bodies, and BCAA, metabolites resulting from perturbations of metabolic pathways linked to kidney dysfunction (3-indoxyl sulfate), lipid metabolism (glycerophospholipids, free fatty acids), and bile acid metabolism. Additional metabonomics investigations suggested that the catabolism of BCAAs was tightly intertwined with the levels of insulin resistance, while greater levels of BCAAs were detected in the obese and IR phenotype [69, 70]. Several by-products of BCAA catabolism, such as glutamate, α-ketoglutarate, propionylcarnitine, and 2-methylbutyryl and isovalerylcarnitines, were showing a very strong contribution to the metabolic signature for obesity and insulin resistance (IR) phenotype. The authors further tested their hypothesis by supplementing BCAAs in a diet-induced obesity rat model. However, while having reduced food intake and weight gain, no improvement of the IR levels was detected. Very recently, blood plasma profiling was successfully employed to provide predictive markers of the development of diabetes in prospective human studies [71, 72]. In a first report, five branched-chain and

aromatic amino acids were strongly associated with IR, namely, isoleucine, leucine, valine, tyrosine, and phenylalanine. The authors demonstrated that a combination of three amino acids (isoleucine, phenylalanine, tyrosine) could predict future diabetes (>5-fold higher risk for individuals in top quartile) [72]. Together these key findings demonstrate a critical role of BCAA metabolism in the early onset of IR and T2D development. In a second report, specific interrelationships between dyslipidemia and IR development were evaluated [71]. Interestingly, this work reports how lipids of lower carbon number and double bond content were associated with an increased risk of T2D, unlike higher carbon number and double bond content lipids [71]. In particular, a combination of two triacylglycerols further improved diabetes prediction and could aid in clinical risk assessment. Recently, a lipidomics approach was applied to reveal the molecular phenotype in prediction of type 1 diabetes (T1D) [73]. The authors found that T1D progressors are characterized by a distinct cord blood lipidomic profile which includes reduced major choline-containing phospholipids including sphingomyelins and phosphatidylcholines. A molecular signature was developed comprising seven lipids which predicted high risk for progression to T1D, with an odds ratio of 5.94 (95 % confidence interval, 1.07–17.50). Reduction in choline-containing phospholipids in cord blood is therefore specifically associated with progression to T1D but not with development of β-cell autoimmunity in general. Several studies also investigated the interactions between lifestyle, diet, and metabolic disorders associated with IR. In particular, Huffman et al. explored the impact of exercise training on insulin sensitivity (IS) in combination with monitoring of circulating concentrations of metabolic intermediates, hormones, and inflammatory mediators. Improvement in IS was associated with reduced levels of fatty acid oxidation by-products and increased concentrations in glycine and proline [74]. Moreover, metabonomics was also employed to decipher indicators of early onsets of prediabetes status. Zhao et al. investigated the blood plasma composition in normal and impaired glucose-tolerant populations and demonstrated that prediabetes was associated with alterations in fatty acid, tryptophan, uric acid, bile acid, and gut microbial metabolism. In parallel, a great amount of knowledge was also consolidated in the field of T1D, with patients also showing a variety of metabolic abnormalities including hyperglycemia, ketogenesis, and muscle proteolysis [75]. Lanza et al. analyzed plasma from T1D humans during insulin treatment and acute insulin deprivation [75] and provided additional evidence on the disease etiology including protein synthesis and breakdown, gluconeogenesis, ketogenesis, amino acid oxidation, mitochondrial bioenergetics, and oxidative stress. There is increasing evidence that the specific metabolic disturbances preceding β-cell autoimmunity in humans are of relevance for preventive medicine and potential prognosis of children who subsequently progress to T1D [65, 76, 77]. In a series of studies, the specificity of the pre-autoimmune metabolic changes was tested both in non-obese prediabetic mouse models and in prospective human cohorts [65, 76, 77]. Of particular interest is the observation that autoimmune diabetes is preceded by a state of increased metabolic demands from the islets resulting in elevated insulin secretion and suggest alternative metabolic-related pathways as therapeutic targets to prevent diabetes.

Nonalcoholic fatty liver disease (NAFLD) is increasingly considered as a main pathological determinant in various metabolic deregulations such as obesity, insulin resistance, hypertension, dyslipidemia, and cardiovascular disease (CVD) [78, 79]. NAFLD is characterized by fatty acid infiltration in the liver in the absence of alcohol abuse [80]. NAFLD ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), the latest being marked by increased inflammation status [81, 82]. In the absence of validated biomarkers of NAFLD alternative to liver biopsy, metabonomics and lipidomics are foreseen promising to deliver both a new set of minimally invasive clinical classifiers, i.e., biomarkers and metabolic mechanistic insights into the disease etiology and progression. Recently, Vinaixa et al. reported the use of metabonomics for quantitative profiling of liver extracts from LDLr−/− mice [83]. NMR-based metabonomics was used to investigate the metabolic effects and implications of the dietary cholesterol in the etiology of progression from hepatic steatosis to NASH. Dietary cholesterol increased the hepatic concentrations of cholesterol, triglycerides, and oleic acid but also decreased the polyunsaturated fatty acids (PUFAs)/monounsaturated fatty acids ratio as well as the relative amount of long-chain PUFAs in the liver. Changes in hepatic concentration of taurine, glutathione, methionine, and carnitine were also observed. Likewise, Li et al. used a methionine- and choline-deficient diet to describe metabolic changes associated to different stages of NAFLD in male C57BL/6 mice [84]. Four potential biomarkers including serum glucose, lactate, glutamate/glutamine, and taurine were selected and used to stratify NAFLD severity. In addition, using a parallel NAFLD animal model/human design, Barr et al. analyzed 42 serum samples collected from nondiabetic, morbidly obese, biopsy-proven NAFLD patients and 17 animals belonging to the glycine *N*-methyltransferase knockout (GNMT-KO) NAFLD mouse model [85]. MS-based metabonomics revealed similarities in the GNMT-KO and human NAFLD patients with relevant biochemical perturbations linked to liver dysfunction through reduced levels of creatinine and increased concentrations in bile acids as well as in eicosanoids. Metabonomics was also employed by Kalhan et al. [86] to provide potential metabolic steatosis markers in biopsy confirmed NASH subjects. While steatosis and NASH could not be distinguished, NASH metabolic signature was marked by altered levels of bile acids, glutathione, lipids, and amino acids. More recently, Feldstein et al. used a targeted isotope dilution MS-targeted technique to quantify 9- and 13-HODEs and 9- and 13-oxoODEs as circulating biomarkers of NASH [87].

2.6 Clinical Application for Neurological and Psychiatric Disorders

Diagnostic markers of clinical metabonomics can also find applications in sociopsychological and neurodevelopment disorders. Yap et al. displayed, by the use of NMR spectroscopy, biochemical signature of autistic individuals [88]. Urinary metabolic phenotypes of autistic individuals were marked by increased levels of *N*-methyl-2-pyridone-5-carboxamide, *N*-methyl nicotinic acid, *N*-methyl

nicotinamide, taurine, and a lower concentration of glutamate. Abnormalities in gut microbiota metabolism were also suggested through lower levels of urinary dimethylamine, hippurate, and phenylacetylglutamine in autistic children.

Early detection, risk assessment, and therapeutic monitoring of Alzheimer's disease (AD) were also studied with metabonomics [89]. Shotgun lipidomics indicated reductions of sphingomyelin and significant increases in two ceramide species (N16:0 and N21:0) in plasma of AD patients. Lastly, Oresic et al. reported that serum metabolic profiles of persons with schizophrenia had significantly higher metabolite levels in six lipid clusters encompassing saturated triglycerides and in two small-molecule clusters containing BCAAs, phenylalanine, tyrosine, proline, glutamate, lactate, and pyruvate [90]. A GC-MS-based metabonomics profiling approach was used to detect potential biomarkers associated with schizophrenia and risperidone treatment [91]. Here, 22 marker metabolites provided separation of schizophrenic patients from matched healthy controls, with citrate, palmitic acid, myoinositol, and allantoin exhibiting the best combined classification performance. Moreover, 20 markers displayed the complete separation between posttreatment and pretreatment patients, with myoinositol, uric acid, and tryptophan showing the maximum combined classification performance.

A general comprehensive metabonomics population-based study in Finland was applied [90] to determine metabolic differences between persons included in three main psychotic disorders (schizophrenia, $n=45$; other non-affective psychosis (ONAP), $n=57$; affective psychosis, $n=37$) and controls matched by age, sex, and regions. Here, global lipidomics displayed that compared to healthy controls, those with schizophrenia had significantly higher metabolite levels in six lipid clusters containing mainly saturated triglycerides. In addition, a combined GC metabonomics approach revealed, in persons with schizophrenia, two small-molecule clusters containing, among other metabolites, branched-chain amino acids, phenylalanine and tyrosine, and proline, glutamic, lactic, and pyruvic acids. Among these, serum glutamic acid was elevated in all psychoses ($p=0.0020$) compared to controls, while proline upregulation ($p=0.000023$) was specific to schizophrenia.

2.7 Clinical Applications for Cancer Diagnosis

Metabonomics is nowadays foreseen as a promising high-throughput, automated approach in addition to functional genomics and proteomics for analyses of molecular changes in malignant tumors [92–95]. Metabolite profiling approach was, for instance, successfully employed to characterize molecular changes in ovarian tumor tissues [95]. Sixty-six invasive ovarian carcinomas and nine borderline tumors of the ovary were analyzed by GC-MS. A total of 51 metabolites were significantly different between borderline tumors and carcinomas, which encompassed glycerolipid, pyrimidine, purine, amino acid, propanoate, and free fatty acid metabolism [95]. In addition, the potential of applying metabonomics to explore metabolic pathways modulation specific to organ-confined disease or metastatic disease may lead to the identification of new early disease biomarkers. MS-based metabonomics

analysis of prostate cancer patients based on tissue biopsies, urine, and plasma samples was able to distinguish benign prostate, clinically localized prostate cancer, and metastatic disease [92]. Sarcosine, an N-methyl derivative of the amino acid glycine, was identified as a differential metabolite that was highly increased during prostate cancer progression to metastasis and can be detected noninvasively in urine. Pasikant et al. displayed the potential and validity in the staging, grading, and diagnosis capabilities of urinary metabonomics in bladder cancer tumors [96]. Here, 100 % sensitivity in detecting bladder cancer was observed using urinary metabonomics versus 33 % sensitivity achieved by urinary cytology, the current standard for tumor detection and monitoring of recurrence or progression of bladder cancer. Using plasma-free amino acids profiling, Miyag et al. described metabonomics applications for lung, gastric, colorectal, breast, and prostate cancer disease diagnosis [97]. Cancer patients and controls could be discriminated using multivariate analysis where significant alterations in plasma-free amino acids profiles were observed in the disease cancer stage. Interestingly, tryptophan was identified as a key amino acid associated with cancer progression. New breast cancer diagnostic measures had also been developed by high-resolution magic angle spinning (HR-MAS) NMR spectroscopy [98]. This technique provides a means to generate metabolic profiles of intact tissues. HR-MAS MR spectroscopic studies on breast tissue biopsies revealed elevated levels of taurine- and choline-containing compounds, especially phosphocholine in the cancer samples. Moreover, metabolic profiling allowed a clinical prediction with 69 % sensitivity and 94 % specificity in a validation cohort. NMR and MS metabolic profiles were also used to develop a specific prediction model for early detection of recurrent breast cancer [99], displaying capabilities of metabonomics in providing predictive biomarkers. Interestingly, 55 % of the patients could be correctly predicted to have recurrence 13 months before the recurrence was clinically diagnosed. A MS-based quantitative metabonomics method to analyze plasma samples from 55 breast cancer patients and 25 healthy controls was applied [100]. A number of 30 patients and 20 age-matched healthy controls were used as a training dataset to establish a diagnostic model and to identify potential biomarkers. Here, 39 differentiating metabolites were identified, including significantly lower levels of lysophosphatidylcholines and higher levels of sphingomyelins in the plasma samples obtained from breast cancer patients compared with healthy controls. Using logical regression, a diagnostic equation based on three metabolites (LPC16:0, PCae 42:5, and PCaa 34:2) successfully differentiated breast cancer patients from healthy controls, with a sensitivity of 98.1 % and a specificity of 96.0 %.

Recent technological advances facilitate automated analyses of biological samples, and installations of NMR equipment in close proximity to the surgical theaters are in a growing phase. Metabolic profiling thus has the potential to become a method for rapid characterization of cancerous biopsies in the operation theater. Bathen et al. analyzed 328 tissue samples from 228 breast cancer patients using solid-state NMR [101]. Using double cross validation, high sensitivity, and specificity of 91 % and 93 %, respectively, was achieved. Analysis of the loading profiles from both principal component analysis (PCA) and PLS-DA showed the choline-containing metabolites as main biomarkers for tumor content, with

phosphocholine being especially high in tumor tissue. Other indicative metabolites include glycine, taurine, and glucose.

Being independent of prior assumptions, metabonomics approaches also allow hypothesis generation on how nutritional intervention might be beneficial to malignant cancers. NMR-based metabonomics was used to determine the effects of a diet rich in whole grain (WG) rye products on the profile of metabolites in the plasma of prostate cancer (PC) patients [102]. Seventeen PC patients received 485 g rye bran product (RP) or refined white wheat product (WP) in a randomized, controlled, crossover design during a period of 6 week with a 2-week washout period. Metabonomics analysis of plasma showed an increase in 3-hydroxybutyric acid, acetone, betaine, *N,N*-dimethylglycine, and dimethyl sulfone after RP. Plasma homocysteine concentration was lower ($p = 0.017$) and that of leptin tended to be lower ($p = 0.07$) after RP intake compared to WP intake.

2.8 Conclusion

It has been estimated that by 2020 chronic disease in developing countries will account for almost three-quarters of all deaths worldwide with 75 % of death due to stroke and 70 % of death due to diabetes. In such a context, there is a clear need to develop new predictive approaches for preventive medicine and prognostic strategies for personalized therapeutic management and monitoring (Fig. 2.3). The development of systems biology approaches and the new generation of biomarker patterns will provide the opportunity to associate complex metabolic regulations with key biological processes. By opening a direct biochemical window into the metabonome, metabonomics is a unique science perfectly suited for the identification of biomarkers capable of providing better understanding of the complex metabolic phenomenon. Metabonomics is then foreseen to deliver in clinical settings a new generation of endpoints, e.g., biomarkers, to describe healthy and abnormal developmental metabolic trajectory such as in aging studies. This makes clinical metabonomics a very efficient approach for generation of metabolic patterns for the comprehensive characterization of metabolic health, the prognosis and diagnostic of diseases, and the generation of new insights in the understanding of the interactions between diet and metabolism (Table 2.1).

Table 2.1 Overview of metabolomics applications in selected human studies

Metabolomics applications	Methods	Reference
Aging	MS	[64, 65]
Diabetes	MS	[52, 68, 71–77]
Insulin resistance	MS	[69, 70]
Nonalcoholic fatty liver disease (NAFLD)	MS, NMR	[83–87]
Autism	NMR	[88–91]
Cancer-ovarian tumor	MS, NMR	[92, 95–102]

References

1. Tiret L. Gene-environment interaction: a central concept in multifactorial diseases. Proc Nutr Soc. 2002;61(4):457–63.
2. Nicholson JK, Wilson ID. Opinion: understanding 'global' systems biology: metabonomics and the continuum of metabolism. Nat Rev Drug Discov. 2003;2(8):668–76.
3. Nicholson JK, Lindon JC, Holmes E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica. 1999;29(11):1181–9.
4. Trujillo E, Davis C, Milner J. Nutrigenomics, proteomics, metabolomics, and the practice of dietetics. J Am Diet Assoc. 2006;106(3):403–13.
5. Ordovas JM, Shen J. Gene-environment interactions and susceptibility to metabolic syndrome and other chronic diseases. J Periodontol. 2008;79(8 Suppl):1508–13.
6. Ordovas JM. Integrating environment and disease into "omic" analysis. Rev Esp Cardiol. 2009;62 Suppl 2:17–22.
7. van der Greef J, Hankemeier T, McBurney RN. Metabolomics-based systems biology and personalized medicine: moving towards n = 1 clinical trials? Pharmacogenomics. 2006;7(7): 1087–94.
8. van der Greef J. Systems biology, connectivity and the future of medicine. Syst Biol (Stevenage). 2005;152(4):174–8.
9. Nicholson JK, Lindon JC. Systems biology: metabonomics. Nature. 2008;455(7216): 1054–6.
10. Holmes E, Wilson ID, Nicholson JK. Metabolic phenotyping in health and disease. Cell. 2008;134(5):714–7.
11. Keurentjes JJ. Genetical metabolomics: closing in on phenotypes. Curr Opin Plant Biol. 2009;12(2):223–30.
12. Saito K, Matsuda F. Metabolomics for functional genomics, systems biology, and biotechnology. Annu Rev Plant Biol. 2010;61:463–89.
13. Weckwerth W. Metabolomics in systems biology. Annu Rev Plant Biol. 2003;54:669–89.
14. Beckonert O, Keun HC, Ebbels TMD, Bundy J, Holmes E, Lindon JC, Nicholson JK. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. Nat Protocol. 2007;2(11):2692–703.
15. Keun HC, Beckonert O, Griffin JL, Richter C, Moskau D, Lindon JC, Nicholson JK. Cryogenic probe 13C NMR spectroscopy of urine for metabonomic studies. Anal Chem. 2002;74(17):4588–93.
16. Beckonert O, Coen M, Keun HC, Wang Y, Ebbels TM, Holmes E, Lindon JC, Nicholson JK. High-resolution magic-angle-spinning NMR spectroscopy for metabolic profiling of intact tissues. Nat Protoc. 2010;5(6):1019–32.
17. Dumas ME, Maibaum EC, Teague C, Ueshima H, Zhou B, Lindon JC, Nicholson JK, Stamler J, Elliott P, Chan Q, Holmes E. Assessment of analytical reproducibility of 1H NMR spectroscopy based metabonomics for large-scale epidemiological research: the INTERMAP Study. Anal Chem. 2006;78(7):2199–208.
18. Álvarez-Sánchez B, Priego-Capote F, Castro MDLD. Metabolomics analysis II. Preparation of biological samples prior to detection. TrAC Trends Anal Chem. 2010;29(2):120–7.
19. Bojko B, Cudjoe E, Pawliszyn J, Wasowicz M. Solid-phase microextraction. How far are we from clinical practice? TrAC Trends Anal Chem. 2011;30(9):1505–12.
20. Bruce SJ, Tavazzi I, Parisod V, Rezzi S, Kochhar S, Guy PA. Investigation of human blood plasma sample preparation for performing metabolomics using ultrahigh performance liquid chromatography/mass spectrometry. Anal Chem. 2009;81(9):3285–96.
21. Römisch-Margl W, Prehn C, Bogumil R, Röhring C, Suhre K, Adamski J. Procedure for tissue sample preparation and metabolite extraction for high-throughput targeted metabolomics. Metabolomics. 2011;8(1):133–42.

22. Sellick CA, Hansen R, Maqsood AR BW, Stephens GM, Goodacre R, Dickson AJ, Dunn WB. Effective quenching processes for physiologically valid metabolite profiling of suspension cultured mammalian cells effective quenching processes for physiologically valid metabolite profiling of suspension cultured mammalian cells. Anal Chem. 2009;81:174–83.
23. Villas-Bôas SG, Højer-Pedersen J, Åkesson M, Smedsgaard J, Nielsen J. Global metabolite analysis of yeast: evaluation of sample preparation methods. Yeast. 2005;22(14):1155–69.
24. Buescher JM, Moco S, Sauer U, Zamboni N. Ultrahigh performance liquid chromatography-tandem mass spectrometry method for fast and robust quantification of anionic and aromatic metabolites. Anal Chem. 2010;82(11):4403–12.
25. Wu L, Mashego MR, Van Dam JC, Proell AM, Vinke JL, Ras C, Van Winden WA, Van Gulik WM, Heijnen JJ. Quantitative analysis of the microbial metabolome by isotope dilution mass spectrometry using uniformly 13C-labeled cell extracts as internal standards. Anal Biochem. 2005;336(2):164–71.
26. Kostiainen R, Kauppila TJ. Effect of eluent on the ionization process in liquid chromatography mass spectrometry. J Chromatogr A. 2009;1216(4):685–99.
27. Nordström A, Want E, Northen T, Lehtiö J, Siuzdak G. Multiple ionization mass spectrometry strategy used to reveal the complexity of metabolomics. Anal Chem. 2008;80(2):421–9.
28. Bruce SJ, Breton I, Decombaz J, Boesch C, Scheurer E, Montoliu I, Rezzi S, Kochhar S, Guy PA. A plasma global metabolic profiling approach applied to an exercise study monitoring the effects of glucose, galactose and fructose drinks during post-exercise recovery. J Chromatogr B. 2010;878(29):3015–23.
29. Tolstikov VV, Fiehn O. Analysis of highly polar compounds of plant origin: combination of hydrophilic interaction chromatography and electrospray ion trap mass spectrometry. Anal Biochem. 2002;301(2):298–307.
30. Soga T, Igarashi K, Ito C, Mizobuchi K, Zimmermann HP, Tomita M. Metabolomic profiling of anionic metabolites by capillary electrophoresis mass spectrometry. Anal Chem. 2009;81(15):6165–74.
31. Griffiths WJ, Ogundare M, Williams CM, Wang Y. On the future of "omics": lipidomics. J Inherit Metab Dis. 2011;34(3):583–92.
32. Wenk MR. The emerging field of lipidomics. Nat Rev Drug Discov. 2005;4(7):594–610.
33. Liebisch G, Binder M, Schifferer R, Langmann T, Schulz B, Schmitz G. High throughput quantification of cholesterol and cholesteryl ester by electrospray ionization tandem mass spectrometry (ESI-MS/MS). Biochim Biophys Acta. 2006;1761(1):121–8.
34. Liebisch G, Drobnik W, Reil M, Trumbach B, Arnecke R, Olgemoller B, Roscher A, Schmitz G. Quantitative measurement of different ceramide species from crude cellular extracts by electrospray ionization tandem mass spectrometry (ESI-MS/MS). J Lipid Res. 1999;40(8):1539–46.
35. Liebisch G, Lieser B, Rathenberg J, Drobnik W, Schmitz G. High-throughput quantification of phosphatidylcholine and sphingomyelin by electrospray ionization tandem mass spectrometry coupled with isotope correction algorithm. Biochim Biophys Acta. 2004;1686(1–2):108–17.
36. Schuhmann K, Herzog R, Schwudke D, Metelmann-Strupat W, Bornstein SR, Shevchenko A. Bottom-up shotgun lipidomics by higher energy collisional dissociation on LTQ Orbitrap mass spectrometers. Anal Chem. 2011;83(14):5480–7.
37. Scherer M, Gnewuch C, Schmitz G, Liebisch G. Rapid quantification of bile acids and their conjugates in serum by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2009;877(30):3920–5.
38. Scherer M, Leuthauser-Jaschinski K, Ecker J, Schmitz G, Liebisch G. A rapid and quantitative LC-MS/MS method to profile sphingolipids. J Lipid Res. 2010;51(7):2001–11.
39. Liebisch G, Schmitz G. Quantification of lysophosphatidylcholine species by high-throughput electrospray ionization tandem mass spectrometry (ESI-MS/MS). Methods Mol Biol. 2009;580:29–37.
40. Sumner L, Amberg A, Barrett D, Beale M, Beger R, Daykin C, Fan T, Fiehn O, Goodacre R, Griffin J, Hankemeier T, Hardy N, Harnly J, Higashi R, Kopka J, Lane A, Lindon J, Marriott P,

Nicholls A, Reily M, Thaden J, Viant M. Proposed minimum reporting standards for chemical analysis. Metabolomics. 2007;3(3):211–21.
41. Benton HP, Want E, Keun HC, Amberg A, Plumb RS, Goldfain-Blanc F, Walther B, Reily MD, Lindon JC, Holmes E, Nicholson JK, Ebbels TM. Intra- and interlaboratory reproducibility of ultra performance liquid chromatography-time-of-flight mass spectrometry for urinary metabolic profiling. Anal Chem. 2012;84(5):2424–32.
42. Kamleh MA, Ebbels TM, Spagou K, Masson P, Want EJ. Optimizing the use of quality control samples for signal drift correction in large-scale urine metabolic profiling studies. Anal Chem. 2012;84:2670–7.
43. Brown M, Dunn WB, Dobson P, Patel Y, Winder CL, Francis-McIntyre S, Begley P, Carroll K, Broadhurst D, Tseng A, Swainston N, Spasic I, Goodacre R, Kell DB. Mass spectrometry tools and metabolite-specific databases for molecular identification in metabolomics. Analyst. 2009;134(7):1322–32.
44. Exarchou V, Godejohann M, van Beek TA, Gerothanassis IP, Vervoort J. LC-UV-solid-phase extraction-NMR-MS combined with a cryogenic flow probe and its application to the identification of compounds present in Greek oregano. Anal Chem. 2003;75(22):6288–94.
45. Lommen A, Gerssen A, Oosterink JE, Kools HJ, Ruiz-Aracama A, Peters RJ, Mol HG. Ultrafast searching assists in evaluating sub-ppm mass accuracy enhancement in U-HPLC/Orbitrap MS data. Metabolomics. 2011;7(1):15–24.
46. Moco S, Bino RJ, De Vos RCH, Vervoort J. Metabolomics technologies and metabolite identification. Trends Anal Chem. 2007;26:855–66.
47. Moco S, Forshed J, De Vos RCH, Bino RJ, Vervoort J. Intra- and inter-metabolite correlation spectroscopy of tomato metabolomics data obtained by liquid chromatography-mass spectrometry and nuclear magnetic resonance. Metabolomics. 2008;4:202–15.
48. Tautenhahn R, Bottcher C, Neumann S. Highly sensitive feature detection for high resolution LC/MS. BMC Bioinforma. 2008;9:504.
49. Neumann S, Bocker S. Computational mass spectrometry for metabolomics: identification of metabolites and small molecules. Anal Bioanal Chem. 2010;398(7–8):2779–88.
50. Coughlin SS. Ethical issues in epidemiologic research and public health practice. Emerg Themes Epidemiol. 2006;3:16.
51. Holmes E, Loo RL, Stamler J, Bictash M, Yap IK, Chan Q, Ebbels T, De Iorio M, Brown IJ, Veselkov KA, Daviglus ML, Kesteloot H, Ueshima H, Zhao L, Nicholson JK, Elliott P. Human metabolic phenotype diversity and its association with diet and blood pressure. Nature. 2008;453(7193):396–400.
52. Suhre K, Meisinger C, Doring A, Altmaier E, Belcredi P, Gieger C, Chang D, Milburn MV, Gall WE, Weinberger KM, Mewes HW, Hrabe de Angelis M, Wichmann HE, Kronenberg F, Adamski J, Illig T. Metabolic footprint of diabetes: a multiplatform metabolomics study in an epidemiological setting. PLoS One. 2010;5(11):e13953.
53. He Y, Yu Z, Giegling I, Xie L, Hartmann AM, Prehn C, Adamski J, Kahn R, Li Y, Illig T, Wang-Sattler R, Rujescu D. Schizophrenia shows a unique metabolomics signature in plasma. Transl Psychiatry. 2012;2:e149.
54. Yu Z, Zhai G, Singmann P, He Y, Xu T, Prehn C, Römisch-Margl W, Lattka E, Gieger C, Soranzo N, Heinrich J, Standl M, Thiering E, Mittelstraß K, Wichmann H-E, Peters A, Suhre K, Li Y, Adamski J, Spector TD, Illig T, Wang-Sattler R. Human serum metabolic profiles are age dependent. Aging Cell. 2012;11(6):960–7.
55. Haquin S, Oeuillet E, Pajon A, Harris M, Jones A, Tilbeurgh H, Markley J, Zolnai Z, Poupon A. Data management in structural genomics: an overview. In: Kobe B, Guss M, Huber T, editors. Structural proteomics, vol 426. Methods in Molecular Biology™. Humana Press; 2008. pp 49 – 79. doi:10.1007/978-1-60327-058-8_4.
56. Holland NT, Smith MT, Eskenazi B, Bastaki M. Biological sample collection and processing for molecular epidemiological studies. Mutat Res. 2003;543(3):217–34.
57. Lauridsen M, Hansen SH, Jaroszewski JW, Cornett C. Human urine as test material in ^{1}H NMR-based metabonomics: recommendations for sample preparation and storage. Anal Chem. 2007;79(3):1181–6.

58. Singh R, Kolvraa S, Rattan SI. Genetics of human longevity with emphasis on the relevance of HSP70 as candidate genes. Front Biosci. 2007;12:4504–13.
59. De Benedictis G, Carotenuto L, Carrieri G, De Luca M, Falcone E, Rose G, Cavalcanti S, Corsonello F, Feraco E, Baggio G, Bertolini S, Mari D, Mattace R, Yashin AI, Bonafe M, Franceschi C. Gene/longevity association studies at four autosomal loci (REN, THO, PARP, SOD2). Eur J Hum Genet. 1998;6(6):534–41.
60. Schmitt K, Grimm A, Kazmierczak A, Strosznajder JB, Gotz J, Eckert A. Insights into mitochondrial dysfunction: aging, amyloid-beta and tau – a deleterious trio. Antioxid Redox Signal. 2011;16:1456.
61. Castro MD, Suarez E, Kraiselburd E, Isidro A, Paz J, Ferder L, Ayala-Torres S. Aging increases mitochondrial DNA damage and oxidative stress in liver of rhesus monkeys. Exp Gerontol. 2011;47:29–32.
62. Radak Z, Zhao Z, Goto S, Koltai E. Age-associated neurodegeneration and oxidative damage to lipids, proteins and DNA. Mol Asp Med. 2011;32(4–6):305–15.
63. Xue H, Xian B, Dong D, Xia K, Zhu S, Zhang Z, Hou L, Zhang Q, Zhang Y, Han JD. A modular network model of aging. Mol Syst Biol. 2007;3:147.
64. Lawton KA, Berger A, Mitchell M, Milgram KE, Evans AM, Guo L, Hanson RW, Kalhan SC, Ryals JA, Milburn MV. Analysis of the adult human plasma metabolome. Pharmacogenomics. 2008;9(4):383–97.
65. Nikkila J, Sysi-Aho M, Ermolov A, Seppanen-Laakso T, Simell O, Kaski S, Oresic M. Gender-dependent progression of systemic metabolic states in early childhood. Mol Syst Biol. 2008;4:197.
66. Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, Gordon DJ, Krauss RM, Savage PJ, Smith Jr SC, Spertus JA, Costa F. Diagnosis and management of the metabolic syndrome. An American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. Executive summary. Cardiol Rev. 2005;13(6):322–7.
67. Wirfalt E, Hedblad B, Gullberg B, Mattisson I, Andren C, Rosander U, Janzon L, Berglund G. Food patterns and components of the metabolic syndrome in men and women: a cross-sectional study within the Malmo Diet and Cancer cohort. Am J Epidemiol. 2001;154(12):1150–9.
68. Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF, Haqq AM, Shah SH, Arlotto M, Slentz CA, Rochon J, Gallup D, Ilkayeva O, Wenner BR, Yancy Jr WS, Eisenson H, Musante G, Surwit RS, Millington DS, Butler MD, Svetkey LP. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab. 2009;9(4):311–26.
69. Huffman KM, Shah SH, Stevens RD, Bain JR, Muehlbauer M, Slentz CA, Tanner CJ, Kuchibhatla M, Houmard JA, Newgard CB, Kraus WE. Relationships between circulating metabolic intermediates and insulin action in overweight to obese, inactive men and women. Diabetes Care. 2009;32(9):1678–83.
70. Fiehn O, Garvey WT, Newman JW, Lok KH, Hoppel CL, Adams SH. Plasma metabolomic profiles reflective of glucose homeostasis in non-diabetic and type 2 diabetic obese African-American women. PLoS ONE. 2010;5(12):e15234.
71. Rhee EP, Cheng S, Larson MG, Walford GA, Lewis GD, McCabe E, Yang E, Farrell L, Fox CS, O'Donnell CJ, Carr SA, Vasan RS, Florez JC, Clish CB, Wang TJ, Gerszten RE. Lipid profiling identifies a triacylglycerol signature of insulin resistance and improves diabetes prediction in humans. J Clin Invest. 2011;121(4):1402–11.
72. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, Lewis GD, Fox CS, Jacques PF, Fernandez C, O'Donnell CJ, Carr SA, Mootha VK, Florez JC, Souza A, Melander O, Clish CB, Gerszten RE. Metabolite profiles and the risk of developing diabetes. Nat Med. 2011;17(4):448–53.
73. Oresic M, Gopalacharyulu P, Mykkanen J, Lietzen N, Makinen M, Nygren H, Simell S, Simell V, Hyoty H, Veijola R, Ilonen J, Sysi-Aho M, Knip M, Hyotylainen T, Simell O. Cord serum lipidome in prediction of islet autoimmunity and type 1 diabetes. Diabetes. 2013;62:3268–74.

74. Huffman KM, Slentz CA, Bateman LA, Thompson D, Muehlbauer MJ, Bain JR, Stevens RD, Wenner BR, Kraus VB, Newgard CB, Kraus WE. Exercise-induced changes in metabolic intermediates, hormones, and inflammatory markers associated with improvements in insulin sensitivity. Diabetes Care. 2011;34(1):174–6.
75. Lanza IR, Zhang S, Ward LE, Karakelides H, Raftery D, Nair KS. Quantitative metabolomics by H-NMR and LC-MS/MS confirms altered metabolic pathways in diabetes. PLoS ONE. 2010;5(5):e10538.
76. Sysi-Aho M, Ermolov A, Gopalacharyulu PV, Tripathi A, Seppanen-Laakso T, Maukonen J, Mattila I, Ruohonen ST, Vahatalo L, Yetukuri L, Harkonen T, Lindfors E, Nikkila J, Ilonen J, Simell O, Saarela M, Knip M, Kaski S, Savontaus E, Oresic M. Metabolic regulation in progression to autoimmune diabetes. PLoS Comput Biol. 2011;7(10):e1002257.
77. Oresic M, Simell S, Sysi-Aho M, Nanto-Salonen K, Seppanen-Laakso T, Parikka V, Katajamaa M, Hekkala A, Mattila I, Keskinen P, Yetukuri L, Reinikainen A, Lahde J, Suortti T, Hakalax J, Simell T, Hyoty H, Veijola R, Ilonen J, Lahesmaa R, Knip M, Simell O. Dysregulation of lipid and amino acid metabolism precedes islet autoimmunity in children who later progress to type 1 diabetes. J Exp Med. 2008;205(13):2975–84.
78. Fabbrini E, Sullivan S, Klein S. Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. Hepatology. 2010;51(2):679–89.
79. Johnson NA, Walton DW, Sachinwalla T, Thompson CH, Smith K, Ruell PA, Stannard SR, George J. Noninvasive assessment of hepatic lipid composition: advancing understanding and management of fatty liver disorders. Hepatology. 2008;47(5):1513–23.
80. Tiniakos DG, Vos MB, Brunt EM. Nonalcoholic fatty liver disease: pathology and pathogenesis. Annu Rev Pathol. 2010;5:145–71.
81. James OF, Day CP. Non-alcoholic steatohepatitis (NASH): a disease of emerging identity and importance. J Hepatol. 1998;29(3):495–501.
82. Day CP, James OF. Steatohepatitis: a tale of two "hits"? Gastroenterology. 1998;114(4):842–5.
83. Rull A, Vinaixa M, Angel RM, Beltran R, Brezmes J, Canellas N, Correig X, Joven J. Metabolic phenotyping of genetically modified mice: an NMR metabonomic approach. Biochimie. 2009;91(8):1053–7.
84. Li H, Wang L, Yan X, Liu Q, Yu C, Wei H, Li Y, Zhang X, He F, Jiang Y. A proton nuclear magnetic resonance metabonomics approach for biomarker discovery in nonalcoholic fatty liver disease. J Proteome Res. 2011;10(6):2797–806.
85. Barr J, Vazquez-Chantada M, Alonso C, Perez-Cormenzana M, Mayo R, Galan A, Caballeria J, Martin-Duce A, Tran A, Wagner C, Luka Z, Lu SC, Castro A, Le Marchand-Brustel Y, Martinez-Chantar ML, Veyrie N, Clement K, Tordjman J, Gual P, Mato JM. Liquid chromatography-mass spectrometry-based parallel metabolic profiling of human and mouse model serum reveals putative biomarkers associated with the progression of nonalcoholic fatty liver disease. J Proteome Res. 2010;9(9):4501–12.
86. Kalhan SC, Guo L, Edmison J, Dasarathy S, McCullough AJ, Hanson RW, Milburn M. Plasma metabolomic profile in nonalcoholic fatty liver disease. Metabolism. 2011;60(3):404–13.
87. Feldstein AE, Lopez R, Tamimi TA, Yerian L, Chung YM, Berk M, Zhang R, McIntyre TM, Hazen SL. Mass spectrometric profiling of oxidized lipid products in human nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. J Lipid Res. 2010;51(10):3046–54.
88. Yap IK, Angley M, Veselkov KA, Holmes E, Lindon JC, Nicholson JK. Urinary metabolic phenotyping differentiates children with autism from their unaffected siblings and age-matched controls. J Proteome Res. 2010;9(6):2996–3004.
89. Han X, Rozen S, Boyle SH, Hellegers C, Cheng H, Burke JR, Welsh-Bohmer KA, Doraiswamy PM, Kaddurah-Daouk R. Metabolomics in early Alzheimer's disease: identification of altered plasma sphingolipidome using shotgun lipidomics. PLoS ONE. 2011;6(7):e21643.
90. Oresic M, Tang J, Seppanen-Laakso T, Mattila I, Saarni SE, Saarni SI, Lonnqvist J, Sysi-Aho M, Hyotylainen T, Perala J, Suvisaari J. Metabolome in schizophrenia and other psychotic disorders: a general population-based study. Genome Med. 2011;3(3):19.

91. Xuan J, Pan G, Qiu Y, Yang L, Su M, Liu Y, Chen J, Feng G, Fang Y, Jia W, Xing Q, He L. Metabolomic profiling to identify potential serum biomarkers for schizophrenia and risperidone action. J Proteome Res. 2011;10(12):5433–43.
92. Sreekumar A, Poisson LM, Rajendiran TM, Khan AP, Cao Q, Yu J, Laxman B, Mehra R, Lonigro RJ, Li Y, Nyati MK, Ahsan A, Kalyana-Sundaram S, Han B, Cao X, Byun J, Omenn GS, Ghosh D, Pennathur S, Alexander DC, Berger A, Shuster JR, Wei JT, Varambally S, Beecher C, Chinnaiyan AM. Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. Nature. 2009;457(7231):910–4.
93. Sawyers CL. The cancer biomarker problem. Nature. 2008;452(7187):548–52.
94. Kind T, Tolstikov V, Fiehn O, Weiss RH. A comprehensive urinary metabolomic approach for identifying kidney cancer. Anal Biochem. 2007;363(2):185–95.
95. Denkert C, Budczies J, Kind T, Weichert W, Tablack P, Sehouli J, Niesporek S, Konsgen D, Dietel M, Fiehn O. Mass spectrometry-based metabolic profiling reveals different metabolite patterns in invasive ovarian carcinomas and ovarian borderline tumors. Cancer Res. 2006;66(22):10795–804.
96. Pasikanti KK, Esuvaranathan K, Ho PC, Mahendran R, Kamaraj R, Wu QH, Chiong E, Chan EC. Noninvasive urinary metabonomic diagnosis of human bladder cancer. J Proteome Res. 2010;9(6):2988–95.
97. Miyagi Y, Higashiyama M, Gochi A, Akaike M, Ishikawa T, Miura T, Saruki N, Bando E, Kimura H, Imamura F, Moriyama M, Ikeda I, Chiba A, Oshita F, Imaizumi A, Yamamoto H, Miyano H, Horimoto K, Tochikubo O, Mitsushima T, Yamakado M, Okamoto N. Plasma free amino acid profiling of five types of cancer patients and its application for early detection. PLoS ONE. 2011;6(9):e24143.
98. Li M, Song Y, Cho N, Chang JM, Koo HR, Yi A, Kim H, Park S, Moon WK. An HR-MAS MR metabolomics study on breast tissues obtained with core needle biopsy. PLoS ONE. 2011;6(10):e25563.
99. Gu H, Pan Z, Xi B, Asiago V, Musselman B, Raftery D. Principal component directed partial least squares analysis for combining nuclear magnetic resonance and mass spectrometry data in metabolomics: application to the detection of breast cancer. Anal Chim Acta. 2011;686(1–2):57–63.
100. Qiu Y, Zhou B, Su M, Baxter S, Zheng X, Zhao X, Yen Y, Jia W. Mass spectrometry-based quantitative metabolomics revealed a distinct lipid profile in breast cancer patients. Int J Mol Sci. 2013;14(4):8047–61.
101. Bathen TF, Geurts B, Sitter B, Fjosne HE, Lundgren S, Buydens LM, Gribbestad IS, Postma G, Giskeodegard GF. Feasibility of MR metabolomics for immediate analysis of resection margins during breast cancer surgery. PLoS ONE. 2013;8(4):e61578.
102. Moazzami AA, Zhang JX, Kamal-Eldin A, Aman P, Hallmans G, Johansson JE, Andersson SO. Nuclear magnetic resonance-based metabolomics enable detection of the effects of a whole grain rye and rye bran diet on the metabolic profile of plasma in prostate cancer patients. J Nutr. 2011;141(12):2126–32.

Chapter 3
Adopting Multivariate Nonparametric Tools to Determine Genotype-Phenotype Interactions in Health and Disease

Ivan Montoliu

Abstract This chapter describes the role of machine learning approaches such as random forests in holistic discovery applications and provides a background for its better understanding. Their suitability for feature selection, data integration, and network modelling are also evaluated through recent examples in the literature. These examples cover a variety of fields, ranging from ecology to metabolomics.

Keywords Random forests • Chemometrics • Classification and regression trees • Data integration • Network modelling • Metabolomics

3.1 Metabolomics: Introducing the Paradigm Shift in Data Analysis

The arrival of "-omics" into the scientific scene resulted in a breakthrough for the data analysis community. The generalization of sequencing methodologies, with the introduction of gene expression microarray technology, contributed to the generation of huge datasets covering many aspects related to phenotypic changes present in biological samples. This avenue facilitated a renovation of the bioinformatics concept known so far, moving from the general concept of studying information processes in biological systems to a more complex one: the storing, retrieving, organization, and analysis of biological data. Elements such as control, system, and information theory and statistics were widely introduced in the field to tackle new challenges. In particular, factors such as the dimensionality of datasets and the low number of available samples hardly challenged the established concepts in data analysis. For some applications, such as the analysis of differential gene expression readouts, traditional univariate statistics remained prevalent, being widely used in

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large-scale calculations. In this way, they became, by default, the core of data analysis techniques in the fields of biomarker discovery research. This led to several issues due to the high number of tests performed and the need to address the statistical significance of the results. Research in univariate statistics concepts addressed this point with the introduction of suitable preprocessing methods and concepts such as false discovery rate and multiplicity testing corrections [1, 2].

Metabolomics [3–5] was a late comer to the "-omics" party. Committed to address the metabolic changes associated to a specific phenotype due to intervention, environment, or genetic predisposition showed soon the constraints of the field. The use of advanced analytical chemistry tools, based on MS and NMR technologies, introduced higher complexity in the data structure and higher covariance between variables. Moreover, it became a mandatory step the development of specific tools for preprocessing, in particular for MS holistic approaches [3]. Factors such as analysis time and cost per sample contributed hardly to keep the issue of the curse of dimensionality (low n-to-p ratio), also present in differential gene expression analyses. Furthermore, the indeterminacy on the p-value, strongly dependent on the preprocessing used, questioned the suitability of the established analysis methodologies standardly used in differential gene expression.

Other intrinsic biogenic elements, such as reduced fold changes, created in many cases a more complex framework for biomarker discovery. Being at the lower level of the biochemical expression chain (genome > proteome > metabolome), the inter-individual variability was expected to be high and dependent on cofactors such as the environment. In fact, metabolomics seems to combine environmental and genetic variability, as partially showed by the genetic origin of metabolic responsiveness of human subjects challenged to an intervention [6].

All these elements, data and biology driven, soon faced serious hurdles in providing relevant results using a standard approach rooted in univariate analysis plus multiplicity testing correction. In a univariate approach, chances were that the low number of objects, associated to low fold of changes, would be the source of an eventual high proportion of type II errors (false negatives). As a counterpart, the use of multivariate analysis started providing a summarized view of the simultaneous changes throughout the experiment, disregarding its univariate significance. These characteristics did not help in biomarker discovery by itself, but contributed positively to the understanding of coordinated biochemical changes in complex biological systems.

Chemometrics provide a descriptive or predictive assessment of chemical systems in experimental life sciences using data analysis techniques [7]. It is focused on the analysis of analytical data, and it is rooted in an ensemble of tools coming from different fields such as multivariate statistics, applied mathematics, and computer science. With all those values at hand, chemometrics has become the preferred partner for analyzing metabolomics data.

From the set of techniques driven by a pure chemometrics approach, principal component analysis (PCA) [8], partial least squares regression (PLS) [9, 10], and their derivates, such as orthogonal projection on latent structures (OPLS) [11, 12], soon became the main workhorses of metabolomics data analysis. These are both

soft modelling methods which perform well in low *n*, high *p* datasets through the projection of multivariate data onto a reduced subspace, even with highly covarying variables. Moreover, in spite of being linear, PLS models handle acceptably well soft nonlinearities. However, because both methods are designed with predictive purposes, a strict validation process is needed to assess its generalization ability (minimize overfit risk) [13].

Most of the metabolomics studies where the PLS methodology was applied were typical case-control studies. In this case, classifier variants based both on PLS and OPLS were used, converting both regression models into classifiers through binary class encoding. From this moment on, partial least squares discriminant analysis (PLS-DA) [14] and orthogonal projection on latent structures discriminant analysis (OPLS-DA) [15] became preferred tools for most of the metabolomics studies handling spectroscopic data [16]. To better cover metabolomics goals, both regression methods were supported with interesting contributions that improved somehow the simplicity of interpretation of its parameters for feature extraction [17]. Both PLS-related techniques were recognized as good performers in the metabolomics area, and its use was generalized to other types of data (semiquantitative and MS data). This generalization was also supported by the availability of chemometrics data analysis packages that implemented, in a convenient way, a variety of algorithms including the most common data pretreatments.

One of the key points of the success of PLS is its utility in providing feature highlighting through the analysis of its parameters. This strong asset imposes constraints when selecting alternatives to this model. Even if it highlights metabolic profiles responding to the design, several aspects, often overlooked, need to be recall in PLS analysis: PLS has an assumption on the variable distribution and linearity of the model and needs of a careful validation.

3.2 Expanding the View: Machine Learning

In the last years, an increasing number of new classification algorithms have been proposed, many of them focused on solving low *n*-to-*p* ratio issues. It is hard to determine which of them have the best performance, and on which conditions. This difficulty in the assessment hinders a lack of consensus for the best one, being likely that the best classifier/regressor does not exist for all conditions. Moreover, chances are the best methodology might be probably problem specific.

In some cases, proposed models are complex, with several parameters to tune, and each model needs to be tailored at hand to be more on target. In situations as nowadays, when huge amounts of data need of feature selection, there is a strong request for models that use few parameters, minimal human input and low computational cost with simple parallelization.

In search of more suitable algorithms for data analysis and interpretation in metabolomics, machine learning provides a wealth of tools highly performing for data representation and generalization (classification and regression). Despite most

of them offer properties of interest, not all provide relevant information for feature extraction. Support vector machines (SVM) [18] and multilayer perceptrons (ANN-MLP) [19] are two examples, where the use of kernels and weight connection layers removes any traceability of the role of the individual variables in the model. There have been other alternatives proposed to find this variable relevance, but they are mostly linked to the application of recursive feature elimination patterns [20] that make the procedure computer intensive for long datasets.

3.3 Data Structure and Inference: Classification and Regression Trees (CART)

When dealing with metabolomics multivariate datasets (typically low number of objects, high intragroup variance, unknown individual variable distribution), the use of decision tree learning appears to be a good option to tackle these issues, still keeping some of the advantages of bilinear methods such PLS. Thus, this family of methods can describe the structure of multivariate datasets while providing predictive models of the outputs given. They are built following certain simple rules that create multiple linear boundaries in the multivariate space. According to their main goal, they are known as classification (categorical output) and regression (quantitative output) trees [21].

Decision tree learning [22, 23] has several advantages and limitations. Trees are simple to interpret, and they need simple or no preprocessing, handle well both numerical and categorical data, are possible to validate (act as predictors of external objects), are robust, and perform well with large datasets. In addition, CARTs are able to handle missing attributes. These methods offer also advantages in visualizing the structure of data: how clusters are built and the distribution of the samples within each cluster. Different data types: categorical, (un-)ordered, and continuous, can be handled and can be related nonlinearly to a response.

Their main limitation comes from how the decisions are taken at each branch of the tree, being usually locally optimal. This point does not warrant the global optimality of the tree. Due to its greedy nature during growth, they are also very dependent on the composition of the training set, which makes them naturally instable. Moreover, they have the risk of overcomplexity in defining the boundaries, even for easy discrimination cases. In such cases, using linear bound classifiers can lead to better performance. At the end, a "pruning" step is needed, where the generalization error is minimized to decrease the risk of overfit.

Briefly, CART algorithm splits the overall group of subjects (so-called main node) in several groups or nodes according to the variable that minimizes a preselected criterion of impurity, which in turn accounts for degree of matching with the target pattern. In principle, each split can be performed in n sub-nodes, but the most frequent are binary splits due to its easiness of computation when optimizing a tree. At each node, there is an impurity factor that needs to be calculated for each variable x_i to perform the right selection.

For numerical data, this step is relatively easy, as hyperplane decision boundaries can be calculated in the form: Is $x_i \leq x_{i\,split}$?. The driving criteria throughout all the tree buildup is to keep the model as sparse (with fewer nodes) as possible. With this purpose, at each node CART looks out for the query that makes the subsequent descending node as pure as possible (minimize impurity).

The calculation of node impurity in regression trees is basically driven by the mean standard error (MSE). Alternatively, in classification of trees, several measures are available:

- Entropy impurity (also called information impurity), which is defined through the following expression: $I(N) = -\sum_j p(\omega_j)\log_2 p(\omega_j)$, where $p(\omega_j)$ accounts for fraction of patterns at node N that are in category ω_j. If all patterns are the same (i.e., the node is pure), then $I(N) = 0$.
- Variance impurity, defined in the two-category case as $I(N) = p(\omega_1)p(\omega_2)$, which gives $I(N) = 0$ when all patterns belong to the same class, either ω_1 or ω_2.
- Gini impurity, which consists in the extension of variance impurity to more than two classes: $I(N) = \sum_{i \neq j} p(\omega_i)p(\omega_j)$. When compositions between ω_i and ω_j are unbalanced, it is necessary to weight $I(N)$ with a weight matrix with γ_{ij} elements, thus giving $I(N) = \Sigma_{i \neq j}\gamma_{ij}p(\omega_i)p(\omega_j)$.
- Misclassification impurity, measuring the minimum probability that a training pattern must be misclassified.

As stated above, the achievement of the minimum node impurity is the driver of the tree optimization. To select the best split, the decrease of impurity after the split is calculated for each variable, and the variable that maximizes this drop is the one selected.

From all the impurity measurements available, the Gini index and entropy impurity are often preferred due to its computational simplicity. However, in spite of the diversity of measures, very often the choice if the impurity functions does not affect excessively the accuracy of the final classifier. This leaves the stopping criterion and the pruning method as main drivers of the model accuracy.

The stopping criterion controls predictive performance, limiting the excessive growth of the tree and thus controlling the overfit risk. It may be determined by using standard validation procedures such as cross-validation and test set. An alternative way to limit the tree growth implies setting up a threshold β, which has the obvious advantage that all samples can be used for training the classifier. This approach often leads to an unbalanced tree, where the leaf nodes lay at different levels. However, finding optimal β is not an easy task, as it has little to say with model performance. An alternative, simpler in concept, is to set a minimum node size of a fixed number or percentage of objects. This procedure has the advantage that it adapts the partition size to the density of objects in that region of the multivariate space. Complexity of the tree can also be used as an alternative to stop the growth of the tree, using a balance between the number of nodes present in the tree and the uncertainty of the tree on the training data. Alternatively, the splitting can be stopped using hypothesis testing on the increase of node impurity between the tree layers (usually a χ^2 test).

Using the stopping criterion directly has important drawbacks. Often it leads to biased trees, because the biggest decreases of impurity are achieved in the nodes closer to the root node. Consequently, tree growth stops too early, ignoring further splits. For this reason, growing full trees and posterior pruning has gained acceptance. In this approach, all pairs of leaf (terminal) nodes connected to the same ancestor (one level above) are considered for elimination. Each pair providing a small increase in node impurity is selected for elimination. At this point, the ancestor becomes a leaf. This is basically the inverse of the splitting process described previously, and commonly delivers unbalanced trees. Its main drawback is that it is computationally intensive, which in turn limits its application in very big datasets.

3.4 Ensemble Classifiers: From Single Trees to Random Forests

If they are grown deep enough, trees are learners with low bias and high variance that reflect the data structure quite well. These characteristics make them suitable for performance improvement using general methodologies such as bootstrap aggregation (bagging) [24]. The main idea of bagging is to reduce the variance of the prediction through averaging (regression) or aggregated voting (classification) among several classifiers. This is achieved through the average of many noisy (and approximately unbiased) models. Models are built taking a bootstrap sample with replacement of the hold out data. This strategy gives a trade-off solution, as this averaging provides a decrease in the variance, but with a little increase in bias. Another positive effect of aggregation and majority voting is the decrease in chances of overfit.

Targets of boosting [25] are weak learning algorithms (as trees). The approach generates m different individual classifiers to create an ensemble classifier $G(X)$. In such approach, weight is given to the individual classifiers $G_m(x)$ according to their accuracy. This weighting is used to provide a final result, weighting accordingly the output of the different classifiers $G(X) = sign[\Sigma_{m=1}^{M} \alpha_m G_m(x)]$. In this way, misclassified observations are scaled by an exponential factor that increases the importance of this pattern in the next $G_{m+1}(x)$ model. One popular version of boosting, with good performance, is ADABOOST [26].

The concept of ensemble classifiers is in the deep roots of the random forests (RF) algorithm [27]. Random forests are an ensemble of classification (regression) trees that are trained using bootstrapped (with replacement) samples of the training data. Trees are fully grown and posteriorly pruned to a certain node level (specified) and the remaining patterns ("out-of-bag," OOB samples) are predicted after passing them through the whole ensemble (forest). As in bagged classifiers, majority voting is used to assign the target class. When used in regression, averaging is used instead. One of the novel points introduced in the approach is the application of a secondary randomization scheme, taking a bootstrap sample of the variables at each splitting node, to infer variable relevance (feature extraction). In this way, RF reduces the

Table 3.1 Algorithm: random forests for regression and classification

In training
For $b = 1$ to B:
Draw a bootstrap sample Z^* of size N from training data
Grow a tree T_b to the bootstrapped data, by recursively repeating the following steps (I–III) for each terminal node of the tree, until minimum node size n_{min} is reached
I. Select m variables at random from the p variables
II. Pick the best variable/split point among the m
III. Split the node into two daughter nodes
Output the ensemble of trees $\{T_b\}_1^B$
In prediction
To make a prediction of the pattern $x \mid x \notin Z$ (out-of-bag samples)
Regression: $\hat{f}_{rf}^B(x) = \frac{1}{B}\Sigma_{b=1}^B T_b(x)$
Classification: Let $\hat{C}_b(x)$ be the class prediction of pattern x at bth tree of the RF. Obtain the prediction for the overall forest through $\hat{C}_{rf}^B = majority\ \ vote\left\{\hat{C}_b(x)\right\}_1^B$

Table modified from Hastie et al. [23], Copyright 2009, with permission from Springer-Verlag New York

variance through bagging, but simultaneously de-correlating the variables. This dual randomization scheme has other interesting properties, such as the reduction of the dependence between trees and the suitability of the OOB error rate of an estimator of the generalization ability (Table 3.1).

Number of parameters to be tuned in RF is relatively small: number of variables selected in the node variable subsampling (n_p), total number of trees (n_t), and tree depth (t_d). Indeed, its sensitivity to them is reduced. Furthermore, the literature on the topic provides good practice recommendations [28]. Thus, a good value of n_p should be around $\sqrt{p}$ for RF in classification and $p/3$ in regression mode. Once set to this suggested values (or around), RF models usually provide a good predictive performance. This parameter may become critical when the dimensionality of the set (p) is small. In this very particular case, it is necessary to consider which the expected ratio between informative and noninformative variables is. Low values of the number of selected variables can lead to a big decrease in predictive performance of the RF, as some of the important variables can be ignored. Even if this ratio is very hard to know at the very beginning of the analysis, it is rarely the case in metabolomics datasets (usually highly p dimensional).

One of the main claims of RF is that they do not overfit [27]. In general, this is a true assumption, but with some remarks. Number of trees (n_t) can be determined with relative simplicity, just plotting the evolution of the OOB error rate according to the number of trees included in the forest. If n_t increases too much, chances are that all variance (relevant or not) is contained in the forest. In these conditions, the model may become too rich and too close to the training data, the right conditions for an overfitted model [23].

Similarly to n_p, proposed tree depths are 1 and 5 depending on the RF mode (classification and regression, respectively). This is a parameter that makes little sense to optimize, as the little gains in predictive performance described in the literature do not justify optimizing one more parameter.

The ensemble nature of the RF limits one of the nice features of trees, which is the convenient representation of the data structure. The average of b_{n_t} trees makes rather complex to determine the relevant structure common to the different bootstrapped samples of the main dataset. However, RF provides key parameters to identify the importance of the variables and to visualize the similarities among samples.

Variable importance measures allow the identification of their role in the structure of the dataset. The two most used in available RF implementations are based on:

I. *Changes in node impurity*. At each split, the improvement criterion (impurity decrease) is stored and cumulated individually for each variable. The procedure is applied to each of the b_{n_t} trees of the forest and averaged. This estimator is mostly used in classification, using the Gini index as improvement criterion.
II. *Changes in the accuracy of the model*. In this estimator, the importance of the variable is linked to its predictive strength. For its calculation, there are used the samples in the OOB, using the following scheme:
    (a) At each b tree, OOB samples are passed down to the tree to get the model accuracy.
    (b) The values of the jth variable are permuted randomly in the OOB samples. Accuracy is recalculated accordingly.
    (c) Decreases of accuracy due to permutation are averaged over all the b trees of the random forest B. This gives an estimation of the importance of the variable j. Importance is then converted to percentage.

In general, this approach provides more gradual variable importance estimations than the changes in node impurity. It is mostly used in regression, estimating the accuracy as mean standard error $MSE_{OOB} = \frac{1}{b}\sum_{1}^{b}\left\{y_i - \hat{y}_i^{OOB}\right\}^2$ (MSE).

On the other side, proximities can be calculated while building the forest with the aim of visualizing similitude patterns between objects. To construct such proximity matrix, for each tree $b(x; \theta_b)$, any pair of observations in the OOB set sharing a leaf (terminal node) have their proximity increased by one. To visualize these proximities between patterns, a multidimensionality scaling (MDS) plot is used. In brief, this approach consists in an eigenvalue decomposition of the distance matrix and is key in approaches such principal coordinate analysis [29].

The interpretation of these plots in RF is often controversial, as their utility is often questioned by some authors. Often they show a similar star shape, with each arm corresponding to an individual subclass. The more pronounced is the separation between groups, the better the performance of the RF is expected. Some numerical experiments show how the classifier boundaries are usually in the center of the star-shaped cloud, while better separated samples lay in the edges. This point makes sense, as similar patterns have more chances to end up in the same terminal node. On the opposite, more dissimilar patterns have much less chances of sharing those kinds of nodes (Fig. 3.1).

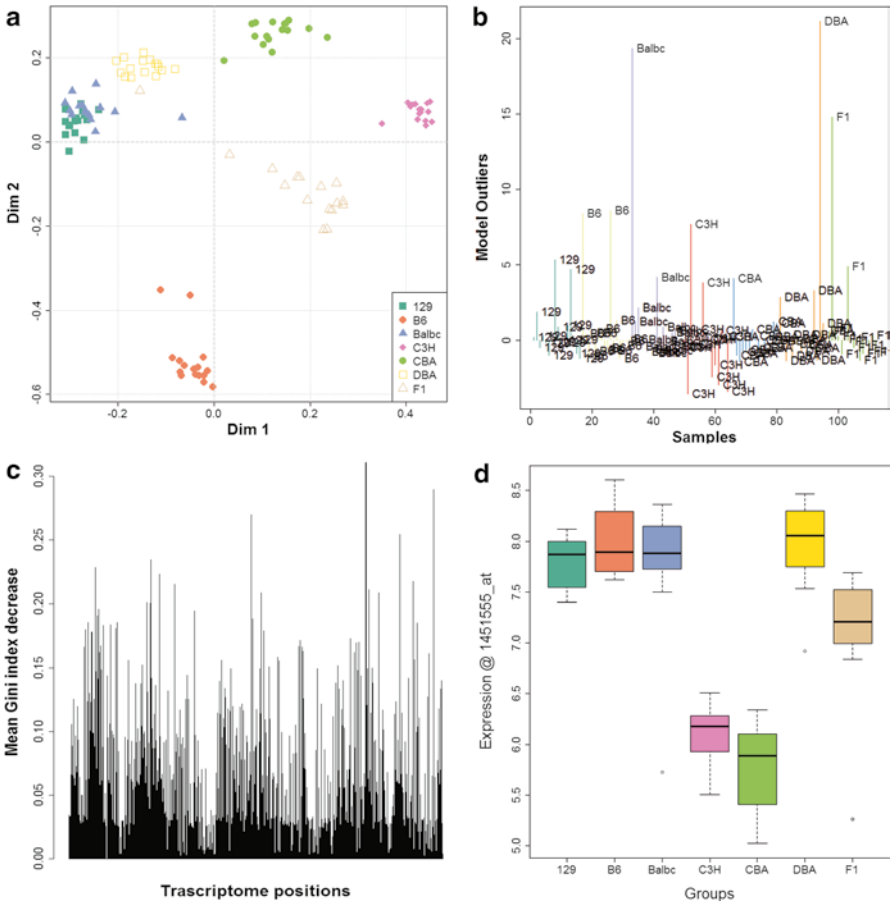

Fig. 3.1 Analysis of transcriptomics data from seven mice strains [73]. Graphical evaluation of Random Forests results based in sample proximities: multidimensional scaling (**a**) shows differences between groups of samples (**b**) Highlighting samples with extreme behavior (outliers). (**c**) Variable importance measure (classification) based in the mean Gini index decrease (scaled) after variable permutation. Expression levels at position expressing a highest mean decrease in the Gini index (**d**)

Other visualizations of the role of the objects in the model can be achieved using the information contained in the proximity matrix. Thus, proximity information may also be used to evaluate the outlier character of the samples in RF. To determine this feature, the reciprocal of the sum of squared proximities is calculated between that observation and the remaining ones within each class. Extreme values will point out those objects with high influence in the overall performance of the model. Often, they correspond to patterns that have been incorrectly classified or with high standard errors.

In principle, RF can handle continuous and categorical discrete variables. However, in these cases, there has been detected possible bias in the assignment of variable importance [30]. There have been identified two major sources for this behavior, mainly the important changes in the measurement scale and the oscillation in the number of categories. Two explanations on the mechanism underlying those

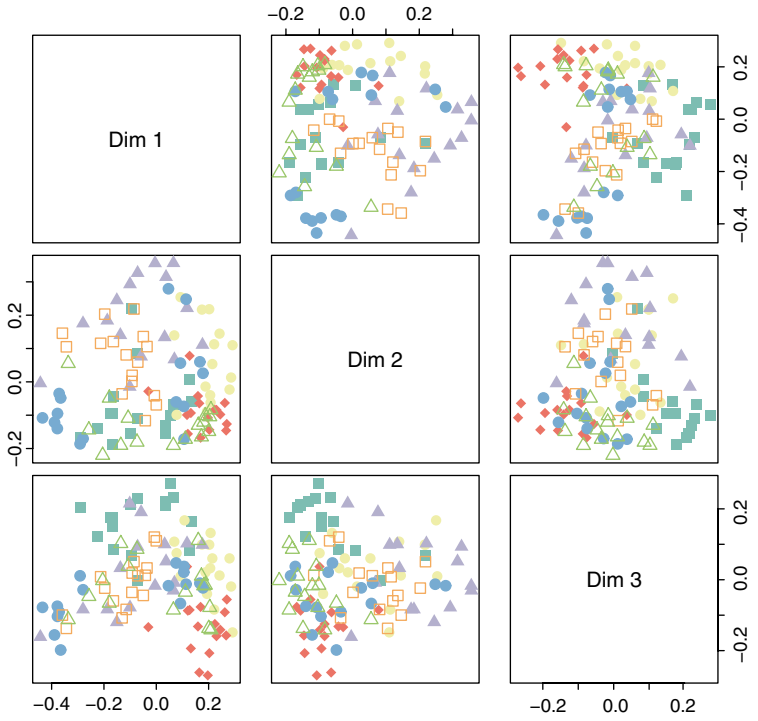

Fig. 3.2 Analysis of transcriptomics data from seven mice strains [73]. Graphical evaluation of unsupervised random forests results based in sample proximities: multidimensional scaling shows the differences between groups of samples on the first three dimensions

deficiencies are the biased variable selection in each individual classification tree and the side effects of a bootstrapping with replacement (as used in RF).

RF can be also used in unsupervised mode to describe the structure of the dataset [28] alike in principal component analysis (PCA) [8]. The key point is to create a two-class case, where one class is the real dataset and the other class is generated synthetically to be as close as possible as the original dataset. Both sets are combined, and a RF model is built to predict both classes. As an outcome, objects that are similar will lay in the same terminal nodes. This information is recorded in the proximity matrix and can be visualized using the MDS plot (Fig. 3.2).

Summarizing, RF can be used in the buildup of supervised models of classification or regression, since they are models with good performance, even in highly complex real data. They are tolerant with missing data and can be easily transformed in an unsupervised model, if used smartly, to describe the structure of the data. They provide efficient tools for variable selection for homogeneous data sets, even if the validity of the approach for heterogeneous datasets (continuous and discrete data) can be argued. Moreover, the implementation of outlier detection features enables their use for event detection.

3.5 Recent Contributions from RF in the Metabolome-Microbiome Arena

Since the publication of the initial algorithm in 2001 [27], the interest in RF has undergone an exponential growth. The amount of publications has increased from less than 10 in 2002 to more than 500 in 2012 (www.scopus.com), covering a wide spectrum of areas of interest in science. From the very beginning, computer science and biochemistry, genetics, and molecular biology publications have been maintaining a sustained lead, partially due to the strong interaction they have through multidisciplinary fields such as bioinformatics. The progressive growth in interest in RF has been especially relevant in the medical community, wide-spreading applications that have gone from support to diagnostics to biomedical signal processing (imaging and multiparametrical monitoring). Other fields such as engineering, agriculture sciences, environmental science, and chemistry have also devoted a remarkable interest in RF as a partner tool for their applications in several fields, such as remote sensing, prediction of environmental parameters, and quantitative structure activity relationships in chemistry. In parallel to the exponential increase in publications using the RF approach, it is also remarkable a qualitative aspect: the progressive increase of heterogeneity of fields of application. This growth that can be considered normal in a new coming procedure has stabilized at a high number of fields. One possible interpretation of this fact is the awareness of the global scientific community on the performance of the algorithm, which is in the process of its consolidation as a standard tool of machine learning.

3.6 Integration Between Information Sources: RF and Networks

Data fusion using RF has been performed mostly in engineering on several domains, from medical to environmental applications. The approach is often used in high-dimensional datasets coming from imaging, multisensorial applications and even microarrays. A medical application example of this integration can be found in the integration between transcriptomics and dermatoscopy in cutaneous melanoma patients, which has led to improved classifications using RF [31], or eventually in their key role in handling structural and functional imaging for prostate cancer diagnosis [32]. Environmental sciences have also benefited of the approach, applying RF regression models onto datasets fusing Lidar, Radar, and multispectral remote sensing to predict multiyear bird detections (migratory activity) of eight bird species [33] and structural forest attributes [34].

The integration of random forests as a preliminary feature extraction step in network analysis has become a relevant procedure in several areas such as pathway analysis and data integration and in describing the relationship between chemical structure and interactions. Pathway analysis has benefited from the generalization of

the use of random forests as feature extraction method in microarray data to rank important pathways from externally available databases [35]. The feature extraction properties of RF have been also exploited in feeding networks integrating several "-omics" datasets (metabolomics, gene expression) and phenotypic traits [36–38].

Moreover, RF have demonstrated to be useful, and even better performing than other methods, in describing protein-protein interactions [39, 40], the prediction of long disordered regions within proteins [41], the identification of protein complexes using topological structure from local subgraphs [42], and even predicting binding sites by using structural information [43]. RNA-protein interactions using sequence information were tested with success with two classifiers based in RF and SVM as a basis to further create RNA-protein interaction networks [44]. Still in this area, the integrative analysis of co-expression modules (gene ontology, protein-protein interaction data and literature) using RF enabled finding interesting gene-phenotype associations [45].

In general, the use of structural information is important to understand the interaction mechanisms but can also be relevant to facilitate drug design. A good example of the application with success of RF into this setup can be found in the prediction of sulfotyrosine binding sites, where the RF are winning option to SVM, ANN, and hidden Markov models (HMM) [46]. Their performance was still highly scored in the integration of chemical, genomic [47], and pharmacological information to determine drug-target interactions [48], where they can be compared to other methods such SVM [49], to help in the assessment of the reliability of the results.

Furthermore, a two-stage-based RF analysis was performed to characterize the functional effects of single amino acid variants (SAV) combining sequence, structure, and residue-contact network features [50]. RF models showed their utility not only in highlighting functional regions but also in scoring protein interactions from one organism model into another to be used in protein interaction networks [51]. The antioxidant biological activity of proteins associated to star graph topographical indices was better highlighted with RF, when compared with other methods [52].

Classifier performance is one of the main points of interest in all applications where those models are the working horse to provide insight on data, or they are even the main outcome to be achieved. RF has been explored in combination with other classifiers to provide better overall predictions through using consensual outputs. In these setups, it is necessary to find a precision index measure that summarizes individual performances, incorporating concepts such as maximum posterior probability [53]. Patient treatment strategies can also benefit from this multiclassifier strategy, for instance, the prediction of the coreceptor usage in HIV-1 patients using translated V3 genotypes as input [54].

3.7 Application-Driven Improvements to RF Scheme

The standard RF method based in the routines proposed initially by Breiman is still actual, very performing and widely applied into different fields. Notwithstanding, there have appear modifications to the original algorithm, with the aim of adapting

it better for the purpose of the application [55]. GWAS analyses are a very particular setup, with a very unbalanced n-to-p ratio. Analyzing them using a RF approach should have advantages over other strategies, in particular for the detection of interactions between single nucleotide polymorphisms (SNPs). Despite this, careful evaluation of the RF performance in such huge p, low n analyses has detected limitations in the utilization of RF to detect interactions [56]. Thus, GWAS analysis with RF is an example of special request that needs to be addressed with "ad hoc" customizations, for instance, the modification of the sampling scheme introducing a stratified sampling of the SNPs [57]. The aim of the approach is dual: avoiding highly computational costs derived from an exhaustive analysis and keeping enough informative SNPs at the same time. A similar example of a modification of the RF scheme to limit the number of variables in GWAS analyses is the application of search algorithms based on simulated annealing and genetic programming, basis of the random forests fishing (RFF) model. In this approach, the dimensionality is reduced updating repeatedly a limited set of variables obtained by RF tests to find groups of variables predictive of the target phenotype.

Not only variable subset selection, but also choosing the number of trees to be included in the classifier has received attention. In this way, there has been proposed a dynamic determination of the number of trees during the growing of the forest [58]. This approach goes in the opposite direction to growing an excess of trees and select afterwards their optimal number checking the model performance. Furthermore, changes in the voting mechanism based on weighting [59], feature selection, clustering, nearest neighbors, and optimization techniques have been proposed [60].

In this process of revisiting some of the key aspects of RF, variable importance measures were also reevaluated focusing in their performance on extreme cases of recognized limited performance of RF, such in highly unbalanced datasets [61]. With this aim, a more robust variable importance indicator was proposed, now using a variant at the variable permutation step based in the area under the curve (AUC) [30]. The application of clustering techniques (partition around medoids, PAM) to the RF proximity matrix also enabled the identification of regulatory cliques in transcriptomics data from yeast [62]. When assessing the performance of variable importance measures in feature ranking, RF showed a limited performance in some cases. With this purpose, average gain measure and the similarity-weighted estimate were introduced with success to replace information gain and maximum likelihood estimates [63].

More from an algorithmically point of view, improvements in predictive performance have been achieved by proven variable selection schemes, such backward variable elimination, and introducing changes in the tree induction procedure that attempt to complement the trees in the ensemble [64]. The application of backward variable elimination outperforms both single classification trees and standard RF and provides similarity measures that successfully cluster samples per molecular pattern [65]. Moreover, some of the algorithm basics implemented in RF methodology (bagging and model aggregation) have been also a source for inspiring new modelling approaches, such the stabilization of recursive partitioning models [66]

and the adaptation of RF into semi-supervised learning [67]. In a kind of loop-back, boosting approaches have also benefited from RF, through their use as weak learners in ADABOOST to decrease chances in over fit [68].

3.8 New Challenges to RF: Network Inference

The possibilities of random forests have been extensively tested in many environments in biology, even as part of complex strategies comprising ensembles of data, models, and methodologies. Aside from those cases in which the predictive step is key for the application, their properties in feature extraction have become extremely relevant with a strong impact in the field, feeding networks for further graph-based modelling. Even if useful, this kind of approach reduces RF to a filtering step to select the relevant variables from a set of measurements. Then, the challenge now is to determine if RF could be directly incorporated into a model to benefit from their statistical properties for network inference.

One possible approach of network inference using RF goes through the decomposition of the regulatory network of p variables onto p regression problems and its posterior evaluation with RF in regression [69]. Edge's estimation is performed building a RF model of $p_{i \neq k}$ variables to predict p_k and using the variable importance measure to estimate the importance of the link. These putative link strengths are further used to build the network model. Using this setup, properties from RF are inherited into the network: no assumptions on the nature of the variable (both linear and nonlinear interactions are allowed), and they provide directed graphs, are fast to compute, and are relatively easy to scale up. Furthermore, this is an approach able to generalize easily up to many types of data and even capable to integrate data from different sources (microbiome-metabolome).

Pathway analysis is a powerful approach to add interesting insights to the outcome of genome-wide association study (GWAS) analyses. Most of the pathway methods are based in testing the cumulative main effects associated to a phenotype (disease). However, gene-gene interactions are also expected to have relevance on the etiology of disease. To tackle this information, a two-stage RF-based algorithm has been proposed, which is a restricted variant of a previously published one [70]. In this algorithm, RF is performed twice in the set of SNPs to reduce the variable size and thus increase the power in classification. In a first round, all SNPs corresponding to a user-specified pathway are modelled according to the target stratification, and SNPs above a certain threshold in their variable importance are kept to be used in a second round. During this step, the dataset is reanalyzed using just the relevant SNPs, and the prediction error rate of the model is used to generate a score of the pathway. The significance of the score is assessed empirically using a permutation test [71].

Integrating pathway information to identify similarities among them has been also explored using RF [72]. In this setup, RF is used to build pathway clusters using tight clustering, which are supposed to agglomerate pathways sharing similar

functions (even if they do not share specific variables). The approach models the phenotype using sets of variables from different pathways, and their prediction errors are used for further clustering. In some way, the approach is linking similitude between RF models with pathway similitude. To sustain these proximities, OOB errors rate the performance of the pathway to describe a specific phenotype, thus giving an idea of RF model – pathway potential interest. In this approach, the handling of the OOB errors is quite interesting, as it uses class votes to define pathway distances that can be clustered, later on. These class votes are defined using the proportion of votes of each class, for a specific subject, along all trees in the forest. An interesting point of the approach is that enables the comparison between pathways, even if they do not share variables (genes, metabolites). This is an interesting point for data integration, as it enables the comparisons between pathways of different organisms (host, microbiome) sharing same phenotype.

3.9 Conclusions and Perspective

Since their introduction, RF has become an alternative for all those cases in which reliability of other models are compromised. Their nonparametric character, its capability in handling low n, high p datasets, its predictive performance, and its low tendency to over fit have been largely in favor of its consolidation as a general purpose tool.

This consolidation does not imply this is a completely frozen approach. New application-driven developments, improvements in the internals of the algorithm, and changes in the mechanisms for feature selection reflect the research area is fully active and still under development. Moreover, the validity of the approach is sustained by proposed enhancements to tailor other analysis setups, as network modelling or data integration. These ongoing proposals make integration between data from different organisms/compartments and their association with phenotype highly susceptible to benefit from RF approach.

References

1. Dudoit S, Shaffer JP, Boldrick JC. Multiple hypothesis testing in microarray experiments. Stat Sci. 2003;18(1):71–103.
2. Shaffer JP. Multiple hypothesis testing. Annu Rev Psychol. 1995;46(1):561–84.
3. Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G. XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. Anal Chem. 2006;78(3):779–87.
4. Nicholson JK, Lindon JC, Holmes E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica. 1999;29(11):1181–9.
5. Fiehn O. Metabolomics – the link between genotypes and phenotypes. Plant Mol Biol. 2002;48(1–2):155–71.

6. Montoliu I, Genick U, Ledda M, Collino S, Martin FP, Le Coutre J, et al. Current status on genome-metabolome-wide associations: an opportunity in nutrition research. Genes Nutr. 2013;8(1):19–27.
7. Massart DL, Vandeginste BGM, Buydens LMC, De Jong S, Lewi PJ, Smeyers-Verbeke J. Handbook of chemometrics and qualimetrics. Amsterdam: Elsevier Science B.V.; 1997.
8. Jolliffe IT. Principal component analysis. New York: Springer; 2002.
9. Wold S, Sjöström M, Eriksson L. PLS-regression: a basic tool of chemometrics. Chemom Intell Lab Syst. 2001;58(2):109–30.
10. Geladi P, Kowalski BR. Partial least-squares regression: a tutorial. Anal Chim Acta. 1986;185(C):1–17.
11. Trygg J, Wold S. Orthogonal projections to latent structures (O-PLS). J Chemom. 2002;16(3):119–28.
12. Trygg J, Wold S. O2-PLS, a two-block (X-Y) latent variable regression (LVR) method with an integral OSC filter. J Chemom. 2003;17(1):53–64.
13. Westerhuis JA, Hoefsloot HCJ, Smit S, Vis DJ, Smilde AK, Velzen EJJ, et al. Assessment of PLSDA cross validation. Metabolomics. 2008;4(1):81–9.
14. Barker M, Rayens W. Partial least squares for discrimination. J Chemom. 2003;17(3):166–73.
15. Bylesjö M, Rantalainen M, Cloarec O, Nicholson JK, Holmes E, Trygg J. OPLS discriminant analysis: combining the strengths of PLS-DA and SIMCA classification. J Chemom. 2006;20(8–10):341–51.
16. Westerhuis JA, van Velzen EJJ, Hoefsloot HCJ, Smilde AK. Multivariate paired data analysis: multilevel PLSDA versus OPLSDA. Metabolomics. 2010;6(1):119–28.
17. Cloarec O, Dumas ME, Craig A, Barton RH, Trygg J, Hudson J, et al. Statistical total correlation spectroscopy: an exploratory approach for latent biomarker identification from metabolic 1H NMR data sets. Anal Chem. 2005;77(5):1282–9.
18. Cortes C, Vapnik V. Support-vector networks. Mach Learn. 1995;20(3):273–97.
19. Hagan MT, Menhaj MB. Training feedforward networks with the Marquardt algorithm. IEEE Trans Neural Netw. 1994;5(6):989–93.
20. Guyon I, Weston J, Barnhill S, Vapnik V. Gene selection for cancer classification using support vector machines. Mach Learn. 2002;46(1–3):389–422.
21. Breiman L, Friedman J, Olshen R, Stone C. Classification and regression trees. Boca Raton: CRC Press LLC; 1984.
22. Duda RO, Hart PE, Stork DG. Pattern classification. 2nd ed. New York: Wiley; 2001.
23. Hastie T, Tibshirani R, Friedman J. The elements of statistical learning. Data mining, inference and prediction. 2nd ed. New York: Springer; 2009. p. 588.
24. Breiman L. Bagging predictors. Mach Learn. 1996;24(2):123–40.
25. Friedman J, Hastie T, Tibshirani R. Additive logistic regression: a statistical view of boosting. Ann Stat. 2000;28(2):337–407.
26. Schapire RE, Singer Y. Improved boosting algorithms using confidence-rated predictions. Mach Learn. 1999;37(3):297–336.
27. Breiman L. Random forests. Mach Learn. 2001;45(1):5–32.
28. Liaw A, Wiener M. Classification and regression by randomForest. R News. 2002;2(3):18–22.
29. Borg I, Groenen P. Modern multidimensional scaling: theory and applications. New York: Springer Verlag; 2005.
30. Janitza S, Strobl C, Boulesteix AL. An AUC-based permutation variable importance measure for random forests. BMC Bioinforma. 2013;14:119.
31. Moutselos K, Maglogiannis I, Chatziioannou A, editors. Heterogeneous data fusion and selection in high-volume molecular and imaging datasets. IEEE 12th conference on Bioinformatics and Bioengineering proceedings 2012;407–412.
32. Viswanath S, Bloch BN, Rosen M, Chappelow J, Toth R, Rofsky N, et al. Integrating structural and functional imaging for computer assisted detection of prostate cancer on multi-protocol in vivo 3 tesla MRI. SPIE Medical Imaging 2009;7260.
33. Swatantran A, Dubayah R, Goetz S, Hofton M, Betts MG, Sun M, et al. Mapping migratory bird prevalence using remote sensing data fusion. PLoS ONE. 2012;7(1):e28922.

34. Latifi H, Nothdurft A, Straub C, Koch B. Modelling stratified forest attributes using optical/LiDAR features in a central European landscape. Int J Digit Earth. 2012;5(2):106–32.
35. Pang H, Lin A, Holford M, Enerson BE, Lu B, Lawton MP, et al. Pathway analysis using random forests classification and regression. Bioinformatics. 2006;22(16):2028–36.
36. Acharjee A, Kloosterman B, de Vos RCH, Werij JS, Bachem CWB, Visser RGF, et al. Data integration and network reconstruction with -omics data using Random Forest regression in potato. Anal Chim Acta. 2011;705(1–2):56–63.
37. Chen Z, Zhang W. Integrative analysis using module-guided random forests reveals correlated genetic factors related to mouse weight. PLoS Comput Biol. 2013;9(3):e1002956.
38. Tang X, Xiao J, Li Y, Wen Z, Fang Z, Li M. Systematic analysis revealed better performance of random forest algorithm coupled with complex network features in predicting microRNA precursors. Chemom Intell Lab Syst. 2012;118:317–23.
39. Lin N, Wu B, Jansen R, Gerstein M, Zhao H. Information assessment on predicting protein-protein interactions. BMC Bioinforma. 2004;5:154.
40. Lee J, Lee J. Hidden information revealed by optimal community structure from a protein-complex bipartite network improves protein function prediction. PLoS ONE. 2013;8(4):e60372.
41. Han P, Zhang X, Norton RS, Feng ZP. Large-scale prediction of long disordered regions in proteins using random forests. BMC Bioinforma. 2009;10:8.
42. Li ZC, Lai YH, Chen LL, Zhou X, Dai Z, Zou XY. Identification of human protein complexes from local sub-graphs of protein-protein interaction network based on random forest with topological structure features. Anal Chim Acta. 2012;718:32–41.
43. Zheng C, Wang M, Takemoto K, Akutsu T, Zhang Z, Song J. An integrative computational framework based on a two-step random forest algorithm improves prediction of zinc-binding sites in proteins. PLoS ONE. 2012;7(11):e49716.
44. Muppirala UK, Honavar VG, Dobbs D. Predicting RNA-protein interactions using only sequence information. BMC Bioinforma. 2011;12:489.
45. Mehan MR, Nunez-Iglesias J, Dai C, Waterman MS, Zhou XJ. An integrative modular approach to systematically predict gene-phenotype associations. BMC Bioinforma. 2010;11 Suppl 1:S62.
46. Yang ZR. Predicting sulfotyrosine sites using the random forest algorithm with significantly improved prediction accuracy. BMC Bioinforma. 2009;10:361.
47. Cao DS, Liang YZ, Deng Z, Hu QN, He M, Xu QS, et al. Genome-scale screening of drug-target associations relevant to Ki using a chemogenomics approach. PLoS ONE. 2013;8(4):e57680.
48. Heider D, Verheyen J, Hoffmann D. Predicting Bevirimat resistance of HIV-1 from genotype. BMC Bioinforma. 2010;11:37.
49. Yu H, Chen J, Xu X, Li Y, Zhao H, Fang Y, et al. A systematic prediction of multiple drug-target interactions from chemical, genomic, and pharmacological data. PLoS ONE. 2012;7(5):e37608.
50. Wang M, Zhao XM, Takemoto K, Xu H, Li Y, Akutsu T, et al. FunSAV: predicting the functional effect of single amino acid variants using a two-stage random forest model. PLoS ONE. 2012;7(8):e43847.
51. Pesch R, Zimmer R. Complementing the eukaryotic protein interactome. PLoS ONE. 2013;8(6):e66635.
52. Fernandez-Blanco E, Aguiar-Pulido V, Robert Munteanu C, Dorado J. Random forest classification based on star graph topological indices for antioxidant proteins. J Theor Biol. 2013;317:331–7.
53. Ko D, Windle B. Enriching for correct prediction of biological processes using a combination of diverse classifiers. BMC Bioinforma. 2011;12:189.
54. Masso M, Vaisman II. Accurate and efficient gp120 V3 loop structure based models for the determination of HIV-1 co-receptor usage. BMC Bioinforma. 2010;11:494.
55. Liu S, Chen Y, Wilkins D. Large margin classifiers and random forests for integrated biological prediction. Int J Bioinforma Res Appl. 2012;8(1–2):38–53.
56. Winham SJ, Colby CL, Freimuth RR, Wang X, de Andrade M, Huebner M, et al. SNP interaction detection with Random Forests in high-dimensional genetic data. BMC Bioinforma. 2012;13:164.

57. Wu Q, Ye Y, Liu Y, Ng MK. SNP selection and classification of genome-wide SNP data using stratified sampling random forests. IEEE Trans Nanobiosci. 2012;11(3):216–27.
58. Tripoliti EE, Fotiadis DI, Manis G. Automated diagnosis of diseases based on classification: dynamic determination of the number of trees in random forests algorithm. IEEE Trans Inf Technol Biomed. 2012;16(4):615–22.
59. Robnik-Sikonja M. Improving random forests. 2004.
60. Tripoliti EE, Fotiadis DI, Manis G. Modifications of the construction and voting mechanisms of the random forests algorithm. Data Knowl Eng. 2013;87:41–65.
61. Anaissi A, Kennedy PJ, Goyal M, Catchpoole DR. A balanced iterative random forest for gene selection from microarray data. BMC Bioinforma. 2013;14:261.
62. Xiao Y, Segal MR. Identification of yeast transcriptional regulation networks using multivariate random forests. PLoS Comput Biol. 2009;5(6):e1000414.
63. Jiang L. Learning random forests for ranking. Front Comput Sci China. 2011;5(1):79–86.
64. Bernard S, Adam S, Heutte L. Dynamic random forests. Pattern Recogn Lett. 2012;33(12):1580–6.
65. Li S, Fedorowicz A, Singh H, Soderholm SC. Application of the random forest method in studies of Local Lymph Node Assay based skin sensitization data. J Chem Inf Model. 2005;45(4):952–64.
66. Garge NR, Bobashev G, Eggleston B. Random forest methodology for model-based recursive partitioning: the mobForest package for R. BMC Bioinforma. 2013;14:125.
67. Leistner C, Saffari A, Santner J, Bischof H, editors. Semi-supervised random forests. 2009.
68. Zeng JY, Cao XH, Gan JY. An improvement of AdaBoost for face detection with random forests. ed. CCIS; 2010;93: 22–9.
69. Huynh-Thu VA, Irrthum A, Wehenkel L, Geurts P. Inferring regulatory networks from expression data using tree-based methods. PLoS ONE. 2010;5(9):e12776.
70. Chang JS, Yeh RF, Wiencke JK, Wiemels JL, Smirnov I, Pico AR, et al. Pathway analysis of single-nucleotide polymorphisms potentially associated with glioblastoma multiforme susceptibility using random forests. Cancer Epidemiol Biomarkers Prev. 2008;17(6):1368–73.
71. Chung RH, Chen YE. A two-stage random forest-based pathway analysis method. PLoS ONE. 2012;7(5):e36662.
72. Pang H, Zhao H. Building pathway clusters from Random Forests classification using class votes. BMC Bioinforma. 2008;9:87.
73. Collino S, Martin F-P, Montoliu I, Barger J, Da Silva L, Prolla T, et al. Transcriptomics and metabonomics identify essential metabolic signatures in calorie restriction (CR) regulation across multiple mouse strains. Metabolites. 2013;3(4):881–911. PubMed PMID: doi:10.3390/metabo3040881.

Chapter 4
Metabonomics in Translational Research for Personalized Medicine and Nutrition

Guoxiang Xie and Wei Jia

Abstract Personalized medicine promises prediction, prevention, and treatment of illness that is targeted to individuals' needs. New technologies for detailed biological profiling of individuals at the molecular level have been crucial in initiating the move to personalized medicine. Metabonomics is promising to contribute significantly to the characterization of various disease phenotypes and to the identification of personal metabolic features that can predict response to therapies. Based on analytical platforms such as mass spectrometry or nuclear magnetic resonance spectroscopy, the metabonomic approach enables a comprehensive overview of the metabolites, leading to the characterization of the metabolic profiles of a given sample. These metabolic profiles can then be used to distinguish between different disease phenotypes and to predict a drug's effectiveness and/or toxicity. Metabonomics has tremendous potential to advance our understanding of human health and disease and to inform the development of personalized approaches to disease prevention, diagnosis, and treatment.

Keywords Gut microbiota • Metabonomics • Personalized medicine • Personalized nutrition • Pharmacometabonomics • Pharmacogenetics • Biomarkers • Mass spectrometry • Nuclear magnetic resonance

4.1 Metabolomics and Metabonomics

The "-omics" sciences (Fig. 4.1), including genomics, transcriptomics, proteomics, and metabonomics, have emerged over the last two decades as a systems biology approach to obtain important insights into the role of host–gut microbial metabolic interactions in an individual's susceptibility to disease and treatment outcomes [1]. In particular, metabonomics will have a particular role with respect to other "-omics" sciences because of its ability to detect, in real time, the adaptive multiparametric responses of an organism to pathophysiological stimuli or genetic modifications [2].

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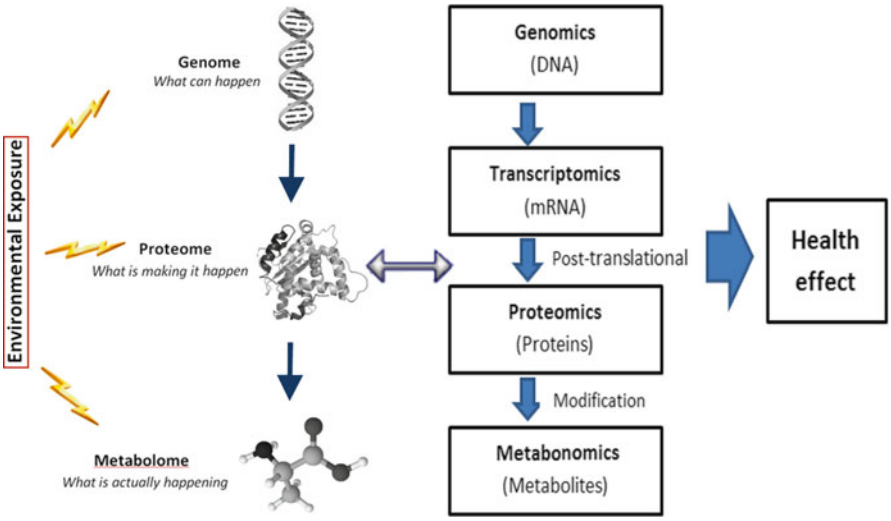

Fig. 4.1 The flow of the "omics" sciences: genomics, transcriptomics proteomics, and metabonomics technologies in individualized medicine

Metabolomics [3], or metabonomics [4], which is the quantitative measurement of dynamic metabolic changes of living systems using nuclear magnetic resonance (NMR) spectroscopy and/or mass spectrometry (MS), offers new opportunities to explore individual needs, foods, and nutrients functionalities and to formulate tangible biological hypotheses that can be tested at the individual and population scales. Monitoring the metabolic variations provides a unique insight into intra- and extracellular regulatory processes involved in our metabolic regulation and homeostasis. Application of metabonomics to diagnostics, drug research, and nutrition might be integral to improved health and personalized medicine [5]. To date, numerous metabolic profiling studies involving both animal models and human subjects have been reported in the field of personalized medicine or nutrition [6–9]. For more details, see Chaps. 1 and 2.

Metabonomics has been applied in clinical studies in two major areas. First area is the early diagnosis and characterization of disease phenotypes, where metabonomic analysis can detect a panel of metabolites that discriminate between groups of subjects, enabling the metabolic characterization of a disease, or of a disease phenotype. This is an exploratory process, since unexpected or even unknown metabolites may turn out to be important in this discrimination, paving the way to the formulation of new pathophysiological hypotheses [10, 11]. The second area of application is the identification of individual metabonomic characteristics able to predict drug effectiveness and/or toxicity – an approach termed as "pharmacometabonomics." The pharmacometabonomics is believed as a promising metabonomic approach for screening human populations, leading to a concrete possibility of a genuinely individualized approach to treatment [12, 13].

4.2 Metabonomics: The Link Between Genotypes and Phenotypes

Generally, the phenotype is not necessarily predicted by the genotype. The gap between the genotype and the phenotype is spanned by many biochemical processes, each with individual dependencies on various influences, including drugs, nutrition, and environmental factors. In this chain of biomolecules from the genes to the phenotype, metabolites are the quantifiable molecules with the closest link to the phenotype [14]. Many phenotypic and genotypic states, such as a toxic response to a drug, are predicted by differences in the concentrations of functionally relevant metabolites in biological fluids and tissues. Personalized medicine is currently based on the concept of pharmacogenomics that studies the influence of an individual's genotype and/or single-nucleotide polymorphisms (SNPs) on their response to a drug or medical treatment. Despite enormous efforts, pharmacogenomics has had limited success in clinical pharmacology to predict drug response with absolute certainty using single or multiple SNPs as biomarkers [15–18]. The main reason for the limitations is that the response is dependent upon the phenotype of an individual, which is determined by both genotype and the complex interactions between genes and other environmental factors [19, 20]. These environmental factors include diet, lifestyle, gut microbiota, nutrition, medications, age, and exposures to toxins or dietary supplements, as well as the individual physical and pathological conditions (e.g., type 2 diabetes and obesity). Therefore, it is critical to be able to assess an individual's metabolic phenotype, which will provide useful information for determining the correct drug and dose treatment and predicting the individual response following a therapeutic intervention.

The metabolic phenotype (metabotype) is a result of the overall influences of the patient's physiological status, gut microbiome status, and chemical, genetic, and other environmental factors. Changes in the metabotype reflected in the biofluid or tissue evaluated occur downstream of alterations in gene and protein expression. As such, the metabotype, which comprises the genotype and phenotype, represents the ultimate biological endpoint and can provide useful information about an individual's current physiological status that can be used for predicting the outcome prior to a therapeutic intervention.

The "-omics" technologies have been used to obtain a more holistic view of how biological systems work and underpin the base of functional genomics and systems biology. They are also widely employed to identify biomarkers for use in the diagnosis and monitoring of human disease. While "-omics" technologies such as transcriptomics and proteomics are now well established and widely used across the biological sciences, each has limitations and only provides part of the picture. Metabonomics is clearly complementary to other "-omics" approaches but may have a special role in bridging the phenotype–genotype gap, since metabonomics provides the capability to analyze large arrays of metabolites for extracting biochemical information that reflects true functional endpoints of overt biological events, whereas other functional genomics technologies such as transcriptomics and

proteomics merely indicate the potential cause for phenotypic response. Metabonomics bridges this information gap by depicting, in particular, such functional information because metabolite differences in biological fluids and tissues provide the closest link to the various phenotypic responses. Such changes in the biochemical phenotype are of direct interest to pharmaceutical, biotech, and health industries once appropriate technology allows the cost-efficient mining and integration of this information. Metabonomic approaches can also provide unique insights into metabolic dynamics. Specifically, analysis of metabolite changes over time, and stable isotope labeling experiments can be used to infer metabolic fluxes that cannot be deduced by any of the other "-omics" methods. Therefore, they can necessarily predict drug effects, toxicological response, or disease states at the phenotype level unless functional validation is added.

Over the last few years, genome-wide association studies (GWAS) have been carried out and reported an extraordinary harvest of new genetic associations with metabolic traits as phenotypic traits [21]. GWAS technology has been particularly productive in the area of human autoimmune disorders, where over 100 confirmed associations have been reported in diseases including type 1 diabetes [22], depression [23], inflammatory bowel disease [24, 25], and rheumatoid arthritis [26]. The metabotypes of individuals result from gene, environment, lifestyle, food, and host–gut microbial interactions have been identified by simultaneous measurements of SNP and blood concentrations of endogenous metabolites in human population [27]. Individuals with polymorphisms in genes coding for well-characterized enzymes of the lipid metabolism have significantly different metabolic capacities with respect to the synthesis of some polyunsaturated fatty acids, the beta-oxidation of short- and medium-chain fatty acids, and the breakdown of triglycerides [21]. Thus, the concept of "genetically determined metabotype" as an intermediate phenotype provides a measurable quantity in the framework of GWAS with metabonomics and might help to better understand the pathogenesis of common diseases and gene–environment interactions. The metabotypes, in interactions with environmental factors such as nutrition and lifestyle, may influence the susceptibility of an individual for certain phenotypes. For example, there are potential links between long-chain fatty acid metabolism and attention-deficit hyperactivity syndrome [28]. Understanding these connections, in turn, may eventually lead to more targeted nutrition or therapies and more refined disease risk stratification. These could result in a critical step towards personalized health care and nutrition based on a combination of genotyping and metabolic characterization.

Personalized medicine promises prediction, prevention, and treatment of illness that is targeted to individuals' needs. A primary goal of personalized medicine is to provide the best medical treatment for each individual patient by determining which drug will have the best efficacy with the least toxicity and/or adverse effects [29]. Figure 4.2 describes the different flows from clinical presentation through treatment for standard clinical practice and a personalized medicine approach. Moving forward, the use of a combination of "-omics" technologies aiming to develop biomarkers tailored for individual responses will provide a more personalized approach to patient treatment with a more positive outcome by diagnosing not only the disease but also the disease phenotype [29].

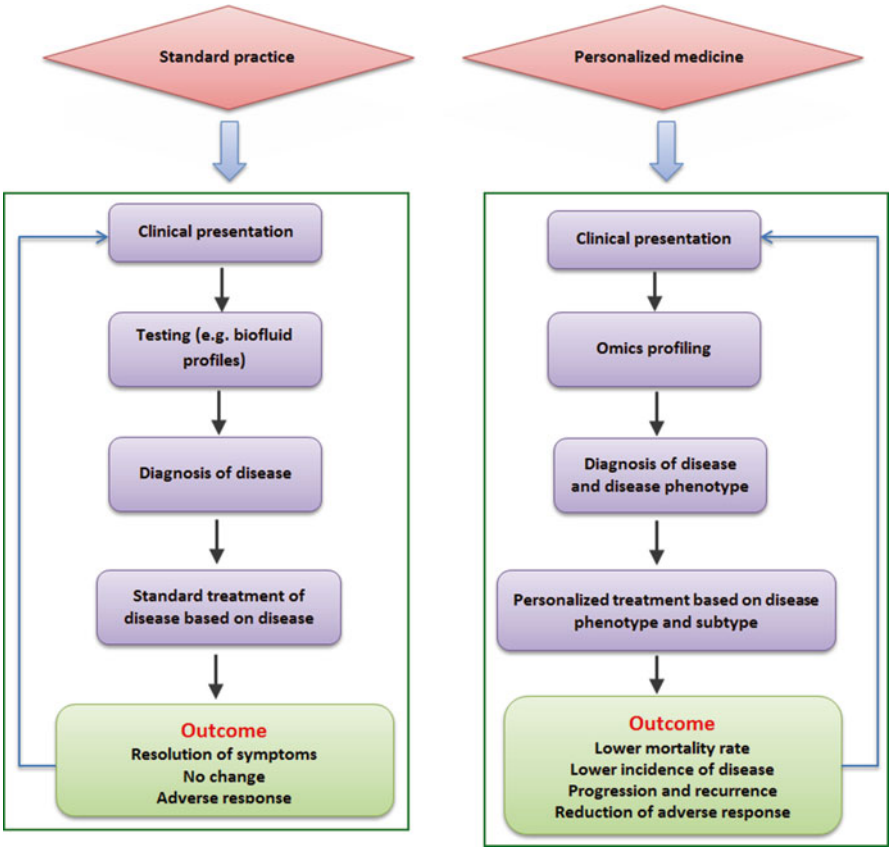

Fig. 4.2 Differences between standard clinical practice and a personalized medicine approach to diagnosis and treatment of disease (Adapted from Ref. [30])

4.3 Role of Pharmacometabonomics in Personalized Medicine

In order to choose the right therapy for the right patient at the right time, more accurate tests that can diagnose and classify a patient's disease and their response to medical treatment are needed. Pharmacometabonomics, focusing on small molecule metabolite profiles and concerning the inherent different metabotypes, is an emerging approach that combines metabolic profiling and bioinformatics to link the inherent variation of a metabotype to the prediction of drug efficacy or toxicity in patients [30]. Nicholson [30, 31] described the potential for pharmacometabonomics in clinical trials and in longitudinal studies of individual patients or groups of patients prescribed with particular therapies.

The main potential application for pharmacometabonomics is in personalized health care. The advantages of pharmacometabonomics over the other targeted

"-omics" technologies are its unbiased opportunity for finding nonpreselected, and hence unexpected, biomarkers and biomarker combinations, as multiple analytes are quantified simultaneously from biofluids. The metabolic profile represents the phenotype of the organism and reflects the overall biological influences, including interactions between multiple genomes (e.g., genomes from animals or humans and their gut microbiome). Pharmacometabonomics uses the pre-dose metabolite profiling in the biofluids or fecal extracts to predict the responses of an individual to a drug/nutritional intervention and to identify surrogate markers for subsequent drug administration. Furthermore, pharmacometabonomics is capable of providing useful drug pharmacokinetic and drug metabolite information for an individual, which can provide a mechanistic understanding of varied responses between individuals to the efficacy, side effects, and toxicity of a drug. Thus, it needs not be limited by prior biological understanding or hypotheses and can indeed be a powerful hypothesis-generating scenario (Fig. 4.3) [3].

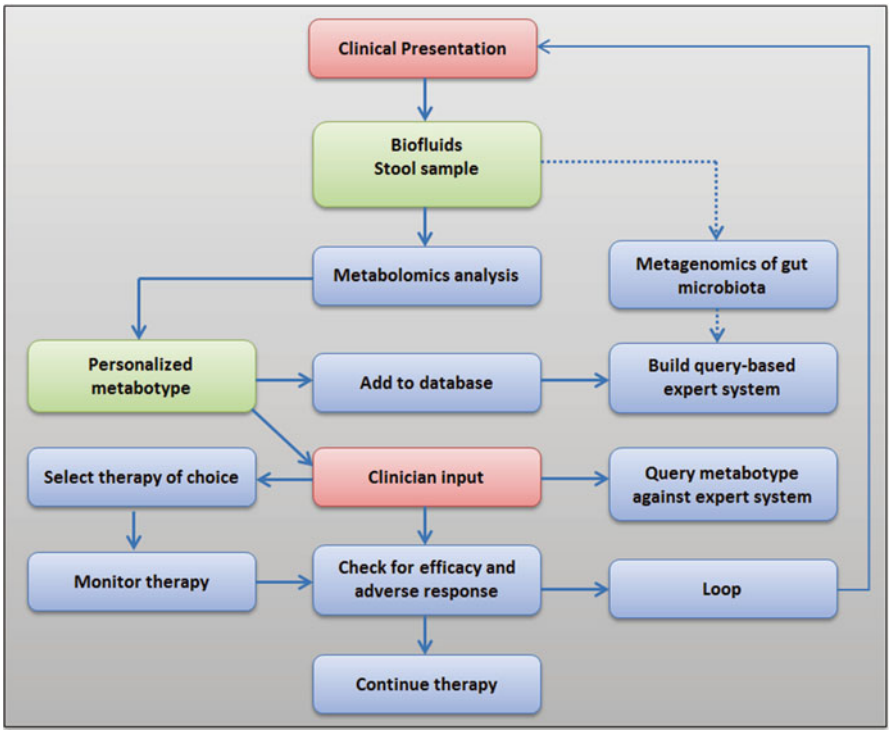

Fig. 4.3 An illustration of how pharmacometabonomics could be incorporated into clinical operations after suitable validation. In addition, metagenomic data on the gut microbiota can be included, as shown by the *dotted arrows* (Adapted from Ref. [31])

4.4 Methodologies Used in Metabonomics

Analysis of the metabolome is challenging, not only because of the chemical diversity and dynamic range of cellular metabolites, but also the redundancy of cellular metabolic pathways that complicate data interpretation. However, recent advances in analytical platforms, particularly MS, as well as bioinformatic and computational tools, have made metabonomics much more accessible to researchers.

In view of the chemical and physical diversity of small biological molecules, the challenge remains in developing protocols to gather the whole "metabolome." It is generally accepted that no single analytical technique such as NMR, gas chromatography–MS (GC–MS), and liquid chromatography–MS (LC–MS) could capture the entire composition of endogenous metabolites, and therefore, multiple complementary analytical platforms for global metabolic profiling are needed for an enhanced metabolic measurement and visualization.

In general, analytical techniques such as NMR spectroscopy or MS are the primary analytical methods employed in metabolite profiling. NMR metabonomics is characterized by a higher throughput potential and more reliable determination of metabolite concentrations: the intensities of the peaks in NMR spectra are proportional to the metabolite concentrations, making it a true quantitative technique [11]. The basic workflow for NMR-based as well as MS-based studies is as follows: quenching/extraction of metabolites → data collection → data processing/analysis [32–34]. Metabonomics is usually carried out on samples that are available either noninvasively (e.g., urine, feces, saliva, sweat, or exhaled breath condensate) or minimally invasively (e.g., serum or plasma from blood). Tissues, intact cells, or extracts thereof can also be analyzed.

NMR spectroscopy [34, 35] is a nondestructive technique that is highly effective for structural elucidation. Compared to MS, it is less sensitive, but recent development in operating methods and technology has increased its sensitivity and spectral dispersion considerably. NMR data acquisition on intact tissue samples using a high-resolution ^{1}H magic angle spinning NMR spectroscopy is also possible. All of the metabonomic datasets generated from different instruments can be, and should be, combined if possible so that an integrated metabonomic approach across different types of samples is possible.

MS is an effective analytical technique for the identification of metabolic biomarkers [32]. It is inherently more sensitive than NMR spectroscopy but, to ensure maximal metabolome coverage and minimize technical problems associated with ion suppression, it requires a separation technique prior to MS. Both GC and LC directly coupled to MS have been employed for metabolic profiling, but in most studies MS has been coupled to HPLC or UPLC for metabonomic studies on biofluids and tissue extracts [36]. UPLC, which employs sub-2-μm particle columns, provides superior sensitivity and resolution compared to conventional LC [37, 38]. Moreover, the coupling of this technique to tandem MS (MS/MS) further enhances specificity, provides an improved signal-to-noise ratio compared to single-stage MS, and has radically improved the ability to obtain robust and comprehensive

metabolite profiles such as bile acids [39–41]. The combination of NMR and MS data may improve the identification of unknown metabolites [42].

Both NMR and MS spectra are highly complex, and the biological information can only be extracted by applying bioinformatic or computational tools, such as pattern recognition methods. Simple methods such as principal component analysis (PCA) allow visualization of clustering of similar samples and the determination of aberrant or outlier samples. The supervised methods, such as partial least squares (PLS), use a training set of samples (of known classification) to create a mathematical model that is then used to test an independent dataset, which enables us to predict to which group a new sample belongs on the strength of the characteristics of its spectra [43].

Later, new methods for improving the structural identification of metabolites from NMR data have been established. Statistical total correlation spectroscopy (STOCSY) takes advantage of the colinearity of the intensity variables for the multiple peaks of a metabolite in a set of NMR spectra, so that correlations from NMR peaks belonging to the same molecule can be identified [44]. This is particularly useful for biomarker identification from complex biofluid analysis. An extension of STOCSY, statistical heterospectroscopy, allows for the coanalysis of datasets obtained by both NMR spectroscopy and MS [45]. Moreover, the principles of this approach have the potential to correlate data from any two spectroscopic techniques or, by extension, to correlate such data with that from other "-omics" fields such as proteomics. These two tools, and various extensions of the methods that have been published recently, may become major approaches for biomarker identification and biochemical pathway information in "-omics" sciences and systems biology. Other metabonomic and lipidomics strategies are being discussed in Chap. 2.

4.5 Metabonomic Technologies for Toxicology Studies

Metabonomics evaluation is nontargeted, enabling users to gain a comprehensive evaluation of the systemic response of the subject (preclinical or clinical) to pathophysiological stimuli or genetic modification [2]. Metabonomics studies demonstrate its potential impact on the drug discovery process by enabling the incorporation of safety endpoints much earlier in the drug discovery process, reducing the likelihood (and cost) of later stage attrition [14]. The advantage of metabonomics to convey phenotype and to be obtained from peripheral samples makes it very attractive from a translation standpoint for rapid throughput in vivo toxicity screening particularly within the pharmaceutical industry [46]. To maximize the information obtainable from multivariate datasets, a high-throughput technology is desirable so that the data matrices produced can fully define both the variations associated with a disorder and the innate variations associated with the biological system, while minimizing false positives associated with such global multivariate analyses. A metabolic profile needs not be a comprehensive survey of composition, nor needs it be completely resolved and assigned, although these are all desirable attributes.

For the profile to be useful across a range of problems, however, it must be amenable to quantitative interpretation, and it should be relatively unbiased in its scope. A further requirement for the analytical platforms used to generate profiles is that the analytical variations introduced after collection be less than the typical variation in the normal population of interest, so as not to reduce significantly the opportunity to detect treatment/group-related differences. Fulfilling this condition is dependent on the actual system and questions to be addressed, in addition to the means of quality control used in the analytical methods.

In a study conducted by Clayton et al., NMR-based metabonomics was applied to profile pre- and post-dose urine samples from 65 rats given a single toxic dose of acetaminophen [13]. The metabolic profile of the pre-dose urine samples can predict both individual susceptibility to acetaminophen-induced toxicity and liver injury and also can predict the relative excretion levels of acetaminophen metabolites in the forms of glucuronide and sulfate conjugates. Later, the same group provided the first demonstration of pharmacometabonomics in humans by demonstrating a clear connection between an individual's basal urinary metabolic phenotype and the metabolic fate of a standard dose of the widely used analgesic acetaminophen [47]. NMR-based metabonomics approaches were employed to profile pre- and post-dose urinary metabolites and discovered that human subjects with high pre-dose levels of *p*-cresol (one of the metabolites related to an individual's gut microbiome) had lower concentrations of acetaminophen metabolites. From post-dose urine samples, it was possible to determine the proportions of the various drug metabolites excreted by each subject, which was known to show considerable inter-subject variation. The findings indicate that each individual, colonized by a unique assortment of trillions of microbes, responds to a drug differently, either beneficially or adversely. It provides the information of how a particular drug is metabolized and excreted by each individual. Such information may have a major influence on the drug safety and efficacy. This study demonstrates that evaluation of a metabolic phenotype by metabolic profiling could play an important role in drug metabolism and toxicity, as well as in personalized health care.

One other study in animals reported by Li et al., who used two established experimental models, the streptozotocin-induced diabetic model and a high-energy, diet-induced obesity model, both in rats, demonstrated that the different outcomes of streptozotocin-induced diabetes or dietary intervention could be correlated to variations in pre-dose urinary metabolites of the rats, mainly those from gut microbiota [48]. It appears that these predispose the animals to different pathophysiological outcomes upon diet alteration or chemical stimulus. They also surmised that, based on their animal model observations, for obese/diabetic human subjects, subtle variations in metabolic phenotype may predetermine their responses to xenobiotic perturbation, ultimately leading to variability in pathophysiological processes.

Cisplatin has been one of the most widely used anticancer agents, but its nephrotoxicity remains a dose-limiting complication. The idiopathic nature and the pre-dose prediction of cisplatin-induced nephrotoxicity were evaluated using a NMR-based pharmacometabonomic approach [49]. Significant individual difference was observed that cisplatin produced serious toxic responses in some animals

(toxic group) but had little effect in others (nontoxic group). The individual metabolic profiles, assessed by urine NMR spectra, showed large differences between the post-administration profiles of the two groups. Importantly, multivariate analysis of the NMR data showed that the toxic and nontoxic groups can be differentiated based on the pretreatment metabolite profiles. This study provides a working model that can predict the idiopathic toxicity outcome based on metabolite markers found by metabonomics approach. Thus, a pharmacometabonomic approach using pretreatment metabolite profiles may help expedite personalized chemotherapy of anticancer drugs.

In both preclinical screening and mechanistic exploration, metabolic profiling can offer rapid, noninvasive toxicological information that is robust and reproducible, with little or no added technical resources to existing studies in drug metabolism and toxicity. Metabonomics is an integral component of the pharmacogenomics toolbox, especially as its ultimate goal is personalized health. The metabolic profile contains information about the metabotype in addition to the genotype. The information obtained from genotyping and metabotyping will allow a specific course of treatment to be defined that will have the potential for a successful outcome, thus making personalized health care a reality.

4.6 Metabonomics and Personalized Nutrition

The metabolome, or the complete metabolite composition of a system such as a cell or organism, is the end product not only of the genetic blueprint of an organism but also all influential factors to which the organism is exposed, such as nutrition, environmental factors, or treatments. Changes in an individual's metabolome occur immediately or on a more gradual basis, partially due to the constancy of an individual's genetic makeup and lifestyle/environment. Holmes et al. suggest that common diets, gut microbes, medicinal practices, genetics, and other lifestyle and environmental factors give rise to regional metabonomic phenotypes [50]. It has long been understood that nutrition plays a role in human health. However, many of the links between an individual's diet and specific health outcomes are still not completely understood, for example, why one person easily develops obesity and another, with the same diet, does not (responders and nonresponders). Metabonomics, generating profiles of metabolites in biofluids, including urine, plasma, and fecal water, provides a systems approach to understanding global metabolic regulation of an organism and its commensal and symbiotic microbiota. In particular, it focuses on the measurements of metabolite concentrations, fluxes, and secretions in cells and tissues in which there is a direct connection between gene expression, protein activity, and metabolic activity [4]. Metabonomic strategies together with advanced chemometric and bioinformatic tools [44, 51, 52] can help track the interaction between nutrients and human metabolism, as well as the involvement of the genome and the gut microbiome, in overall human health, and can be considered critical measures of function or phenotype [53]. This makes it possible to assess the

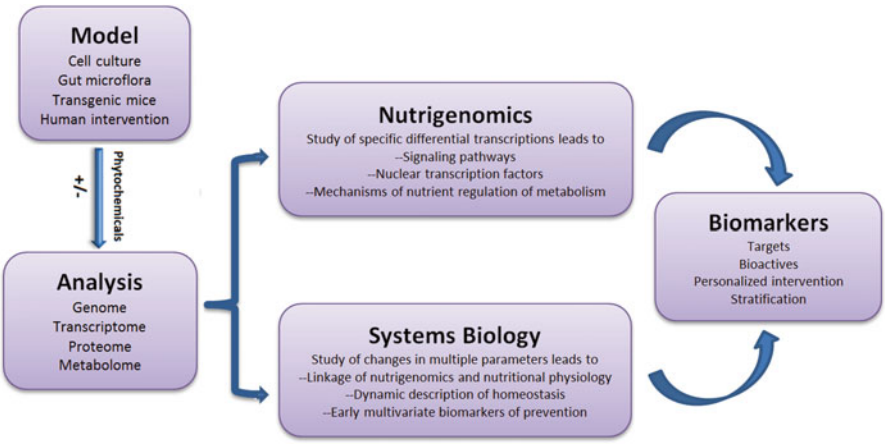

Fig. 4.4 Application of nutrigenomics and systems biology together with new bioinformatics tools to unravel disease mechanisms, define biomarkers, or apply personalized nutrition. The nutrigenomics approach extracts relevant differences, which become leads for further mechanistic research, while the nutritional systems biology approach aims at a complete description of the physiologic response by exploiting the complete data sets, thus targeting a new concept of biomarker (Adapted from Ref. [55])

metabolic component of nutritional phenotypes and will enable individualized dietary recommendations. The relation between diet and metabonomic profiles as well as between those profiles and health and disease needs to be established. In the past three decades, nutritional research has undergone an important shift in the focus from physiology and epidemiology to genetics and molecular biology. Many progresses are made through a systematic inventory of all relevant parameters by using different "-omics" technologies and application of new bioinformatics tools together with extensive data warehousing to unravel disease mechanisms, define biomarkers, or apply personalized medication (Fig. 4.4).

Personalized nutrition is the outcome for individuals who will adapt their diet and lifestyle according to the knowledge about their current or future healthy status, and their subsequent nutritional requirements [54]. The knowledge could be built around the characterization of different metabolic phenotypes in human population. The pharmacometabonomics, discussed in this chapter, is sensitive to both genetic and environmental influences, and addresses the metabolic response at the individual level. This concept could be alternatively applied to nutritional research as a means of assessing individual response to diets or phytochemicals. In the future, researchers could use such metabolic profiling to measure, predict, and optimize the metabolic response of individual response to dietary interventions or modulations [55]. Likely, in cases of impairment of human homeostasis, the patients would thus develop a coordinated approach to reestablish a metabolic trajectory for the individual consistent with their metabolic phenotype.

A depletion–repletion study of choline conducted by Zeisel et al. showed that an individual's metabonomics profile at baseline could predict whether or not the

individual would develop liver dysfunction as a result of inadequate choline intake [56]. A study funded through the European Nutrigenomics Organisation (NuGO) found that it is possible to characterize individual responses to fasting and to take a "snapshot" of small metabolic changes after 36 h of fasting [57]. A number of metabolites and hormones not previously associated with fasting were identified and high variability between individuals for certain markers, including leptin, the satiety hormone, which shows how it is possible to use metabonomics to characterize the different responses of individuals to nutritional or physiological stress.

A metabonomics study by Wang et al. [58] was performed to investigate the human metabolic response to nutritional intervention with chamomile, an important alternative and functional food. The results showed a clear clustering of the subjects as a function of chamomile tea intake characterized by a decreased urinary creatinine level and an increase in glycine and hippurate. Samples are obtained 2 weeks after daily chamomile intake deviating from the samples collected before chamomile intervention, which was partially ascribed to chamomile-induced changes in gut microbial metabolism. The results of this study highlight the diversity of physiological variations of human metabolism and emphasize the effect of nutritional phytochemicals in modulating human metabolism and maintaining homeostasis of human gut eco-system.

Xie et al. [59] performed a study on 20 volunteers to investigate the human metabolic response to drinking Pu-erh tea over a 6-week period, using a UPLC-QTOFMS-based metabonomics approach. The final metabolic profile was greatly altered by Pu-erh tea consumption. The trajectory of the PCA scores plot based on urine data revealed a clear separation tendency of samples obtained before (days 1 and 7), during (days 16, 21, and 28), and after tea ingestion (washout period; days 30, 36, 42). Interestingly, the metabolic patterns of samples obtained 2 weeks after tea intake are still distinct from the pre-dose pattern, probably due to the possibility that Pu-erh tea may change the structure of the resident gut microbiota.

This was followed by a more in-depth study of Pu-erh tea in human subjects [60]. Urine samples were collected at 0, 1, 3, 6, 9, 12, and 24 h within the first 24 h of tea intake and once a day during a 2-week daily Pu-erh tea ingestion phase and a 2-week "washout" phase. The dynamic concentration profile of bioavailable plant molecules (due to in vivo absorption and the hepatic and gut bacterial metabolism) and the human metabolic response profile were identified and correlated with each other, highlighting the great potential of metabonomic strategy to unravel the complex interactions between multicomponent nutraceuticals and human metabolic system in nutritional studies.

A metabonomics study by Rezzi et al. [61] demonstrated that metabonomics can be used to predict whether an individual will respond to a certain dietary treatment. Twenty-two healthy male volunteers selected from 75 volunteers based on their chocolate preferences (chocolate loving or chocolate hating) underwent a 1-week double-cross-over study in which they consumed either chocolate or a bread placebo on the two test days and followed a standardized diet throughout the study. NMR analysis of 24 h blood and urine samples revealed that the chocolate preference of the individual could be predicted from both biofluid samples even in the absence of

the chocolate stimulus. Such a prediction of the dietary preference of the individual indicates that the metabolic profile may indeed contain a wealth of information relating to the diet of an individual, and it may be possible to predict dietary response, thus proposing a role for metabonomics in personalized nutrition [62].

4.7 Gut Microbiota and Phytochemical Profiling in Nutrition Science

It is widely accepted that many major human diseases have significant genetic and environmental factors and that the impairment of human homeostasis in an individual or population is a complex result of the conditional probabilities of certain genes interacting with a variety of environmental triggers.

Diet (nutrients) has a major influence on many diseases and modulates the complex intercommunity of gut microbiota [63]. Understanding the relationships between the host genome, nutrient (phytochemicals) intervention, and the highly variable gut microbiota with their genomes is a sophisticated challenge in modern nutritional science. In the search for new natural bioactive phytochemicals (drugs) and therapies, most consideration in toxicology and efficacy is given to the genetic components of the host (animal model or human). Little attention, however, is directed to the individual microbiome or species variation in the microbiome that might contribute to the interaction of the potent phytochemicals or drugs within the human global system. Although the application of some "functional foods" and "nutraceuticals" has been promoted by food companies, and probiotics are specifically designed to nourish gut microbiota, the identification and function of many gut microbial species remain largely unknown. There is an urgent need to understand the global function of these gut organisms in terms of their impact on human health, taking into account host–gut microbiota interactions at gene, protein, and metabolite expression levels. Bioanalytical profiling, such as metabonomics, of human urinary or fecal samples can be utilized to provide holistic and dynamic information to assist dietary and medical researchers on disease diagnosis, stratification, and personalized gut microbiota targeted treatment. On the other hand, elucidating the molecular mechanisms underlying host–gut microbial interactions during both healthy and pathological conditions should be pursued in order to obtain a deeper understanding to aid nutritional intervention and drug development.

The goal of nutrition has extended beyond just ameliorating or curing diseases and now aims to achieve an overall objective in preventing diseases and improving health. Therefore, the pivotal scientific objective has become understanding the relationship between diet (both macro- and micronutrients) and health/diseases. The comprehensive analysis of the metabolome via metabonomics will serve as the bioinformational base for modern nutritional science. Biomarkers and/or patterns of expression will undoubtedly have the potential to be used for human health assessment (Fig. 4.5). Together this indicates that the future goal of nutritional research

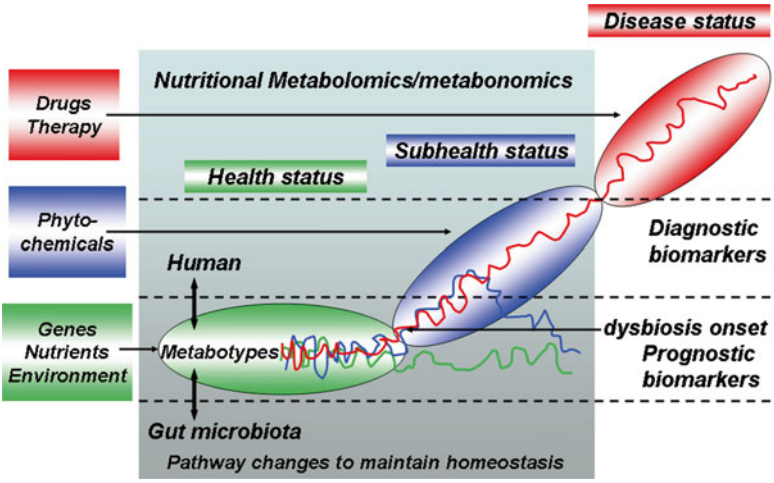

Fig. 4.5 Conceptualization of nutritional metabonomics/nutrimetabonomics for health and risk management. Integration of nutritional metabonomics/nutrimetabonomics and systems biology at the population scale may lead to enhanced use of nutrients to prevent or delay the onset of disease and to optimize human health at an extensive scale. The metabotypes of individuals result from gene, environment, lifestyle, food, and host–gut microbiota interactions. Different metabotypes (represented by *green*, *blue,* and *red lines/ellipse*) are under homeostasis that aims to maintain metabolic fluctuations within a healthy range (*green ellipse*). Metabonomics-generated prognostic biomarkers can be used to assess homeostasis loss and likelihood for future diseases. Nutritional metabonomics/nutrimetabonomics aims at optimizing nutrition for health maintenance and to restore homeostasis as illustrated by the *blue line/ellipse* (Adapted from Refs. [54, 55, 73])

will be to predict the likelihood of future diseases within the context of an individual's overall heath and identify causal risk factors, leading to recommendations for appropriate intervention, such as to change dietary habits or to avoid homeostasis loss and maintain healthy status.

4.8 The Importance of the Gut Microbiota on Metabolic Profiles

Symbiotic relationships between microbes and the mammalian and plant hosts shape our world. The symbiotic microbiome tremendously increases the diversity of metabolic pathways accessible to mammalian hosts, enabling them to metabolize many things that they otherwise could not. As a result, gut microbes have been associated with various essential biological functions in humans through a "network" of microbial–host co-metabolism to process nutrients and drugs and modulate the activities of multiple pathways in a variety of organ systems [64–66]. The studies by Nicholson et al. [64–67] demonstrate that the metabolic variations in GI compartments such as duodenum, jejunum, and ileum, mammalian tissues such as

kidney and liver, and biofluids such as blood and urine are directly related to the activities of various microorganisms that coexist in the gut. Perhaps most importantly, gut microbes enable us to digest cellulose, the single largest nutritional energy source on the planet, and to survive on diets with low levels of particular nutrients and high levels of particular toxins. For example, gut microbes metabolize unabsorbed carbohydrates to short-chain fatty acids (SCFA), CO_2, and H_2 in the colon. SCFAs are monocarboxylic acids with a chain length up to six carbon atoms, i.e., acetic, propionic, butyric, iso-valeric, valeric, iso-caproic, and caproic acids. SCFAs function both as an energy source and as a signaling molecule, and their abundance and type (e.g., butyric, propionic, acetic acids) are directly related to the speciation of the microbiota and their syntrophic interactions. Other signaling pathways (e.g., such as through the SCFA receptor GPR43) are similarly involved in host energy balance, and different microbial communities interact differently with these molecules [68]. Most mammals can obtain essential amino acids, such as lysine, from their diet, but there is evidence that they also obtain them from their gut microbes [69]. Many amino acids and perhaps other nitrogen-containing compounds may be cycling between humans and their microbiota, a process that could reduce dietary requirements for those nutrients [69]. However, whether the fluxes of those amino acids or other essential nutrients between microbes and humans are sufficient in quantity and diversity to meet the nutritional requirements is yet to be resolved.

Diversity in gut microbial communities and function creates differences in nutrient milieu, digesta retention times, and temperatures that create diverse microbial niches and inhabitants. With the recent advances in the new molecular profiling technologies such as metagenomics and metabonomics [69, 70], the direct correlation of global metabolic changes with gut microbiome becomes increasingly important to decipher the host–microbe relationships and to gain mechanistic understanding of nutritional and drug intervention in the "gateway" of host–microbe co-metabolism. Scientists from different disciplines are working together and beginning to determine the details of gut microbial diversity and manipulate the complex interactions between the host metabolism and its symbionts for improved nutrition and disease treatment [9, 70, 71].

4.9 Conclusion and Future Perspectives

Metabonomics is a novel approach that promises to enable the detection of states of disease, to stratify patients based on metabolic profiles and to monitor disease progression. Metabonomic analysis may also be able to orient the choice of therapy, identify responders, and predict toxicity (pharmacometabonomics), paving the way to a personalized therapy.

Whereas the human genome is the set of all genes in a human being, the human metabolome is the set of all metabolites in a human being. Metabonomics bridges the gap between the genotype and the phenotype and is an important basis of

personalized medicine. Metabonomics has been used to identify biomarkers for disease and the effects of drugs. Various metabonomic technologies including NMR and MS have been intensively applied to metabonomics study. Pharmacometabonomic approach to personalizing drug treatment uses a combination of pre-dose metabolite profiling and chemometrics to model and predict the responses of individual subjects. Metabonomics also has a role to play in assessing drug toxicity and in guiding nutrition.

The advantage of the personalized medicine approach includes the fact that the "-omics"-derived biomarkers will potentially provide better, earlier diagnosis of disease and disease phenotype, leading to a better clinical outcome for the patient [29]. Recent efforts that have correlated GWAS data with downstream metabolite concentrations or ratios [21] will further drive these technologies towards personalized medicine. Such associations provide a unique approach to stratify an individual's genotype and phenotype. On a population basis, such associations will also provide more descriptive information about a gene's function as well as pathway-specific information about various diseases that will ultimately lead to the development of more targeted therapies. An example of how the "-omics" datasets may move forward the field of personalized medicine has been reported by the group of Chen et al. [72]. An approach referred to as integrative personal "-omics" profile evaluated genomic, transcriptomic, proteomic, metabonomic, and antibody profiles from a single individual over a 14-month period. The study revealed changes in the "-omics" profiles between healthy and viral states and between nondiabetic and diabetic states throughout the study period. Furthermore, it was noted that disease risk could be assessed from the individual and maternal genome sequences. This study demonstrated that the integration of genomics data with other dynamic "-omics" datasets can be used to predict various medical risks and the health status of an individual. Such datasets for many individuals may provide a database that can be used for enhancing diagnostics, monitoring, and treatment in the future with metabonomics playing a critical role.

References

1. Nicholson JK. Global systems biology, personalized medicine and molecular epidemiology. Mol Syst Biol. 2006;2:52.
2. Nicholson JK, Lindon JC, Holmes E. Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica. 1999;29(11):1181–9 [Review].
3. Fiehn O. Metabolomics – the link between genotypes and phenotypes. Plant Mol Biol. 2002;48(1–2):155–71.
4. Nicholson JK, Connelly J, Lindon JC, Holmes E. Metabonomics: a platform for studying drug toxicity and gene function. Nat Rev Drug Discov. 2002;1(2):153–61.
5. Hunter P. Reading the metabolic fine print. The application of metabolomics to diagnostics, drug research and nutrition might be integral to improved health and personalized medicine. EMBO Rep. 2009;10(1):20–3.

6. German JB, Roberts MA, Watkins SM. Personal metabolomics as a next generation nutritional assessment. J Nutr. 2003;133(12):4260–6.
7. Scalbert A, Brennan L, Fiehn O, Hankemeier T, Kristal BS, van Ommen B, et al. Mass-spectrometry-based metabolomics: limitations and recommendations for future progress with particular focus on nutrition research. Metabolomics. 2009;5(4):435–58.
8. Martin F-PJ, Rezzi S, Pere-Trepat E, Kamlage B, Collino S, Leibold E, et al. Metabolic effects of dark chocolate consumption on energy, gut microbiota, and stress-related metabolism in free-living subjects. J Proteome Res. 2009;8(12):5568–79.
9. Li M, Wang B, Zhang M, Rantalainen M, Wang S, Zhou H, et al. Symbiotic gut microbes modulate human metabolic phenotypes. Proc Natl Acad Sci U S A. 2008;105(6):2117–22 [Research Support, Non-U.S. Gov't].
10. Sauer U, Heinemann M, Zamboni N. Genetics. Getting closer to the whole picture. Science. 2007;316(5824):550–1 [Comment].
11. Nicholson JK, Lindon JC. Systems biology: metabonomics. Nature. 2008;455(7216):1054–6 [Historical Article News].
12. Meyer JM, Ginsburg GS. The path to personalized medicine. Curr Opin Chem Biol. 2002;6(4):434–8 [Review].
13. Clayton TA, Lindon JC, Cloarec O, Antti H, Charuel C, Hanton G, et al. Pharmaco-metabonomic phenotyping and personalized drug treatment. Nature. 2006;440(7087): 1073–7.
14. Jain KK. Textbook of personalized medicine. New York: Springer; 2009.
15. Becquemont L, Alfirevic A, Amstutz U, Brauch H, Jacqz-Aigrain E, Laurent-Puig P, et al. Practical recommendations for pharmacogenomics-based prescription: 2010 ESF-UB Conference on Pharmacogenetics and Pharmacogenomics. Pharmacogenomics. 2011;12(1): 113–24 [Congresses].
16. Cecchin E, Agostini M, Pucciarelli S, De Paoli A, Canzonieri V, Sigon R, et al. Tumor response is predicted by patient genetic profile in rectal cancer patients. Pharmacogenomics J. 2011;11(3):214–26.
17. Tabernero J, Cervantes A, Rivera F, Martinelli E, Rojo F, von Heydebreck A, et al. Pharmacogenomic and pharmacoproteomic studies of cetuximab in metastatic colorectal cancer: biomarker analysis of a phase I dose-escalation study. J Clin Oncol. 2010;28(7):1181–9 [Clinical Trial, Phase I Comment].
18. Benhaim L, Labonte MJ, Lenz HJ. Pharmacogenomics and metastatic colorectal cancer: current knowledge and perspectives. Scand J Gastroenterol. 2012;47(3):325–39 [Review].
19. Loscalzo J, Kohane I, Barabasi AL. Human disease classification in the postgenomic era: a complex systems approach to human pathobiology. Mol Syst Biol. 2007;3:124.
20. Wilson ID. Drugs, bugs, and personalized medicine: pharmacometabonomics enters the ring. Proc Natl Acad Sci U S A. 2009;106(34):14187–8 [Comment].
21. Gieger C, Geistlinger L, Altmaier E, Hrabe de Angelis M, Kronenberg F, Meitinger T, et al. Genetics meets metabolomics: a genome-wide association study of metabolite profiles in human serum. PLoS Genet. 2008;4(11):e1000282 [Research Support, Non-U.S. Gov't].
22. Barrett JC, Clayton DG, Concannon P, Akolkar B, Cooper JD, Erlich HA, et al. Genome-wide association study and meta-analysis find that over 40 loci affect risk of type 1 diabetes. Nat Genet. 2009;41(6):703–7 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].
23. Shyn SI, Shi J, Kraft JB, Potash JB, Knowles JA, Weissman MM, et al. Novel loci for major depression identified by genome-wide association study of sequenced treatment alternatives to relieve depression and meta-analysis of three studies. Mol Psychiatry. 2011;16(2):202–15 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].
24. Barrett JC, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD, et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nat Genet. 2008;40(8):955–62 [Meta-Analysis Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].

25. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature. 2012;491(7422):119–24. doi:10.1038/nature11582.
26. Gregersen PK, Olsson LM. Recent advances in the genetics of autoimmune disease. Annu Rev Immunol. 2009;27:363–91 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Review].
27. Martin FP, Collino S, Rezzi S, Kochhar S. Metabolomic applications to decipher gut microbial metabolic influence in health and disease. Front Physiol. 2012;3:113.
28. Burgess JR, Stevens L, Zhang W, Peck L. Long-chain polyunsaturated fatty acids in children with attention-deficit hyperactivity disorder. Am J Clin Nutr. 2000;71(1 Suppl):327S–30 [Review].
29. Sun J, Beger RD, Schnackenberg LK. Metabolomics as a tool for personalizing medicine: 2012 update. Pers Med. 2013;10(2):149–61. 2013/03/01.
30. Nicholson JK, Wilson ID, Lindon JC. Pharmacometabonomics as an effector for personalized medicine. Pharmacogenomics. 2011;12(1):103–11.
31. Nicholson JK, Everett JR, Lindon JC. Longitudinal pharmacometabonomics for predicting patient responses to therapy: drug metabolism, toxicity and efficacy. Expert Opin Drug Metab Toxicol. 2012;8(2):135–9 [Editorial].
32. Want EJ, Masson P, Michopoulos F, Wilson ID, Theodoridis G, Plumb RS, et al. Global metabolic profiling of animal and human tissues via UPLC-MS. Nat Protocs. 2013;8(1):17–32. doi:10.1038/nprot.2012.135.
33. Dunn WB, Broadhurst D, Begley P, Zelena E, Francis-McIntyre S, Anderson N, et al. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. Nat Protocs. 2011;6(7):1060–83. doi:10.1038/nprot.2011.335.
34. Beckonert O, Keun HC, Ebbels TMD, Bundy J, Holmes E, Lindon JC, et al. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. Nat Protocs. 2007;2(11):2692–703. doi:10.1038/nprot.2007.376.
35. Lindon JC, Nicholson JK. Spectroscopic and statistical techniques for information recovery in metabonomics and metabolomics. Annu Rev Anal Chem (Palo Alto, Calif). 2008;1:45–69 [Review].
36. Lenz EM, Wilson ID. Analytical strategies in metabonomics. J Proteome Res. 2007;6(2):443–58 [Review].
37. Russo R, Guillarme D, TTN D, Bicchi C, Rudaz S, Veuthey JL. Pharmaceutical applications on columns packed with sub-2 microm particles. J Chromatogr Sci. 2008;46(3):199–208.
38. Xie G, Plumb R, Su M, Xu Z, Zhao A, Qiu M, et al. Ultra-performance LC/TOF MS analysis of medicinal Panax herbs for metabolomic research. J Sep Sci. 2008;31(6–7):1015–26.
39. Want EJ, Coen M, Masson P, Keun HC, Pearce JT, Reily MD, et al. Ultra performance liquid chromatography-mass spectrometry profiling of bile acid metabolites in biofluids: application to experimental toxicology studies. Anal Chem. 2010;82(12):5282–9.
40. Huang JG, Bathena SPR, Csanaky IL, Alnouti Y. Simultaneous characterization of bile acids and their sulfate metabolites in mouse liver, plasma, bile, and urine using LC-MS/MS. J Pharm Biomed Anal. 2011;55(5):1111–9.
41. Xie GX, Zhong W, Li HK, Li Q, Qiu YP, Zheng XJ, et al. Alteration of bile acid metabolism in the rat induced by chronic ethanol consumption. FASEB J. 2013;27:3583–93.
42. Pan Z, Raftery D. Comparing and combining NMR spectroscopy and mass spectrometry in metabolomics. Anal Bioanal Chem. 2007;387(2):525–7 [Review].
43. Baraldi E, Carraro S, Giordano G, Reniero F, Perilongo G, Zacchello F. Metabolomics: moving towards personalized medicine. Ital J Pediatr. 2009;35(1):30 [Editorial].
44. Cloarec O, Dumas ME, Craig A, Barton RH, Trygg J, Hudson J, et al. Statistical total correlation spectroscopy: an exploratory approach for latent biomarker identification from metabolic 1H NMR data sets. Anal Chem. 2005;77(5):1282–9 [Research Support, Non-U.S. Gov't].

45. Crockford DJ, Holmes E, Lindon JC, Plumb RS, Zirah S, Bruce SJ, et al. Statistical heterospectroscopy, an approach to the integrated analysis of NMR and UPLC-MS data sets: application in metabonomic toxicology studies. Anal Chem. 2006;78(2):363–71 [Research Support, Non-U.S. Gov't].
46. Harrigan GG, Yates LA. High-throughput screening, metabolomics and drug discovery. IDrugs. 2006;9(3):188–92.
47. Clayton TA, Baker D, Lindon JC, Everett JR, Nicholson JK. Pharmacometabonomic identification of a significant host-microbiome metabolic interaction affecting human drug metabolism. Proc Natl Acad Sci U S A. 2009;106(34):14728–33 [Clinical Trial Research Support, Non-U.S. Gov't].
48. Li H, Ni Y, Su M, Qiu Y, Zhou M, Qiu M, et al. Pharmacometabonomic phenotyping reveals different responses to xenobiotic intervention in rats. J Proteome Res. 2007;6(4):1364–70 [Comparative Study Research Support, Non-U.S. Gov't].
49. Kwon HN, Kim M, Wen H, Kang S, Yang HJ, Choi MJ, et al. Predicting idiopathic toxicity of cisplatin by a pharmacometabonomic approach. Kidney Int. 2011;79(5):529–37 [Research Support, Non-U.S. Gov't].
50. Holmes E, Loo RL, Stamler J, Bictash M, Yap IK, Chan Q, et al. Human metabolic phenotype diversity and its association with diet and blood pressure. Nature. 2008;453(7193):396–400 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].
51. Holmes E, Foxall PJ, Nicholson JK, Neild GH, Brown SM, Beddell CR, et al. Automatic data reduction and pattern recognition methods for analysis of 1H nuclear magnetic resonance spectra of human urine from normal and pathological states. Anal Biochem. 1994;220(2):284–96.
52. Wang Y, Bollard ME, Keun H, Antti H, Beckonert O, Ebbels TM, et al. Spectral editing and pattern recognition methods applied to high-resolution magic-angle spinning 1H nuclear magnetic resonance spectroscopy of liver tissues. Anal Biochem. 2003;323(1):26–32.
53. Go VL, Nguyen CT, Harris DM, Lee WN. Nutrient-gene interaction: metabolic genotype-phenotype relationship. J Nutr. 2005;135(12 Suppl):3016S–20 [Research Support, N.I.H., Extramural Review].
54. Rezzi S, Ramadan Z, Fay LB, Kochhar S. Nutritional metabonomics: applications and perspectives. J Proteome Res. 2007;6(2):513–25.
55. Xie G, Li X, Li H, Jia W. Toward personalized nutrition: comprehensive phytoprofiling and metabotyping. J Proteome Res. 2013;12:1547–59.
56. Sha W, da Costa KA, Fischer LM, Milburn MV, Lawton KA, Berger A, et al. Metabolomic profiling can predict which humans will develop liver dysfunction when deprived of dietary choline. FASEB J. 2010;24(8):2962–75.
57. Rubio-Aliaga I, de Roos B, Duthie SJ, Crosley LK, Mayer C, Horgan G, et al. Metabolomics of prolonged fasting in humans reveals new catabolic markers. Metabolomics. 2011;7(3):375–87.
58. Wang Y, Tang H, Nicholson JK, Hylands PJ, Sampson J, Holmes E. A metabonomic strategy for the detection of the metabolic effects of chamomile (Matricaria recutita L.) ingestion. J Agric Food Chem. 2005;53(2):191–6.
59. Xie G, Ye M, Wang Y, Ni Y, Su M, Huang H, et al. Characterization of pu-erh tea using chemical and metabolic profiling approaches. J Agric Food Chem. 2009;57(8):3046–54.
60. Xie G, Zhao A, Zhao L, Chen T, Chen H, Qi X, et al. Metabolic fate of tea polyphenols in humans. J Proteome Res. 2012;11(6):3449–57.
61. Rezzi S, Ramadan Z, Martin FP, Fay LB, van Bladeren P, Lindon JC, et al. Human metabolic phenotypes link directly to specific dietary preferences in healthy individuals. J Proteome Res. 2007;6(11):4469–77 [Research Support, Non-U.S. Gov't].
62. Brennan L. Session 2: personalised nutrition. Metabolomic applications in nutritional research. Proc Nutr Soc. 2008;67(4):404–8 [Research Support, Non-U.S. Gov't Review].
63. Nicholson JK, Holmes E, Lindon JC, Wilson ID. The challenges of modeling mammalian biocomplexity. Nat Biotechnol. 2004;22(10):1268–74.

64. Claus SP, Tsang TM, Wang Y, Cloarec O, Skordi E, Martin FP, et al. Systemic multicompartmental effects of the gut microbiome on mouse metabolic phenotypes. Mol Syst Biol. 2008;4:219 [Research Support, Non-U.S. Gov't].
65. Martin FP, Dumas ME, Wang Y, Legido-Quigley C, Yap IK, Tang H, et al. A top-down systems biology view of microbiome-mammalian metabolic interactions in a mouse model. Mol Syst Biol. 2007;3:112 [Research Support, Non-U.S. Gov't].
66. Martin FP, Wang Y, Yap IK, Sprenger N, Lindon JC, Rezzi S, et al. Topographical variation in murine intestinal metabolic profiles in relation to microbiome speciation and functional ecological activity. J Proteome Res. 2009;8(7):3464–74 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].
67. Martin FP, Wang Y, Sprenger N, Yap IK, Rezzi S, Ramadan Z, et al. Top-down systems biology integration of conditional prebiotic modulated transgenomic interactions in a humanized microbiome mouse model. Mol Syst Biol. 2008;4:205 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].
68. Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, et al. Evolution of mammals and their gut microbes. Science. 2008;320(5883):1647–51.
69. Metges CC, Eberhard M, Petzke KJ. Synthesis and absorption of intestinal microbial lysine in humans and non-ruminant animals and impact on human estimated average requirement of dietary lysine. Curr Opin Clin Nutr Metab Care. 2006;9(1):37–41 [Research Support, Non-U.S. Gov't Review].
70. Pang X, Hua X, Yang Q, Ding D, Che C, Cui L, et al. Inter-species transplantation of gut microbiota from human to pigs. ISME J. 2007;1(2):156–62 [Evaluation Studies Research Support, Non-U.S. Gov't].
71. Wei H, Dong L, Wang TT, Zhang MH, Hua WY, Zhang CH, et al. Structural shifts of gut microbiota as surrogate endpoints for monitoring host health changes induced by carcinogen exposure. FEMS Microbiol Ecol. 2010;73(3):577–86.
72. Chen R, Mias GI, Li-Pook-Than J, Jiang L, Lam HY, Miriami E, et al. Personal omics profiling reveals dynamic molecular and medical phenotypes. Cell. 2012;148(6):1293–307 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].
73. van der Greef J, Stroobant P, van der Heijden R. The role of analytical sciences in medical systems biology. Curr Opin Chem Biol. 2004;8(5):559–65.

Chapter 5
Can We Use Metabolomics to Understand Changes to Gut Microbiota Populations and Function? A Nutritional Perspective

Sofia Moco and Alastair B. Ross

Abstract Food is an integral part of human life, and the composition of our diet is an important determinant of our health and well-being. Food is also the main source of energy and nutrients for the gut microbiota, the 100 trillion cells that coexist inside us. The impact of macronutrients (protein, fat, carbohydrates, and fiber) and specific non-nutrient food components (polyphenols) will be reviewed in the context of gut microbial function and interaction with the host. Colonic microbiota provides diverse enzymatic activities differing from our own, which lead to the production of metabolites essential for key metabolic functions, including carbohydrate and amino acid metabolism. Certain gut metabolites are specific to microbial activity and confer functionalities beyond energy production, such as signalling cascades across cells, tissues, and organs. Metabolomics has proven to be a useful tool to measure the effects of food on the gut microbiota and its interaction with host metabolism.

Keywords Nutrition • Gut • Metabolomics • Digestion • Phase II metabolism • Food • One carbon metabolism • Polyphenols • Fiber • Microbiota • Diet • Fat • Protein • Carbohydrate • Choline • Short-chain fatty acids • Phenolic and phenyl metabolites • Indole metabolites • Hippurate • *p*-Cresol sulfate • Trimethylamine oxide • Metabolism • Metabolomics • Colon • Intestine • Butyrate • Gut-liver • Gut-brain • Pathways • Chocolate • Whole-grain cereals • Carnitine • Branched-chain fatty acids • Prebiotics

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5.1 Introduction

The sum of all small molecules in a system (i.e. the metabolome) not only reflects the metabolic response of the subject of interest but also the organisms living in symbiosis with the subject – in the case of humans, the gut microbiota is an example. The gut microbiota produces thousands of metabolites through their reproduction, interaction with other microorganisms, the host and with partially digested food. Many of these metabolites are specific for microbial metabolism, and cannot be synthesized by mammalian enzymes. These specific microbial metabolites can be absorbed from the gut, adding to the diversity of the metabolome, and at the same time providing a window into the interaction between host, food and gut microbiota. In this chapter, we examine what dietary components are known to have an impact on gut microbial metabolism, which biochemical classes of gut metabolites are produced from different diets, and how metabolomics can be a powerful tool to measure the effect of food on the gut microbiota, and its interaction with mammalian metabolism.

5.2 Colonic Digestion

The large intestine is a digestive organ where dietary substrates not absorbed in the small intestine, are further broken down by anaerobic bacteria (Fig. 5.1). The major substrates for colonic fermentation include carbohydrates that have escaped digestion in the upper gut (mainly dietary fibers: resistant starch and non-starch polysaccharide such as celluloses, pectins and gums, and non-digestible oligosaccharides). The main products of carbohydrate fermentation are short-chain fatty acids (SCFAs), such as butyrate, propionate, and acetate, which are then absorbed and used as an energy source. In Western diets, SCFAs contribute less than 10 % to the total energy obtained from food, although in some cases this value can be up to 30 % [1]. Bacteria well adapted for fermenting carbohydrate come from the Prevotella and Xylanibacter genuses [2, 3].

In addition, residual amounts of protein (such as elastin, collagen and albumin), peptides and amino acids can also reach the colon. Proteolytic bacteria in human feces are predominantly Bacteroides and Propionibacterium, with lesser numbers of the genera Streptococcus, Clostridium, Bacillus and Staphylococcus [4]. Low levels of the amino acid fermentation products ammonia and branched-chain fatty acids (BCFA) are found in ileal contents indicating that little amino acid fermentation occurs in the small intestine, underlining the importance of the gut microbiota for producing these compounds. Protein fermentation leads not only to the production of BCFAs but also to relatively low amounts of a variety of products, such as branched-chain amino acids (BCAAs), phenols, and amines which are both absorbed into the host as well as excreted.

The proportion of carbohydrates to protein in the colon has been estimated as 3–4:1. Regional differences occur in the gut, where the right (proximal) gut has a

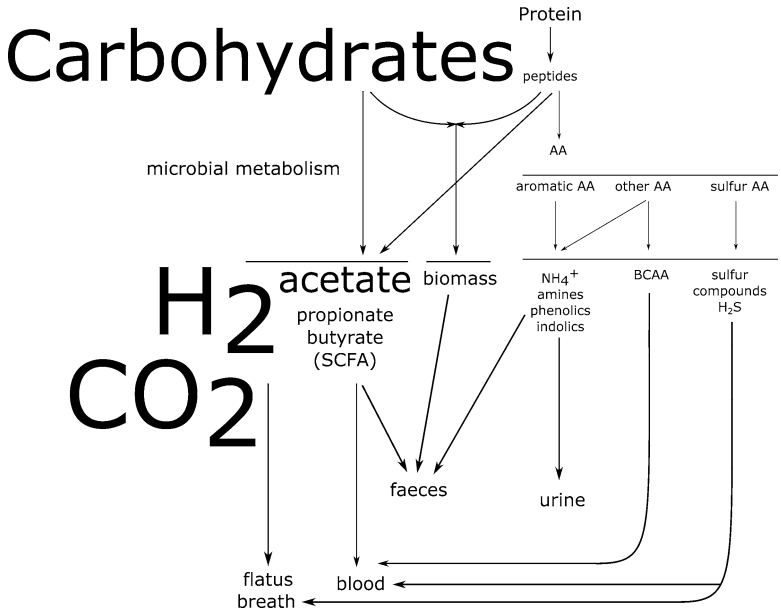

Fig. 5.1 Fermentation in the large bowel (size of compounds' font indicate approximate relative concentration)

higher saccharolytic activity while the left (distal) is more proteolytic. These fermentation processes provide the nutrients required for gut microbiota biomass growth while at the same time producing waste products hydrogen, carbon dioxide, ammonia, and methane which are excreted via flatus. More complex chemical structures such as polyphenols or alkaloids, mostly from plant foods, are also degraded during colonic fermentation [1].

Beyond its role in digestion and absorption, the large intestine contributes to health in a variety of ways: as a physical barrier preventing invasion of pathogenic bacteria and stimulating immune function and as a site for biosynthesis of vitamins and metabolism of xenobiotics.

5.3 Gut Modulation by Foods and Diet

5.3.1 How Do Different Foods Alter Gut Microbiota and Their Metabolism?

There is little controversy in the idea of using food or food ingredients to alter both gut microbiota populations and gut microbiota metabolism. Directly or indirectly, food is the main source of energy and nutrients for the gut microbiota and evolutionary pressure to adapt to the gastrointestinal environment and a major determinant of which

microbial genes are upregulated. Technological developments over the past two decades in the areas of genetic sequencing, to determine the gut microbiome from fecal samples without the need for culture techniques, have led to a rapid explosion of our understanding of the importance of the gut microbiota and how it changes with changing diet. An example of this are pre- and probiotics, where fermentable fiber sources or live bacterial cultures (often in dairy products), are given with the aim to positively alter the gut microbiota.

There is still discussion about what constitutes an "ideal" gut microbiota population, though favorable changes to gut microbiota are generally described towards bacterial genus or species that succeed when carbohydrate is the abundant energy source, while "negative" bacterial species are those that are well adapted to fermenting protein. Arguably, quantifying the population of different bacterial families or species provides little direct information about actual gut bacterial metabolism: many can switch between proteolytic and saccharolytic metabolism. It may be that the end products of microbial metabolism are able to help build the best possible picture of how gut microbiota are collectively responding to different diets or conditions. Some metabolites of dietary substrates are well known and are summarized in Table 5.1.

5.3.2 Microbial Metabolism and a Carbohydrate-Rich Diet

Carbohydrates are an important energy source for both humans and our gut microbiota. They are found in foods in several different forms, including monosaccharides (e.g., glucose), disaccharides (e.g., sucrose or lactose), starch, and a range of different types of dietary fiber, carbohydrates not broken down by human digestive enzymes, but are often fermentable by gut microbiota. While traditional diets are generally rich in complex carbohydrates (e.g., starch) and high in diverse forms of fiber, in "Western" pattern diets, simple sugars (e.g., glucose and sucrose) dominate the carbohydrate fraction of the diet, with low diversity in the small amount of fiber present [2]. It is likely that the difference between traditional and Western dietary patterns also leads to an impact on the gut microbiota and intestinal milieu leading to an increased risk of gastrointestinal disorders including large bowel cancer, gall stones, and Crohn's disease. Diet intervention studies high in refined sugar have found an altered gut metabolism, increased mouth-to-cecum transit time, and increased production of secondary bile acids [5].

One of the main sources of dietary fibers is cereal-based foods. Cereal grains that have the bran and germ fractions removed (refined or "white" flour) are also largely depleted in dietary fiber. Whole grains are cereal grains that still have all the three grain components in their correct proportions (bran, germ, and endosperm) and are generally rich in both insoluble and soluble fibers [6]. Soluble dietary fibers are by definition water soluble and tend to be highly fermentable by the gut microbiota, producing SCFAs. SCFAs appear to have a wide range of roles, including as an energy source and for reducing gut inflammation [2]. Diets high in fiber such as

Table 5.1 Common gut microbial metabolites of dietary substrates detected using metabolomics (urine, feces, blood). oxidation is typical of phase i metabolism and glycine, glutamine and sulfate conjugations are typical of phase ii metabolism occurring in the liver. glycine or glutamine conjugation is pathway- and species-specific (differences may occur between mice, rats and humans)

Microbial metabolite	Dietary precursors	Specific bacterial species	References
Phenolic, benzoyl, and phenyl derivatives	Phenolic compounds; flavonoids; protein (phenylalanine, tyrosine)	*Lactobacillus*	[34, 43, 44, 75–77]
Hippurate		*Clostridium difficile*	
Cinnamoyl (glycine)		*Clostridium scatologenes*	
		Proteus vulgaris	
Phenol (sulfate)		*E. coli*	[77]
		Clostridium bifermentans	
		Clostridium specticum	
		Bacteroids fragilis	
		Bifidobacterium longum	
p-Hydroxyphenylacetate		*Clostridium difficile*	[77]
		Bacteroides ovatus	
		Bifidobacterium bifidum	
		Bifidobacterium adolescentis	
		Bifidobacterium infantis	
		Bifidobacterium longum	
		Bifidobacterium pseudolongum	
p-Hydroxyphenylpropionate		*Clostridium bifermentans*	[77]
		Clostridium paraputrificum	
		Clostridium specticum	
		Bacteroides thetaiotaomicron	
		Bifidobacterium infantis	
p-Cresol (sulfate)		*Clostridium difficile*	[77]
		Clostridium paraputrificum	
		Clostridium perfringens	
		Bacteroids fragilis	
		Bacteroides thetaiotaomicron	
		Bifidobacterium bifidum	
		Bifidobacterium adolescentis	
		Bifidobacterium infantis	
		Bifidobacterium pseudolongum	
		Bacteroides thetaiotaomicron	

(continued)

Table 5.1 (continued)

Microbial metabolite	Dietary precursors	Specific bacterial species	References
Phenylacetate (glycine)		*Clostridium bifermentans*	[77]
		Clostridium difficile	
		Bacteroids fragilis	
		Bacteroides ovatus	
Phenylpropionate (glycine)		*Clostridium difficile*	[77]
		Peptostreptococcus asaccharolyticus	
		Bacteroides ovatus	
Phenyllactate (glycine)		*Clostridium perfringens*	[77]
		Bacteroides ovatus	
		Bifidobacterium longum	
Indole derivatives	Protein (tryptophan)	*E. coli*	[43, 44, 65, 78]
Tryptamine		*Clostridium bifermentans Proteus vulgaris*	
Serotonin		*Paracolobactrum coliforme*	
		Achromobacter liquefaciens	
		Bacteroides spp	
		Clostridia	
Indole (sulfate)		*E. coli*	[77]
		Bacteroides ovatus	
Indole pyruvate		*E. coli*	[77]
		Clostridium perfringens	
		Peptostreptococcus asaccharolyticus	
		Bacteroides ovatus	
		Bifidobacterium bifidum	
		Bifidobacterium adolescentis	
		Bifidobacterium infantis	
		Bifidobacterium pseudolongum	
Indole lactate		*E. coli*	[77]
		Clostridium perfringens	
		Peptostreptococcus asaccharolyticus	
		Bacteroides ovatus	
		Bacteroides thetaiotaomicron	
		Bifidobacterium bifidum	
		Bifidobacterium adolescentis	
		Bifidobacterium infantis	
		Bifidobacterium longum	
		Bifidobacterium pseudolongum	

(continued)

Table 5.1 (continued)

Microbial metabolite	Dietary precursors	Specific bacterial species	References
Indole-3-acetate (glutamine)		*E. coli*	[77]
		Clostridium difficile	
		Clostridium paraputrificum	
		Clostridium perfringens	
		Peptostreptococcus asaccharolyticus	
		Bacteroides fragilis	
		Bacteroides thetaiotaomicron	
		Bifidobacterium pseudolongum	
		Bifidobacterium longum	
Indole-3-propionate		*Clostridium paraputrificum*	[77]
		Peptostreptococcus asaccharolyticus	
		Bacteroides fragilis	
		Bifidobacterium longum	
		Bifidobacterium bifidum	
		Bifidobacterium adolescentis	
		Bifidobacterium infantis	
		Bifidobacterium pseudolongum	
Choline metabolites	Choline	*Bacteroides fragilis*	[15, 79]
Methylamine	Carnitine	*Clostridium perfringens*	
Dimethylamine		*Faecalibacterium prausnitzii*	
Trimethylamine (-*N*-oxide)			
Flavones		*Lactobacillus mucosae*	[43, 80]
Equol (sulfate)	Flavonoids	*Enterococcus faecium*	
Methyl equol (sulfate)		*Finegoldia magna*	
Short-chain fatty acids		*Bifidobacterium*	[81]
Acetate	Glucose and starch	*Propionibacterium*	
Propionate	Polysaccharides	*Lactobacilllus*	
Butyrate	Fiber	*Clostridium*	
Branched-chain fatty acids	Protein (branched-chain amino acids: leucine, isoleucine, valine)	*Bacteroides ruminicola*	[82]
Isobutyrate		*Megasphaera elsdenii*	
Isovalerate			

those rich in whole grains can alter gut microbiota populations [7–9] and gut microbiota metabolism [10, 11]. Of the few metabolomics studies that compared intake of whole grains with refined grains, one rat study found that urinary hippurate was increased on a whole grain diet, along with the aromatic amino acids phenylalanine, tryptophan, and tyrosine [12]. This finding was not replicated in human urine samples after a whole grain intervention, though other biomarkers of gut microbiota activity were decreased on a whole grain diet, including 4-hydroxyphenylacetate, a microbial metabolic product of aromatic amino acid metabolism and trimethylamine, a microbial metabolic product of choline and carnitine [11]. As in many areas of nutritional science, results on the impact of whole grains on gut microbiota are variable, with some studies not finding any changes to gut microbial species measured [13]. This variation in gut microbial response to an admittedly broad and heterogeneous food group may explain in part some of the variation in results between intervention studies, an area that will be further explored as more advanced microbial sequencing techniques become routine [9].

5.3.3 Fat-Rich Diet Interactions with Gut Microbiota

High-fat diets are frequently used in metabolic studies for testing diet-induced metabolic syndrome (increased risk of developing cardiovascular disease and diabetes), especially in rodent models. A direct relationship has been established between high-fat feeding and metabolic disorders, where altered gut flora is causal in inducing gut permeability, increasing lipopolysaccharide (LPS) absorption, and inflammation [14, 15]. Given this association between diet and gut microflora, specific strategies for modifying gut microbiota may be a useful means to reduce the impact of high-fat feeding on the occurrence of metabolic diseases. However, as these results mainly stem from rodent models, where high-fat diets represent a far greater proportion of energy intake than would normally be found among humans, caution is required until definitive clinical studies are performed.

5.3.4 Choline Metabolism: An Interaction Node Between Diet, Host, and Gut Microbiota?

Recent studies from a cohort of non-Caucasians based in Cleveland, United States, have highlighted that gut microbial metabolism of specific dietary components may result in toxic metabolites that lead to cardiovascular disease. Using LC-MS metabolomics, Wang et al. found that high plasma concentrations of a microbial metabolite of choline, trimethylamine (TMA), was related to cardiovascular disease risk, concluding that whether the gut microbiota converted choline into TMA or not was a key modifiable risk factor for development of cardiovascular disease [16]. The active molecule mediating increased disease risk was identified as trimethylamine oxide

(TMAO), a toxic metabolite of liver metabolism of TMA. Choline, and its related metabolite, TMA, and betaine (a downstream mammalian metabolite of choline) were related to cardiovascular disease in this cohort. These metabolomics results were cross validated, and biomarkers confirmed in mouse models of cardiovascular disease, though do not fully explain other findings with the same biomarkers. For example, a comprehensive study on the sources of TMA in humans found that very little choline was converted into TMA [17]. While this could be explained by differences in gut microbiota, the intake of fish and shellfish led to extremely high production of TMA [17], with some fish species leading to an excretion of over 4,000 μmol of TMA and TMAO in urine over 8 h. Meat, eggs, and dairy products conversely did not lead to more TMA and TMAO excretion compared to a control diet [17]. If these results in urine are reflected in plasma, any increase in TMA due to nonoptimal gut microbiota metabolism of choline from fatty foods would be "drowned" out by that due to fish intake. Thus, TMAO being a biomarker for cardiovascular disease risk would be at odds with a high intake of fish being associated with a decrease in cardiovascular disease risk [18–20], which would suggest that TMAO is not a good biomarker of cardiovascular disease in populations where fish intake is common. Similarly, the finding that elevated betaine may be associated with cardiovascular disease risk goes against other work finding that betaine is substantially associated with a decreased risk of cardiovascular disease risk factors [21]. The same study however also found that plasma choline was associated with risk factors for cardiovascular disease [21]. Betaine is one of the main phytochemicals present in whole grain wheat [22], and fasting betaine concentrations can be increased on a whole grain-rich diet [8], and both oral choline and betaine lead to decreased circulating homocysteine [23, 24], a cholesterol-independent risk factor for cardiovascular disease. In the context of these other findings, it is possible that elevated choline and TMAO are biomarkers of cardiovascular disease risk in this population, if fish intake is low.

In a follow-up study using stable isotope-labelled phosphatidylcholine, the role of gut microbiota in the formation of TMAO from choline was clearly established, along with choline being the main source of circulating betaine [25]. So in this population, elevated betaine probably comes from a high intake of choline, rather than a high intake of betaine-containing foods. The complementary analysis of food records and use of dietary biomarkers of intake (e.g., alkylresorcinols for whole grains [26] or omega-3 fatty acids for fish intake [27, 28]) along with gut microbiota measurements and metabolomics may be instructive for determining if elevated concentrations of these biomarkers are related to disease risk or diet.

A second study by the same group hypothesized that another TMA, L-carnitine, may also be a risk factor for cardiovascular disease as it can also be metabolized by gut microbiota to TMAO [29]. Carnitine, which is a major component of red meat and is conditionally essential for fatty acid transport for mitochondria, was found to lead to increased TMAO production that depended on gut microbiota. Of interest for metabolomic methodology is that in the initial screening of the same cohort where choline was suggested to be a risk factor for CVD [16], carnitine was only found to be a significant risk factor if correction for multiplicity was not used in the statistical analysis. While statistical considerations are important, the possibility

that associations of interest may be lost when using stringent tests should not be overlooked. A recent study comparing a whole grain diet to a refined grain diet found that urinary excretion of carnitine and acetylcarnitine was reduced when consuming whole grains, along with a decrease in the TMAO precursor TMA [11], in a study where gut microbial populations were also altered due to the whole grain intervention [8]. It is clear from this work on precursors of TMAO that while there may be several complexities in assigning biomarkers to disease risk that are also derived from diet, the one carbon metabolism pathway and phospholipid metabolism are likely key areas of interaction between diet, physiology, gut microbiota, and cardiovascular disease.

5.3.5 *Protein-Rich Diets and Gut Microbiota*

While human protein digestion and amino acid absorption are efficient, some proteins and free amino acids still reach the colon and are associated to increased production of potentially toxic substances such as volatile sulfur compounds, ammonia, and *p*-cresol [30, 31]. Experimental evidence from animal models and in vitro data shows that dietary proteins can influence cancer expression. Increased dietary protein consumption can cause increased colonic DNA damage and thinning of the colonic mucosal barrier [32]. Production of microbial metabolites from amino acids can be reduced by dietary fiber (via increasing the proportion of carbohydrate reaching the colon), as carbohydrate appears to be a preferred substrate for most gut microbiota species [32].

The molar ratios of the BCFAs isovalerate and isobutyrate, compounds resulting from the bacterial fermentation of valine and leucine, were found to be increased relative to total fecal SCFAs with high-protein diets. A marked increase in fecal nitrogenous organic compounds (NOC) amounts was also found when subjects consumed high-protein diets. NOCs are carcinogens in vitro; although the toxicological significance of increased fecal NOCs is uncertain, NOCs, at concentrations present in the colonic lumen, contribute to DNA damage in the colon and rectum and possibly to increased risk of human cancer [33]. Broadly speaking, much evidence suggests that interaction between protein and amino acids is negative for the host, though secondary roles of these metabolites on gene signalling and immune function have not been researched.

5.3.6 *Interaction of a Polyphenol-Rich Diet with the Gut Microbiota*

While not a nutrient in the strict sense, polyphenols, or at least polyphenol-rich foods may also lead to a change in gut microbiota metabolism, notable examples being coffee and chocolate [34]. This metabolic interaction may lead to many

downstream effects and it has even been suggested that there is a link between preference for chocolate and gut microbiota, depending on how cocoa polyphenols are metabolized [35]. While conceptually it makes sense that people who regularly consume chocolates have gut microbiota more readily adapted to metabolizing cocoa polyphenols compared to those who avoid chocolate, it remains an intriguing question as to whether there are wider effects beyond gut microbial metabolism and into the realm of "gut-host interactions." Certainly recent studies in both humans [36] and rodents [37] clearly demonstrate that cocoa polyphenols can alter gut microbiota populations. In vitro colon model studies find that cocoa polyphenols increase butyric acid production and formation of 3-hydroxyphenylpropionic acid from cocoa flavanols [38]. Consumption of dark chocolates also increases 3-hydroxyphenylpropionic acid and hippurate excretion in urine [35]. As will be addressed below, phenolic compounds may also be metabolized into hippuric acid [39], and this convergence with amino acid metabolism may lead to some problems in interpreting metabolomics data relating to polyphenols and amino acid intake. To be confident of identifying changes in gut microbial metabolism, several related changes may need to be identified, preferably with concurrent changes to gut microbiota populations.

While it is clear that there is a relationship between diet, gut microbiota, and certain metabolites resulting from gut microbial metabolism, the link between gut microbial metabolites and systemic effects remains largely unclear. Are they simply markers, or also mediators? A number of dietary phenolic compounds act as signalling molecules for regulating various metabolic cascades [40], though no data exists on whether of the common aromatic metabolites identified as being produced by gut microbiota have any role in influencing gene expression.

There is much that remains to be studied in terms of the diet and gut microbiota – protein-rich or sulfur-rich diets have received relatively little attention compared to high-fat diets or different sources of carbohydrates and prebiotics. Beyond specific pre- and probiotics, several different food groups are also known to have an effect on the gut microbiota, with consequent possible effects on gut microbial metabolism, though to date whether these effects have long-term effects on the host is less clear. This is further complicated by the apparent resistance of gut microbial populations to long-term change, as demonstrated by fecal transplantation studies, where host populations frequently revert towards pretransplantation levels [41].

5.4 Nutritional Metabolomics: A Methodology Well Suited for Understanding the Effects of Food on Gut Microbiota

Metabolomics is the study of multiple metabolites (small molecules, generally <1,500 Da) in response to different stimuli or conditions and generally involves the measuring of several to hundreds of metabolites or thousands of features in a metabolic profile [42]. This is followed by multivariate analysis to determine what metabolite(s) best explain(s) the research question. Metabolomics is

complementary to other omics such as genomics and proteomics. So while genetics is often seen as a "blueprint," genetic disposition is often not reflected in phenotype. As metabolites are the end product of genotype differences, they reflect how a system is responding to different stimuli. Simplistically, metabolites can be seen as the result of genotype + epigenetic modification + posttranslational modification of proteins + interaction with the environment. Chapters 2 and 3 elaborate on general metabolomics methodologies and data modelling.

5.4.1 Metabolomics Methods to Study Gut Activity Effects on Metabolism

Metabolomics is mainly based on two technologies: nuclear magnetic resonance (NMR) and mass spectrometry (MS). Among a wide variety of applications, it has been used in characterizing the metabolic fingerprint of mammalian hosts under conditions designed to alter the microbial communities in the gastrointestinal tract. While a wealth of studies have found associations between metabolic patterns and diseases to (deregulated) gut microbiota, the full biochemical characterization of the gut microbial activity is yet to be defined. To define the metabolome of the gut microbiome, Wikoff et al. [43] used an untargeted MS-based strategy to compare plasma extracts of germ-free mice to conventional mice. Indole-containing metabolites, phenylated-organic acids, and phase II metabolites of these (sulfated and glucuronidated species) were found in conventional mice and either absent or poorly represented in germ-free animals. The absence of phase II metabolites in germ-free mice suggests a direct impact of the gut microflora on the drug metabolism capacity of the host, where interplay between gut (microbes) and liver (mammal) takes place.

Other strategies to investigate the function of the metabolite influenced by the gut microbiota have included urinary and fecal MS-based profiling of metabolites from Wistar rats exposed to a broad-spectrum β-lactam antibiotic (imipenem/cilastatin sodium) and were compared before and after exposure [44]. An apparent metabolic switchover is observed within 24 h of antibiotic exposure and affecting a wide range of central metabolic pathways (mainly amino acid metabolism, but also organic acid metabolism, oligopeptides, carbohydrate metabolism, purine and pyrimidine metabolism, and the TCA cycle). Benzene- and indole-containing substances, including tryptophan and hippuric acid, were dramatically reduced by the antibiotic treatment.

These two studies [43, 44], using different strategies to remove the influence of the gut microbiota, lead to consistent results on the chemical nature of metabolites produced by gut microbiota activity. The fact that different biological matrices were used for metabolomics analysis (plasma [43] and urine and feces [44]) suggests comparable effects in all systemic biofluids, at least in rodents.

While most metabolomics studies have focused on metabolite analysis of plasma, urine, and fecal water, there are other potential methods for understanding gut microbiotal metabolism that to date have not had widespread use. These include

headspace GC-MS analysis of volatile organic compounds from feces [45], which can measure up to 90 compounds present in the fecal headspace. Breath analysis may also be a fruitful area for understanding gut microbiota activity; several breath analyses are already used in nutrition to measure gut microbiota activity including breath hydrogen to monitor gut fermentation and the urea breath test for *Helicobacter pylori* infection [46]. Gasses produced by gut bacterial fermentation not only exit via flatus but can also be measured in breath – especially those that are active in the stomach, as is the case with *H. pylori*. The lactose breath test is another practical example, where lactose tolerant people can break down lactose before reaching the intestine, while in lactose-intolerant people, lactose reaches the intestine and is rapidly fermented, leading to the production of hydrogen gas. Hydrogen is normally present in low concentrations in breath, so any spike in breath hydrogen is clearly linked to lactose intolerance. The same concept is also used for measuring the fermentation of dietary fibers by gut microbiota in clinical trials [47].

5.5 Metabolites of Gut Activity

The ensemble of bacterial species in the gut can modulate metabolic reactions essential to the host's metabolism and health. There are a set of metabolites that consistently directly or indirectly stand out in association studies on diseases such as obesity, insulin resistance, type II diabetes, cancer, cardiovascular disease, chronic systemic inflammation, and autism and related neurological conditions [15]. These metabolites include SCFAs, bile acids, choline metabolites, phenolic, benzoyl, and phenyl derivatives and indole derivatives (Figs. 5.1 and 5.2). Given the range of conditions where these metabolites may be involved, there is now little doubt about the contribution of the gut microbiota to host metabolism and maintenance.

5.5.1 Short-Chain Fatty Acids

Possibly the best known examples of gut microbiota metabolites are the SCFAs acetate, propionate, and butyrate, which result from bacterial fermentation of carbohydrates. These are water-soluble and readily absorbed respiratory fuels used by the colonic epithelial cells (colonocytes) produced by anaerobic bacteria. Luminal fatty acids are the preferred fuels of colonocytes and the order of preference is SCFAs > ketone bodies > amino acid > glucose [48]. Butyrate is the preferred source of energy for colonic epithelial cells. Butyrate is transported into colonocytes, enters the mitochondria, and undergoes β oxidation to acetyl-CoA, which enters the TCA cycle resulting in the reduction of NAD^+ to NADH. NADH enters the electron transport chain culminating in ATP production with CO_2 as a by-product [49]. Butyrate is associated with a decreased risk of colon cancer proliferation, modulation of

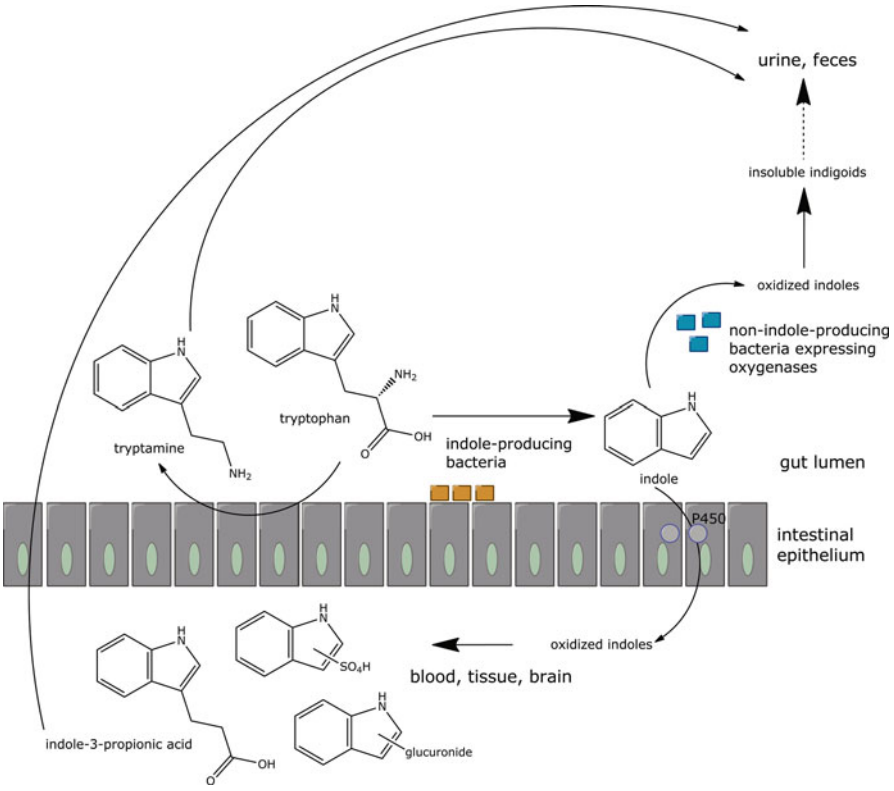

Fig. 5.2 Proposed indole signalling and metabolism in the intestine (inspired by [43, 44, 65])

inflammation, and an increase in satiety [50–54]. Absorbed acetate and propionate are delivered to hepatocytes, which consume most of the propionate for gluconeogenesis. Although acetate can be used for lipogenesis in colonocytes, hepatocytes and adipocytes are the principal sites for de novo lipogenesis, at least in rodents. SCFAs also act as signalling molecules. Propionate, acetate, and to a lesser extent butyrate and pentanoate are ligands for at least two G protein-coupled receptors (GPCRs), Gpr41 and Gpr43. Both GPCRs are broadly expressed, including in the distal small intestine, colon, and adipocytes. SCFAs (C1–C6), which are ligands for Gpr41, stimulate expression of leptin in mouse-cultured adipocytes [55]. Leptin is a polypeptide hormone with pleiotropic effects on appetite and energy metabolism. This possible link between fiber intake, gut microbiota, and satiety has opened up a new area of possibilities for nutrition research. Clostridia are saccharolytic and amino acid fermenting species and are able to produce the three main SCFAs in the colon. Many other bacteria such as Bacteroides, Eubacteria, and Propionibacteria are known for producing specific SCFA from various substrate sources [4, 49]. Most SCFAs are produced from the fermentation of carbohydrates, with smaller amounts by microbial protein degradation in the large intestine. In addition to providing energy for the colon, SCFAs are important energy sources for muscle, kidneys, heart, and brain.

5.5.2 *Phenolic and Phenyl Metabolites*

Hippuric acid is a conjugate of glycine with benzoic acid and is a common end product of metabolism excreted in urine. Dietary sources of protein and polyphenols (fruits, vegetables, coffee, tea, chocolate) ultimately lead to the degradation into quinic acid and benzoic acid by colonic microflora which are then oxidized to hippurate by hepatic mitochondrial function, in a CoA-dependent fashion [34, 39, 56]. Therefore, diets rich in protein and polyphenols lead to increased excretion of hippurate and other phenol-related metabolites such as *p*-cresol, phenol, *p-hydroxybenzoic* acid, and *p*-hydroxyphenylacetic acid [34, 57]. *p-Hydroxyphenylacetic* acid has been identified as an intermediate of *p*-cresol production from tyrosine and is elevated in a variety of conditions [34, 57]. Hippurate is possibly the most recognized gut microbial co-metabolite and has been associated with a variety of conditions or disease status, such as obesity, high blood pressure, Crohn's disease and ulcerative colitis, autism (decreased excretion), type I and II diabetes, and anxiety (increased excretion) [39]. As an example of the relative amount of hippurate precursors, Table 5.2 summarizes different foods and their potential to form hippurate. The amount of potential hippurate from coffee and tea is far greater than the other types of food listed, most notably dark chocolate, also considered a major source of polyphenols in the diet. Aromatic amino acids are also potential sources of hippurate, though the amount of these actually reaching the large intestine is potentially lower than for polyphenols. Caution is needed with the interpretation that elevated hippuric acid is mainly due to polyphenol-rich foods in the diet, as aromatic amino acid-rich foods such as meat and fish could lead to elevated levels in cases of protein malabsorption.

p-Cresol-sulfate is an abundant compound in urine that is obtained from protein fermentation in the human gut, derived from tyrosine and phenylalanine metabolism. Gut bacteria [57, 58] such as the pathogen *Clostridium difficile* [59] are able to convert tyrosine into *p*-cresol. In humans, *p*-cresol is almost completely sulfonated into *p*-cresol sulfate by SULT1A1 (human cytosolic sulfotransferase) which is an enzyme able to sulfonate various substrates, including xenobiotics [60]. High amounts of *p*-cresol in urine are found in adult celiac disease patients [57]. *p*-Cresol can exert a variety of effects such as activation of leukocyte free radical production [61] and blocking the conversion of dopamine into noradrenaline [62]. It is argued that given the high amount of *p*-cresol produced in the body, depending on the diet and eventual modulation of gut bacterial composition, impaired sulfation and events thereof (drug metabolism) might take place, depending on the individual [60]. In addition to *p*-cresol, phenol is also produced in the gut, mostly attributed to aerobic bacteria (*E. coli*, *Proteus* sp, *S. faecalis*, *Staphylococcus albus*), while *p*-cresol is produced by anaerobic bacteria [58]. As anaerobic bacteria outnumber aerobic bacteria in the gastrointestinal tract, it is expected that there is greater excretion of *p*-cresol than phenol.

Phenylacetylglutamine is derived from β-phenylethylamine formed in the large bowel by decarboxylation of phenylalanine released by bacterial proteolysis of

Table 5.2 Relative amounts of aromatic amino acids and common phenolic precursors for hippurate in different foods. Several of these precursors may be metabolized through different pathways, so this table should only be considered as a relative indication of the hippuric acid potential of different foods

	Phenylalanine (g/100 g)	Tyrosine (g/100 g)	Glycine (g/100 g)	Chlorogenic acids (mg/100 g)	Caffeic acid (mg/100 g)	Catechin and epicatechin (mg/100 g)	Total hippurate mole equivalents (mmol/100 g)	Total glycine equivalents (mmol/100 g)	References
Milk chocolate	0.34	0.23	0.17	–	–	67	3.56	2.26	[83–85]
Dark chocolate	0.2	0.074	0.18	–	–	200	2.31	2.40	[83–85]
Fish (smoked salmon)	0.86	0.72	0.99	–	–	–	9.18	13.19	[83]
Fish (smoked herring)	0.82	0.73	0.92	–	–	–	8.99	12.26	[83]
Beef (rump steak)	0.75	0.62	1.1	–	–	–	7.96	14.65	[83]
Chicken (breast meat)	0.74	0.61	1.1	–	–	–	7.85	14.65	[83]

Refined wheat	0.51	0.22	0.35	–	–	–	4.30	4.66	[83]
Whole grain wheat	0.52	0.29	0.48	–	–	–	4.75	6.39	[83]
Refined rice	0.45	0.24	0.38	–	–	–	4.05	5.06	[83]
Brown rice	0.48	0.26	0.41	–	0.3	6	4.36	5.46	[83, 86]
Coffee (soluble, dry)	0.24	0.16	0.42	8,800	1,740	–	36.83	5.59	[83, 87, 88]
Tea (black, leaves)	Trace	Trace	Trace	100	56.8	3,700	13.34	0.00	[87, 89]
Tea (green, leaves)	Trace	Trace	Trace	150	52	5,960	21.24	0.00	[87, 89]

Trace: Trace amounts found, but close to methodological limit of detection
–: No data found, probably not present in foodstuff

unabsorbed protein residues [63]. Indoxyl sulfate and phenylacetylglutamine have been found in higher concentrations in the plasma of diabetic individuals compared to nondiabetics. Abnormal urinary excretion of phenylacetylglutamine, hippurate, and hydroxyhippurates has been reported in autistic children [64] (see Chap. 16).

5.5.3 Indole Metabolites

Copious amounts of indole are produced by the human body and ultimately excreted in urine, in the form of indoxyl sulfate. Indole has been thus associated to gut microbial activity and is produced by tryptophanase (TnA) that reversibly converts tryptophan into indole, pyruvate, and ammonia [65]. The elimination of tryptophan, instead of indole, in urine can be associated to altered microbial activity in the gut. Over 85 bacterial species are known as indole-producing bacteria [65] and in the gut, indole is a signalling molecule recognized by intestinal epithelial cells and is used to strengthen the host cell barrier, maintain controlled inflammation, and increase resistance to pathogen colonization [66]. It is not known if *E. coli* is able to degrade indole, but many non-indole-producing bacteria encode various oxygenases that can modify or degrade indole, producing indole derivatives, such as indigoid compounds [43, 65]. Independent from gut microbial activity, indole can be further modified into sulfated, glucuronidated, and fatty acid-conjugated species, such as indoxyl sulfate, indoxyl glucuronide, and indole-3-propionic acid (IPA) and indole-2-acetic acid (IAA) [43] (Fig. 5.2). Indole has been compared to the known autoinducer-2 (AI-2), a furanosyl borate diester, from the family of signalling molecules used in quorum sensing, although it is still unclear how the roles of two molecules are connected to each other [65].

Tryptamine is another metabolite in the tryptophan metabolism that is decarboxylated by mammalian L-tryptophan decarboxylyase from tryptophan, as well as degraded into indole-3-acetaldehyde by gut bacteria. Low tryptamine levels in urine have been detected in patients with severe depression [67], while high levels of this molecule in urine and feces have been found in antibiotic-treated subjects [44]. Abnormal tryptophan metabolism is indicated in cognitive disorders, such as depression, and 5-hydroxy-L-tryptophan has been used clinically for decades to increase serotonin production in the brain [68].

IAA is a known phytohormone, which belongs to the auxin class of compounds that regulates cell growth and development. Diverse bacterial strains produce IAA, especially endophytes, which signal biofilm formation. In the gut, indoles have been described to lead to biofilm formation [65] and regulation of virulence in vitro and in vivo [69] and specifically IAA has been identified as a marker of gut activity [44] and enhancer of cellular defense [70]. Thus, it could be speculated that the indole class of compounds may act as inter-kingdom signalling molecules regulating mammalian, bacterial, and plant signalling.

IPA is a potent antioxidant and neuroprotective molecule. IPA completely protected primary neurons and neuroblastoma cells against oxidative damage and

death caused by exposure to Alzheimer β-amyloid protein, by inhibition of superoxide dismutase, or by treatment with hydrogen peroxide [71]. The capacity of IPA to scavenge hydroxyl radicals exceeded that of melatonin, an indoleamine considered to be the most potent naturally occurring scavenger of free radicals [71].

In addition, 6-hydroxymelatonin, an oxidation product of melatonin was also identified as a marker of gut activity, as well as other known neurotransmitters such as DOPA, dopamine, norepinephrine, and epinephrine which play important roles in the brain [44]. Several stress mechanisms have been correlated to alteration of bacterial composition of the gastrointestinal tract (GI), altering epithelial cell function, motility, and mucus secretion. Upon stress, norepinephrine is released into the GI tract, potentially altering gut microbial composition and function [72]. These findings are evidence of the strong association of microbial-mammalian metabolism to the gut-brain axis.

5.5.4 Choline Metabolites

Eggs, milk, liver, red meat, poultry, shell fish, and fish are natural sources of phosphatidylcholine and choline. As described earlier, microbial conversion of dietary phosphatidylcholine and choline (or betaine) leads to the production of TMA in the gut which is oxidized in the liver to TMAO by the hepatic flavin monooxygenase (FMO) family of enzymes, FMO3. A study on mice has shown that dietary supplementation with choline, TMAO, or betaine was found to promote upregulation of multiple receptors linked to atherosclerosis [16]. Increased levels of TMAO were also associated with nonalcoholic fatty liver disease [73].

5.6 Gut-Liver Interplay

A portion of the products of colonic fermentation are absorbed by the colonocytes and via specific pathways lead to the production of ATP, while others undergo biotransformation in the liver entering phase I and phase II types of metabolism (Fig. 5.3). Especially for phenyl, phenol, and indole derivatives, sulfation, glucuronidation, and glycine conjugation occur and have been described [43]. Most commonly these metabolites are more water soluble and increased polarity of conjugates may limit passive partitioning into cells, thus increasing excretion.

Biotransformation capability of the host is dependent on several factors, including age, gender, genetic variability, nutrition, disease, exposure to other chemicals that can inhibit or induce enzymes, and dose levels. For instance, the elderly shows decreased biotransformation capabilities and gender may also influence the efficiency of biotransformation for specific metabolites or xenobiotics, as this is usually limited to hormone-related differences in the oxidizing cytochrome P-450 enzymes. This area is especially deserving of attention as we seek to further our understanding of what role a dynamic gut microbiota may play in the host aging process.

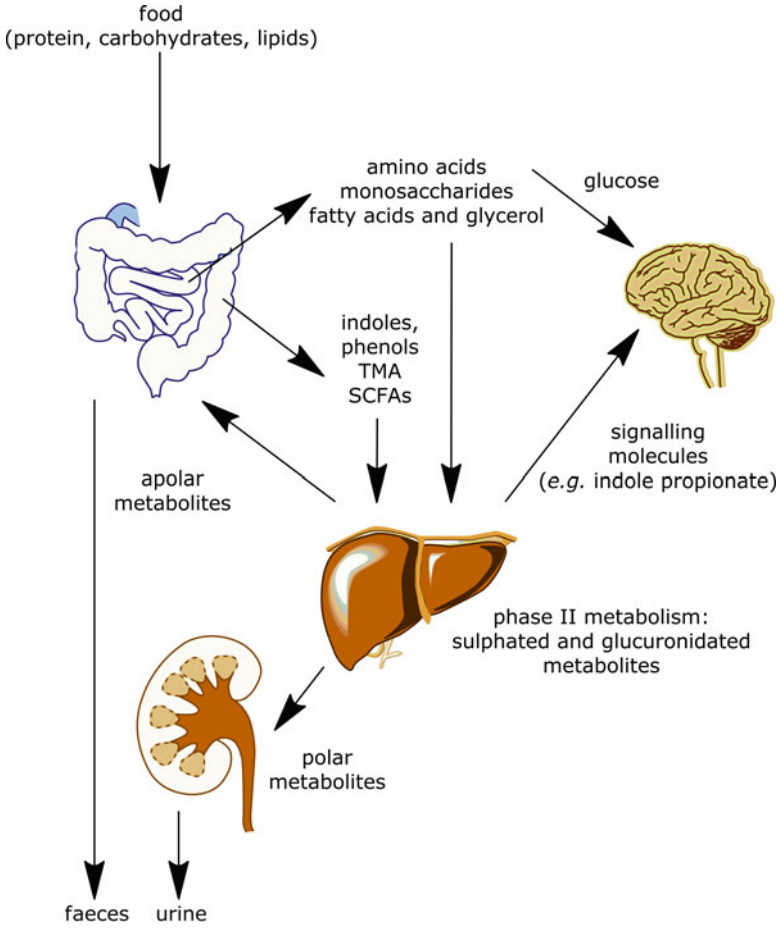

Fig. 5.3 Overview of the interaction between host organs and gut microbiota

5.7 Future Directions

Gut microbiota has relevance for human health and disease beyond the gastrointestinal tract, appearing to have a systemic impact on human metabolism, through interaction with multiple organs. The gut microflora has proven causality in the induction of some metabolic disorders (see Chap. 12), and therapies that target the gut microbiota are at the forefront of nutrition research. Modulation of the gut microbiota is potentially attainable by altering dietary habits; however, we are still far away from understanding either general effects of macronutrients or specific effects of ingredients on gut microbiotal metabolism. There are cautionary tales too – while it is tempting to propose a role for gut microbiota in all observed health benefits related to food, this is not always the case. In one study, cereal fiber changed

gut microbiota composition, but there was no association between these changes and an observed improvement in insulin sensitivity [74]. It is possible that more focus is needed on microbial metabolism, rather than population shifts, an area where metabolomics may be a particularly useful methodology for helping to find answers.

While metabolomics has been instrumental as an exploratory tool to fuel ideas and propose novel hypotheses, we believe that strategies to monitor the gut microbial metabolome will be crucial to define gut activity and its impact on metabolism. To achieve this, targeted metabolomics methods should be implemented to follow the different classes of gut microbial metabolites in health and disease. The quantitation of metabolites will becoming increasingly important to define the kinetics of metabolic fluxes, and to determine mechanisms of action and their association with functionality.

Studying the potential activity of the gut cannot be deduced by solely looking at fecal samples, as fecal transit can vary considerably (12–120 h) and bacterial gradients in the colon exist and thus fecal samples may only be a poor approximation of metabolism along the colon. Gut microbiota metabolites seem to be not only products of digestion, and therefore simpler molecules to be either taken up as energy or discarded by the host, but also signalling functions are being unraveled that prospect a more complex interplay between microbiota and host. It is clear that in terms of our knowledge on the relationship between the diet and nutrition of the host, and our gut microbiota, we are at the beginning of an area that will have a profound impact on our current understanding of human nutrition.

References

1. Salminen S, Bouley C, Boutron-Ruault MC, Cummings JH, Franck A, Gibson GR, Isolauri E, Moreau MC, Roberfroid M, Rowland I. Functional food science and gastrointestinal physiology and function. Br J Nutr. 1998;80 Suppl 1:S147–71.
2. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G, Lionetti P. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A. 2010;107(33):14691–6.
3. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y-Y, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R, Sinha R, Gilroy E, Gupta K, Baldassano R, Nessel L, Li H, Bushman FD, Lewis JD. Linking long-term dietary patterns with gut microbial enterotypes. Science. 2011;334(6052):105–8.
4. Macfarlane GT, Cummings JH, Allison C. Protein degradation by human intestinal bacteria. J Gen Microbiol. 1986;132(6):1647–56.
5. Kruis W, Forstmaier G, Scheurlen C, Stellaard F. Effect of diets low and high in refined sugars on gut transit, bile acid metabolism, and bacterial fermentation. Gut. 1991;32(4):367–71.
6. van der Kamp JW, Poutanen K, Seal CJ, Richardson DP. The HEALTHGRAIN definition of 'whole grain'. Food Nutr Res. 2014;58:22100.
7. Costabile A, Klinder A, Fava F, Napolitano A, Fogliano V, Leonard C, Gibson GR, Tuohy KM. Whole-grain wheat breakfast cereal has a prebiotic effect on the human gut microbiota: a double-blind, placebo-controlled, crossover study. Br J Nutr. 2008;99(1):110–20.

8. Ross AB, Bruce SJ, Blondel-Lubrano A, Oguey-Araymon S, Beaumont M, Bourgeois A, Nielsen-Moennoz C, Vigo M, Fay LB, Kochhar S, Bibiloni R, Pittet AC, Emady-Azar S, Grathwohl D, Rezzi S. A whole-grain cereal-rich diet increases plasma betaine, and tends to decrease total and LDL-cholesterol compared with a refined-grain diet in healthy subjects. Br J Nutr. 2011;105(10):1492–502.
9. Martínez I, Lattimer JM, Hubach KL, Case JA, Yang J, Weber CG, Louk JA, Rose DJ, Kyureghian G, Peterson DA, Haub MD, Walter J. Gut microbiome composition is linked to whole grain-induced immunological improvements. ISME J. 2012;7(2):269–80.
10. McIntosh GH, Noakes M, Royle PJ, Foster PR. Whole-grain rye and wheat foods and markers of bowel health in overweight middle-aged men. Am J Clin Nutr. 2003;77(4):967–74.
11. Ross AB, Pere-Trepat E, Montoliu I, Martin FP, Collino S, Moco S, Godin JP, Cleroux M, Guy PA, Breton I, Bibiloni R, Thorimbert A, Tavazzi I, Tornier L, Bebuis A, Bruce SJ, Beaumont M, Fay LB, Kochhar S. A whole-grain-rich diet reduces urinary excretion of markers of protein catabolism and gut microbiota metabolism in healthy men after one week. J Nutr. 2013;143(6):766–73.
12. Fardet A, Canlet C, Gottardi G, Lyan B, Llorach R, Remesy C, Mazur A, Paris A, Scalbert A. Whole-grain and refined wheat flours show distinct metabolic profiles in rats as assessed by a 1H NMR-based metabonomic approach. J Nutr. 2007;137(4):923–9.
13. Lappi J, Kolehmainen M, Mykkanen H, Poutanen K. Do large intestinal events explain the protective effects of whole grain foods against type 2 diabetes? Crit Rev Food Sci Nutr. 2013;53(6):631–40.
14. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, Burcelin R. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes. 2008;57(6):1470–81.
15. Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W, Pettersson S. Host-gut microbiota metabolic interactions. Science. 2012;336(6086):1262–7.
16. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, Feldstein AE, Britt EB, Fu X, Chung YM, Wu Y, Schauer P, Smith JD, Allayee H, Tang WH, DiDonato JA, Lusis AJ, Hazen SL. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature. 2011;472(7341):57–63.
17. Zhang AQ, Mitchell SC, Smith RL. Dietary precursors of trimethylamine in man: a pilot study. Food Chem Toxicol. 1999;37(5):515–20.
18. Takata Y, Zhang X, Li H, Gao YT, Yang G, Gao J, Cai H, Xiang YB, Zheng W, Shu XO. Fish intake and risks of total and cause-specific mortality in 2 population-based cohort studies of 134,296 men and women. Am J Epidemiol. 2013;178(1):46–57.
19. Li YH, Zhou CH, Pei HJ, Zhou XL, Li LH, Wu YJ, Hui RT. Fish consumption and incidence of heart failure: a meta-analysis of prospective cohort studies. Chi Med J. 2013;126(5):942–8.
20. Raatz SK, Silverstein JT, Jahns L, Picklo MJ. Issues of fish consumption for cardiovascular disease risk reduction. Nutrients. 2013;5(4):1081–97.
21. Konstantinova SV, Tell GS, Vollset SE, Nygård O, Bleie Ø, Ueland PM. Divergent associations of plasma choline and betaine with components of metabolic syndrome in middle age and elderly men and women. J Nutr. 2008;138(5):914–20.
22. Bruce SJ, Guy PA, Rezzi S, Ross AB. Quantitative measurement of betaine and free choline in plasma, cereals and cereal products by isotope dilution LC-MS/MS. J Agric Food Chem. 2010;58(4):2055–61.
23. Zeisel SH, Caudill MA. Choline. Adv Nutr. 2010;1:46–8.
24. Olthof MR, Van Vliet T, Boelsma E, Verhoef P. Low dose betaine supplementation leads to immediate and long term lowering of plasma homocysteine in healthy men and women. J Nutr. 2003;133(12):4135–8.
25. Tang WHW, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, Wu Y, Hazen SL. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med. 2013;368(17):1575–84.
26. Ross AB. Present status and perspectives on the use of alkylresorcinols as biomarkers of wholegrain wheat and rye intake. J Nutr Metab. 2012;12:46297.

27. Markhus MW, Graff IE, Dahl L, Seldal CF, Skotheim S, Braarud HC, Stormark KM, Malde MK. Establishment of a seafood index to assess the seafood consumption in pregnant women. Food Nutr Res. 2013;57:19272.
28. Chien KL, Lee MS, Tsai YT, Chen PR, Lin HJ, Hsu HC, Lee YT, Chen MF. A Taiwanese food frequency questionnaire correlates with plasma docosahexaenoic acid but not with plasma eicosapentaenoic acid levels: questionnaires and plasma biomarkers. BMC Med Res Methodol. 2013;13:23.
29. Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, Britt EB, Fu X, Wu Y, Li L, Smith JD, Didonato JA, Chen J, Li H, Wu GD, Lewis JD, Warrier M, Brown JM, Krauss RM, Tang WH, Bushman FD, Lusis AJ, Hazen SL. Intestinal microbiota metabolism of l-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat Med. 2013;19(5):576–85.
30. Geypens B, Claus D, Evenepoel P, Hiele M, Maes B, Peeters M, Rutgeerts P, Ghoos Y. Influence of dietary protein supplements on the formation of bacterial metabolites in the colon. Gut. 1997;41(1):70–6.
31. Windey K, de Preter V, Verbeke K. Relevance of protein fermentation to gut health. Mol Nutr Food Res. 2012;56(1):184–96.
32. Le Leu RK, Brown IL, Hu Y, Morita T, Esterman A, Young GP. Effect of dietary resistant starch and protein on colonic fermentation and intestinal tumourigenesis in rats. Carcinogenesis. 2007;28(2):240–5.
33. Russell WR, Gratz SW, Duncan SH, Holtrop G, Ince J, Scobbie L, Duncan G, Johnstone AM, Lobley GE, Wallace RJ, Duthie GG, Flint HJ. High-protein, reduced-carbohydrate weight-loss diets promote metabolite profiles likely to be detrimental to colonic health. Am J Clin Nutr. 2011;93(5):1062–72.
34. Moco S, Martin FP, Rezzi S. Metabolomics view on gut microbiome modulation by polyphenol-rich foods. J Proteome Res. 2012;11(10):4781–90.
35. Martin FPJ, Montoliu I, Nagy K, Moco S, Collino S, Guy P, Redeuil K, Scherer M, Rezzi S, Kochhar S. Specific dietary preferences are linked to differing gut microbial metabolic activity in response to dark chocolate intake. J Proteome Res. 2012;11(12):6252–63.
36. Tzounis X, Rodriguez-Mateos A, Vulevic J, Gibson GR, Kwik-Uribe C, Spencer JP. Prebiotic evaluation of cocoa-derived flavanols in healthy humans by using a randomized, controlled, double-blind, crossover intervention study. Am J Clin Nutr. 2011;93(1):62–72.
37. Massot-Cladera M, Perez-Berezo T, Franch A, Castell M, Perez-Cano FJ. Cocoa modulatory effect on rat faecal microbiota and colonic crosstalk. Arch Biochem Biophys. 2012;527(2): 105–12.
38. Fogliano V, Corollaro ML, Vitaglione P, Napolitano A, Ferracane R, Travaglia F, Arlorio M, Costabile A, Klinder A, Gibson G. In vitro bioaccessibility and gut biotransformation of polyphenols present in the water-insoluble cocoa fraction. Mol Nutr Food Res. 2011;55 Suppl 1:S44–55.
39. Lees HJ, Swann JR, Wilson ID, Nicholson JK, Holmes E. Hippurate: the natural history of a mammalian-microbial cometabolite. J Proteome Res. 2013;12(4):1527–46.
40. Mitjavila MT, Moreno JJ. The effects of polyphenols on oxidative stress and the arachidonic acid cascade. Implications for the prevention/treatment of high prevalence diseases. Biochem Pharmacol. 2012;84(9):1113–22.
41. Brandt LJ, Aroniadis OC. An overview of fecal microbiota transplantation: techniques, indications, and outcomes. Gastrointest Endosc. 2013;78(2):240–249.
42. Moco S, Bino RJ, De Vos RCH, Vervoort J. Metabolomics technologies and metabolite identification. Trends Anal Chem. 2007;26:855–66.
43. Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, Peters EC, Siuzdak G. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proc Natl Acad Sci U S A. 2009;106(10):3698–703.
44. Zheng X, Xie G, Zhao A, Zhao L, Yao C, Chiu NH, Zhou Z, Bao Y, Jia W, Nicholson JK. The footprints of gut microbial-mammalian co-metabolism. J Proteome Res. 2011;10(12):5512–22.
45. Dixon E, Clubb C, Pittman S, Ammann L, Rasheed Z, Kazmi N, Keshavarzian A, Gillevet P, Rangwala H, Couch RD. Solid-phase microextraction and the human fecal VOC metabolome. PLoS One. 2011;6(4):e18471.

46. Parra MD, Martinez JA. Nutritional aspects of breath testing based on stable isotopes. Nutr Rev. 2006;64(7 Pt 1):338–47.
47. Rosén LAH, Silva LOB, Andersson UK, Holm C, Östman EM, Björck IM. Endosperm and whole grain rye breads are characterized by low post-prandial insulin response and a beneficial blood glucose profile. Nutr J. 2009;8:42.
48. Roediger WE, Moore A. Effect of short-chaim fatty acid on sodium absorption in isolated human colon perfused through the vascular bed. Dig Dis Sci. 1981;26(2):100–6.
49. Donohoe DR, Garge N, Zhang X, Sun W, O'Connell TM, Bunger MK, Bultman SJ. The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. Cell Metab. 2011;13(5):517–26.
50. Mansour A, Hosseini S, Larijani B, Pajouhi M, Mohajeri-Tehrani MR. Nutrients related to GLP1 secretory responses. Nutrition. 2013;29(6):813–20.
51. Viladomiu M, Hontecillas R, Yuan L, Lu P, Bassaganya-Riera J. Nutritional protective mechanisms against gut inflammation. J Nutr Biochem. 2013;24(6):929–39.
52. Kaczmarczyk MM, Miller MJ, Freund GG. The health benefits of dietary fiber: beyond the usual suspects of type 2 diabetes mellitus, cardiovascular disease and colon cancer. Metab Clin Exp. 2012;61(8):1058–66.
53. Slavin J. Fiber and prebiotics: mechanisms and health benefits. Nutrients. 2013;5(4): 1417–35.
54. Caricilli AM, Saad MJA. The role of gut microbiota on insulin resistance. Nutrients. 2013;5(3):829–51.
55. Samuel BS, Shaito A, Motoike T, Rey FE, Backhed F, Manchester JK, Hammer RE, Williams SC, Crowley J, Yanagisawa M, Gordon JI. Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. Proc Natl Acad Sci. 2008;105(43):16767–72.
56. Cummings JH, Hill MJ, Bone ES, Branch WJ, Jenkins DJ. The effect of meat protein and dietary fiber on colonic function and metabolism. II. Bacterial metabolites in feces and urine. Am J Clin Nutr. 1979;32(10):2094–101.
57. Lord RS, Bralley JA. Clinical applications of urinary organic acids. Part 2: Dysbiosis markers. Alternat Med Rev. 2008;13(4):292–306.
58. Bone E, Tamm A, Hill M. The production of urinary phenols by gut bacteria and their possible role in the causation of large bowel cancer. Am J Clin Nutr. 1976;29(12):1448–54.
59. Dawson LF, Donahue EH, Cartman ST, Barton RH, Bundy J, McNerney R, Minton NP, Wren BW. The analysis of para-cresol production and tolerance in clostridium difficile 027 and 012 strains. BMC Microbiol. 2011;11:86.
60. Clayton TA, Baker D, Lindon JC, Everett JR, Nicholson JK. Pharmacometabonomic identification of a significant host-microbiome metabolic interaction affecting human drug metabolism. Proc Natl Acad Sci U S A. 2009;106(34):14728–33.
61. Schepers E, Meert N, Glorieux G, Goeman J, Van der Eycken J, Vanholder R. P-cresylsulphate, the main in vivo metabolite of p-cresol, activates leucocyte free radical production. Nephrol Dial Transplant. 2007;22(2):592–6.
62. DeWolf WE, Carr SA, Varrichio A, Goodhart PJ, Mentzer MA, Roberts GD, Southan C, Dolle RE, Kruse LI. Inactivation of dopamine Beta-hydroxylase by p-cresol: isolation and characterization of covalently modified active site peptides. Biochemistry. 1988;27(26):9093–101.
63. Seakins JW. The determination of urinary phenylacetylglutamine as phenylacetic acid. Studies on its origin in normal subjects and children with cystic fibrosis. Clin Chem Acta. 1971;35(1):121–31.
64. Holmes E, Li JV, Athanasiou T, Ashrafian H, Nicholson JK. Understanding the role of gut microbiome-host metabolic signal disruption in health and disease. Trends Microbiol. 2011;19(7):349–59.
65. Lee JH, Lee J. Indole as an intercellular signal in microbial communities. FEMS Microbiol Rev. 2010;34(4):426–44.
66. Bansal T, Alaniz RC, Wood TK, Jayaraman A. The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation. Proc Natl Acad Sci U S A. 2010;107(1):228–33.

67. Coppen A, Shaw DM, Malleson A, Eccleston E, Gundy G. Tryptamine metabolism in depression. Br J Psychiatry J Ment Sci. 1965;111(479):993–8.
68. Metzner L, Kottra G, Neubert K, Daniel H, Brandsch M. Serotonin, l-tryptophan, and tryptamine are effective inhibitors of the amino acid transport system PAT1. FASEB J. 2005;19(11):1468–73.
69. Bommarius B, Anyanful A, Izrayelit Y, Bhatt S, Cartwright E, Wang W, Swimm AI, Benian GM, Schroeder FC, Kalman D. A family of indoles regulate virulence and Shiga toxin production in pathogenic *E. coli*. PLoS One. 2013;8(1):e54456.
70. Bianco C, Imperlini E, Calogero R, Senatore B, Amoresano A, Carpentieri A, Pucci P, Defez R. Indole-3-acetic acid improves *Escherichia coli's* defences to stress. Arch Microbiol. 2006;185(5):373–82.
71. Chyan YJ, Poeggeler B, Omar RA, Chain DG, Frangione B, Ghiso J, Pappolla MA. Potent neuroprotective properties against the Alzheimer beta-amyloid by an endogenous melatonin-related indole structure, indole-3-propionic acid. J Biol Chem. 1999;274(31):21937–42.
72. Collins SM, Bercik P. The relationship between intestinal microbiota and the central nervous system in normal gastrointestinal function and disease. Gastroenterology. 2009;136(6): 2003–14.
73. Dumas ME, Barton RH, Toye A, Cloarec O, Blancher C, Rothwell A, Fearnside J, Tatoud R, Blanc V, Lindon JC, Mitchell SC, Holmes E, McCarthy MI, Scott J, Gauguier D, Nicholson JK. Metabolic profiling reveals a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice. Proc Natl Acad Sci U S A. 2006;103(33):12511–6.
74. Weickert MO, Arafat AM, Blaut M, Alpert C, Becker N, Leupelt V, Rudovich N, Möhlig M, Pfeiffer AF. Changes in dominant groups of the gut microbiota do not explain cereal-fiber induced improvement of whole-body insulin sensitivity. Nutr Metab. 2011;8:90.
75. Ward LA, Johnson KA, Robinson IM, Yokoyama MT. Isolation from swine feces of a bacterium which decarboxylates p-hydroxyphenylacetic acid to 4-methylphenol (p-cresol). Appl Environ Microbiol. 1987;53(1):189–92.
76. Yokoyama MT, Carlson JR. Production of Skatole and para-Cresol by a Rumen *Lactobacillus* sp. Appl Environ Microbiol. 1981;41(1):71–6.
77. Smith EA, Macfarlane GT. Enumeration of human colonic bacteria producing phenolic and indolic compounds: effects of pH, carbohydrate availability and retention time on dissimilatory aromatic amino acid metabolism. J Appl Bacteriol. 1996;81(3):288–302.
78. Keszthelyi D, Troost FJ, Masclee AA. Understanding the role of tryptophan and serotonin metabolism in gastrointestinal function. Neurogastroenterol Motil. 2009;21(12):1239–49.
79. Allison C, Macfarlane GT. Influence of pH, nutrient availability, and growth rate on amine production by *Bacteroides fragilis* and *Clostridium perfringens*. Appl Environ Microbiol. 1989;55(11):2894–8.
80. Decroos K, Vanhemmens S, Cattoir S, Boon N, Verstraete W. Isolation and characterisation of an equol-producing mixed microbial culture from a human faecal sample and its activity under gastrointestinal conditions. Arch Microbiol. 2005;183(1):45–55.
81. Nyangale EP, Mottram DS, Gibson GR. Gut microbial activity, implications for health and disease: the potential role of metabolite analysis. J Proteome Res. 2012;11(12):5573–85.
82. Allison MJ. Production of branched-chain volatile fatty acids by certain anaerobic bacteria. Appl Environ Microbiol. 1978;35(5):872–7.
83. Saxholt E, Christensen AT, Møller A, Hartkopp HB, Hess Ygil K, Hels OH. Fødevaredatabanken, version 7 (Danish Food Database, version 7). Afdelning for Enæring, Fødevareinstituttet, Danmarks Tekniske Universitet (2008). Accessed 6 May 2013.
84. Cooper KA, Campos-Gimenez E, Jimenez Alvarez D, Rytz A, Nagy K, Williamson G. Predictive relationship between polyphenol and nonfat cocoa solids content of chocolate. J Agric Food Chem. 2008;56(1):260–5.
85. Actis-Goretta L, Ottaviani JI, Fraga CG. Inhibition of angiotensin converting enzyme activity by flavanol-rich foods. J Agric Food Chem. 2006;54(1):229–34.
86. Irakli MN, Samanidou VF, Biliaderis CG, Papadoyannis IN. Simultaneous determination of phenolic acids and flavonoids in rice using solid-phase extraction and RP-HPLC with photodiode array detection. J Sep Sci. 2012;35(13):1603–11.

87. Mattila P, Hellstrom J, Torronen R. Phenolic acids in berries, fruits, and beverages. J Agric Food Chem. 2006;54(19):7193–9.
88. Renouf M, Marmet C, Guy P, Fraering AL, Longet K, Moulin J, Enslen M, Barron D, Cavin C, Dionisi F, Rezzi S, Kochhar S, Steiling H, Williamson G. Nondairy creamer, but not milk, delays the appearance of coffee phenolic acid equivalents in human plasma. J Nutr. 2010;140(2):259–63.
89. Kyle JA, Morrice PC, McNeill G, Duthie GG. Effects of infusion time and addition of milk on content and absorption of polyphenols from black tea. J Agric Food Chem. 2007;55(12): 4889–94.

Chapter 6
Automated Annotation of Microbial and Human Flavonoid-Derived Metabolites

Velitchka V. Mihaleva, Fatma Yelda Ünlü, Jacques Vervoort, and Lars Ridder

Abstract Flavonoids are a class of natural compounds essentially produced by plants that are part of animal and human diets and have assumed health-promoting benefits. Upon human consumption, these flavonoids are to a modest extent absorbed in the small intestines. The major part arrives in the colon where the microflora utilises and converts the flavonoids to a wide range of products. Many of these products are absorbed in the major intestines and subsequently metabolised by the host. To understand the impact of the microflora on the metabolism and possible effects on human health, complete (and quantitative) identification of the microbial as well as human metabolic conversion products of flavonoids is required. This is a challenging task, as these bioconversion products are often present in relatively small amounts, making classical identification strategies based on (accurate) mass information or nuclear magnetic resonance, not straightforward. In the absence of reference compounds, annotation of a component may be achieved by detailed expert evaluation, e.g. by searching for similar fragmentation patterns in spectral databases of known compounds. However, such manual analysis is a tedious task, and in advanced metabolite profiling experiments, with large numbers of unknown metabolites, this is a major bottleneck. Therefore, new strategies are needed for quick and reliable identification of the diverse range of molecules in complex matrices (faeces, blood, urine). Intelligent software for annotation and identification of unknowns is crucial to fully exploit complex datasets. We developed a new software tool (MAGMA) for (sub)structure-based annotation of LC-MSn datasets which,

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combined with a newly established database for phenolic molecules (MetIDB), enables semiautomated identification of flavonoid derivatives.

Keywords Flavonoids • Identification • Automation • LC-MS • NMR • Microflora • MAGMA • PERCH NMR Software • Microbiota • MetIDB • Profiling • Metabolites • Glucuronide • Sulphate • Valerolactone • Hippuric acid • Epicatechin • Beta oxidation • Alpha oxidation • Lactone hydrolysis • Gallocatechins • Urolithin

6.1 Introduction

Flavonoids are a class of compounds with diverse biological functions in plants and animals. They are almost exclusively biosynthesised in plants and are involved in many biologically relevant functions [1]. Flavonoids are probably most commonly known for their antioxidant activity and assumed health-promoting benefits such as anti-proliferative and antitumour behaviour. The consumption of flavonoid-containing food products (e.g. vegetables, fruit, soybean, olive oil) and drinks (e.g. tea, wine) has been subject of numerous reflections about the link between dietary habits and health effects [2]. Although flavonoids are usually considered to have positive health effects, they have also been assigned potential negative properties. For example, isoflavones were shown to interfere with estradiol binding to the estrogen receptor, and catechins and flavonols were shown to be able to bind to DNA and RNA [3, 4].

About 6,000 flavonoids have been identified from natural sources [5, 6], but due to the known diversity in the plant kingdom, many more are bound to exist. In addition, a large range of flavonoids metabolites are formed by microbial, animal and human biotransformation. There have been numerous nutritional, mechanistic and physiological studies of flavonoids, with large differences in biological outcomes. This diversity in results is probably a consequence of the many (biochemical) modifications flavonoids can be subject to. Flavonoids can be methylated, acylated, sulphated, glycosidated or glucuronidated by humans, but flavonoids are also extensively metabolised by the microbial intestinal flora. These modifications and/or conjugations have impact on the physicochemical characteristics of the flavonoids, such as solubility, receptor-binding abilities and antioxidant or prooxidant behaviour. It is therefore difficult to predict a priori what the effect of flavonoids in a diet for a human individual will be, as the kinetics of modifications and conjugation reactions can be different for each person.

Upon consumption, flavonoids are only to a small extend absorbed in the small intestines [7]. Most of the flavonoids present in the diet are converted by the microbial flora in the colon. In the microbial breakdown pathway of flavonoids, C-ring fission of aglycones is usually observed. This results in hydroxylated aromatic molecules that can be further metabolised to smaller phenylic acids and alcohols, including valerolactones, valeric acids and phenyl-acetic acids [8]. Figure 6.1 shows

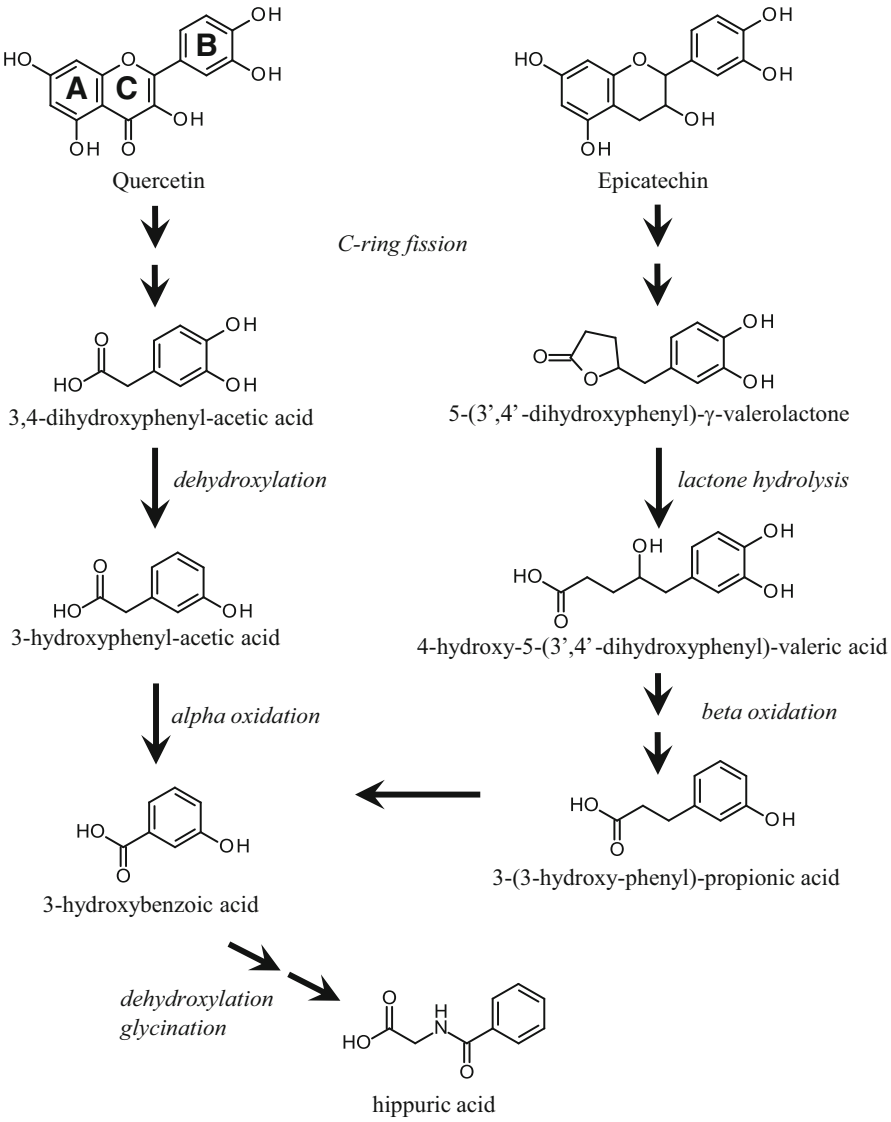


Fig. 6.1 Examples of microbial conversion of flavonoids

the microbial metabolic pathways of quercetin and epicatechin. Microbial conversion of anthocyanins occurs also via C-ring fission, resulting in many different aromatic compounds with hydroxyl, carboxy, methoxy or acetaldehyde functional groups. The products formed reflect the substitution pattern of the original anthocyanins [9]. Flavan-3-ols, like epicatechin in Fig. 6.1, are converted into different valerolactone, valeric acid and aromatic ring structures. The substitution pattern of these products reflects the substitution pattern of the original flavan-3-ols. Ellagitannins are large

polyphenolic molecules which are not absorbed by the intestines but are converted by the microflora into a series of lactones, urolithins [9]. Ellagitannins are abundantly present in different kinds of nuts (walnut, chestnut, pecan nut) and berries (strawberry, raspberry, blueberry) but also in pomegranate, grapes and tea [9]. Due to intra- and interindividual variation in human microbial composition of the colon, major differences are expected to occur in the microbial breakdown pathways of flavonoid molecules [10].

Upon absorption, a large part of the microbiotic conversion products are metabolised by human biotransformation enzymes predominantly present in the gut epithelial cells and in the liver. Xenobiotic metabolism is generally classified into two phases. Phase 1 biotransformation involves a range of oxidation reactions, catalysed primarily by cytochrome P450 enzymes as well as by flavinmonooxygenases, resulting in hydroxylation, epoxidation, heteroatom dealkylation, heteroatom oxidation, alcohol oxidation, oxidative deamination, dehydrogenations, hydrolysis, etc. These reactions generally lead to less lipophilic molecules, which are more susceptible to phase 2 biotransformations. The latter consist of conjugation of a range of (solubilising) moieties mostly to hydroxyl groups, including sulfation, glucuronidation, acetylation, glycination and methylation. Glutathione conjugation has an important role in detoxifying reactive electrophilic species. As these detoxification processes must operate on wide ranges of xenobiotics, they are generally catalyzed by families of enzymes, whose members have different, but broad and partly overlapping, substrate specificities.

While it is thought that the main function of human biotransformation is to solubilise foreign lipophilic compounds, in order to detoxify and excrete them from the body, the same types of reactions occur with common dietary phytochemicals and their microbiotic conversion products. As most polyphenols and phenolic acids discussed in previous paragraphs are only moderately lipophilic and comprising multiple hydroxylic and carboxylic moieties, they are more typical substrates for phase 2 than for phase 1 biotransformation enzymes. Indeed polyphenols and phenolic acids undergo predominantly phase 2 conjugations with glucuronyl, sulphate, glycine and methyl groups. Nevertheless, several studies suggest, mainly on the basis of in vitro experiments, that phase 1 reactions are also involved in hydroxylation of isoflavones and flavonoids [11, 12].

The combination of the variety of metabolic conversions of dietary compounds by the microbiota in the intestinal tract with the large range of human biotransformations reactions results in a vast number of possible metabolites. The actual range and relative quantities of metabolites circulating in blood, and excreted in urine, can vary significantly in time but also between individuals due to differences in composition of the microbiota and the activities of the human biotransformation enzymes. For example, the microbiotic conversion of the isoflavone daidzein to the isoflavan equol was shown to differ significantly between individuals as determined from urine excretion [13]. Equol has an inhibitory effect on topoisomerase [14] which has been associated with preventive effects towards hormone-related cancers [15]. The difference in equol formation was suggested to be a consequence of differences in gut microflora induced by habitual fat versus carbohydrate consumption [16].

Differences in human biotransformation may be caused by genetic polymorphisms. A well-studied example is the effect of glutathione S-transferase polymorphism on the pharmacokinetics of isothiocyanates. Similarly, genetic polymorphisms, especially phase 2 biotransformation enzymes, are indicated to influence the fate of phytochemicals in mammals [12]. While phase 2 conjugation is mainly considered to be important for rapid excretion of xenobiotics, some glucuronyl conjugates have been suggested to have biological activities that can potentially contribute to in vivo effects on health [17].

6.2 Profiling of Metabolites

To understand the impact of diet and the role of the microflora, the full range of metabolites including human biotransformation products need to be identified and quantified [18]. Hitherto the systematic study of the intra- and interindividual variation of flavonoid metabolism has been hampered by the large diversity of products formed and the low amounts of metabolites present in the complex matrices under study. Continuous developments in the area of metabolomics enable comprehensive profiling of metabolites in complex matrices like blood plasma and urine. Mass spectrometry, coupled to gas (GC-MS) or liquid (LC-MS) chromatography, and NMR are the major platforms for metabolite profiling and identification. Due to its sensitivity, mass spectrometry is the principle technology for large-scale metabolite profiling. However, with MS-based platforms, chemical structure elucidation is often only possible on the basis of comparison to reference compounds. As a result, in practice, large fractions of detected features in LC-MS-based metabolomics remain "unknown." This is also the case of flavonoid metabolic products for which reference molecules are not available. Fragmentation can help to go one step further and to (partially) annotate the detected molecules. Characteristic fragmentations may be recognised by comparison to literature reports or databases. GC-MS is commonly based on relatively reproducible "hard" electron impact ionisation, which allows successful searching in available databases for similar spectra of known compounds. Fragmentation data from tandem MS or ion trap-based MSn experiments, based on collision-induced dissociation, are more instrument-specific.

Many flavonoids and their metabolic products occur as different positional and stereochemical isomers, which can usually not be differentiated with mass spectrometry. For example, conjugation with glycosyl groups can occur at different positions on the flavonoid backbone, yielding different molecules with identical mass. NMR can help in the identification of the large number of positional and stereochemical isomers. However, direct ^{1}H-NMR analysis of crude extracts can result in spectral data that are difficult to interpret, due to overlapping NMR signals of different metabolites as well as dominating NMR signals of highly abundant metabolites. For example, hippuric acid [19] which is abundantly present in urine samples can give rise to strong aromatic proton signals which hinder identification of the lower abundant intact phenol conjugates in urine. Therefore, full structure

elucidation by NMR often requires purification of the metabolites using, for example, MS-based SPE trapping procedure, where LC-MS is coupled to NMR [20]. Structure elucidation of purified components is mostly achieved by a combination of mass spectrometry and NMR, preferentially one-dimensional ^{1}H-NMR measurements combined with two-dimensional (or multidimensional) spectra. However, for mass-limited samples, one-dimensional ^{1}H-NMR measurements can easily be achieved if the amount is 1 μm of compound, but two-dimensional NMR spectra are with the current state of technology not feasible for 1 μm of compound. For homonuclear 2D NMR, 10 μm of compound (or more) is requested, for hetero-nuclear 2D NMR, 30 μm of compound (or more) is needed. In addition, extensive purification is often needed, as background signals can obscure the molecule of interest in the NMR spectrum. ^{1}H-NMR spectra combined with MS data on molecular mass, MS/MS fragments and expected molecular formula can be used for structure elucidation and identification of metabolites isolated from complex mixtures [8, 21].

6.3 Automated Structure Elucidation

MS/MS measurements using soft ionisation techniques, such as electro-spray, provide information about the mass of parent molecular ions as well as their fragments, which provide the possibility to interpret the data in the absence of reference spectra. In the case of high-accuracy MS data, the molecular m/z value can be used to derive the likely elemental composition of the molecule. Computational approaches allow automatic retrieval of candidate molecules with matching monoisotopic mass, or elemental formula, from large chemical databases and subsequent assessment of the chemical structures in comparison of the observed fragmentation patterns. A number of methods have been developed that annotate m/z values in a fragmentation spectrum with in silico generated substructures of possible candidate molecules. Methods based on the concept include EPIC [22] (available as MassFragment software from Waters), Mass-Frontier [23], FiD [24], MetFrag [25] and MassMetaSite [26]. We recently described an extended algorithm that can handle multistage spectral trees, obtained from LC-MSn experiments, where $n>2$ [27]. This algorithm results in a hierarchical tree of substructures, where substructure assignments at each MS level take the assignments of the precursor as well as subsequent fragmentations into account. The substructure annotation results in a matching score that can be used to rank different candidate structures for an unknown metabolite, to support its identification. This method has been applied to a complete LC-MSn metabolite profile of a green tea extract, demonstrating that the majority of compounds that had been assigned by rigorous expert analysis [8] were successfully ranked among extensive sets of candidate structures retrieved from the largest public chemical database, PubChem [28]. In addition, the computational annotation of the complex LC-MSn profile resulted in the additional putative annotation of a diverse set of components, some of which had not been reported in tea before. Reliable annotation of metabolites is of great help in subsequent identification efforts, which are commonly based on NMR measurements.

Identification of unknown metabolites based on NMR is facilitated by databases of (already assigned and characterised) ^{1}H-NMR spectra of known molecules, which can be queried for the observed spectral features. The Human Metabolome Database (http://www.hmdb.ca/), the Spectral Database for Organic Compounds SDBS (http://www.aist.go.jp/RIODB/SDBS/cgi-bin/cre_index.cgi), the NMRShiftDB (http://nmrshiftdb.ice.mpg.de/) and the Biological Magnetic Resonance Bank (http://www.bmrb.wisc.edu/) are publicly available databases that contain NMR spectra of metabolites. A major limitation of existing NMR databases is that their content is mostly limited to primary metabolites. Recently we developed a new database, systematically including ^{1}H-NMR spectra of all known glycoside, glucuronide, sulphate and methyl conjugates of the phenolic molecules, supporting more reliable and complete identification (MetIDB, www.metidb.org). The advantage of using ^{1}H-NMR spectra for phenolic compounds is the availability of signals in the 9.0–5.5 ppm range of the aromatic and sp2 photons. These signals are usually well resolved and the chemical shift positions can be easily extracted and used for querying the MetIDB database. The majority of the ^{1}H-NMR spectra in MetIDB are predicted spectra from 3D structure of the molecules with correct stereochemistry and conformation of the molecule and especially of the glycosyl and the glucuronyl fragments. These predicted spectra provide good starting values for structure verification by iterative fit of the predicted and experimental spectrum as we will demonstrate.

6.4 Examples

Tea contains a diverse set of flavonoids, which upon consumption are extensively metabolised by microbiota in the colon and via human biotransformation subsequently are excreted in the urine. Many of these metabolites are not yet present in databases. Therefore, in order to perform automatic annotation to LC-MS profiles of such urine samples, we applied in silico metabolic reaction rules to generate relevant candidate molecule structures, starting from the compounds present in green tea. The reaction rules included basic hydrolysis reactions as catalysed by protease, esterase and glycosidase digestive enzymes, a number of well-studied conversions of flavonoids by microflora resulting in valerolactones and valeric acids, as well as alpha and beta oxidations, para-dehydroxylation and decarboxylation (see Fig. 6.1 for examples). Large molecules with a molecular mass above 600 Da., which are less likely to be absorbed directly from the intestinal tract [29], were removed from the resulting library. To account for human metabolism after uptake, a number of phase 2 biotransformations [30] were applied, resulting in a total set of almost 5,000 compounds. Figure 6.2 illustrates the in silico generation metabolites for (epi)gallocatechin gallate. After formation of 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone systematically, all possible conjugates are generated, of which a subset of confirmed metabolites is shown. The in silico generated metabolites were automatically matched against LC-MS^{n} datasets obtained with urine samples after tea consumption, and fragmentation spectra were annotated by in silico substructures as

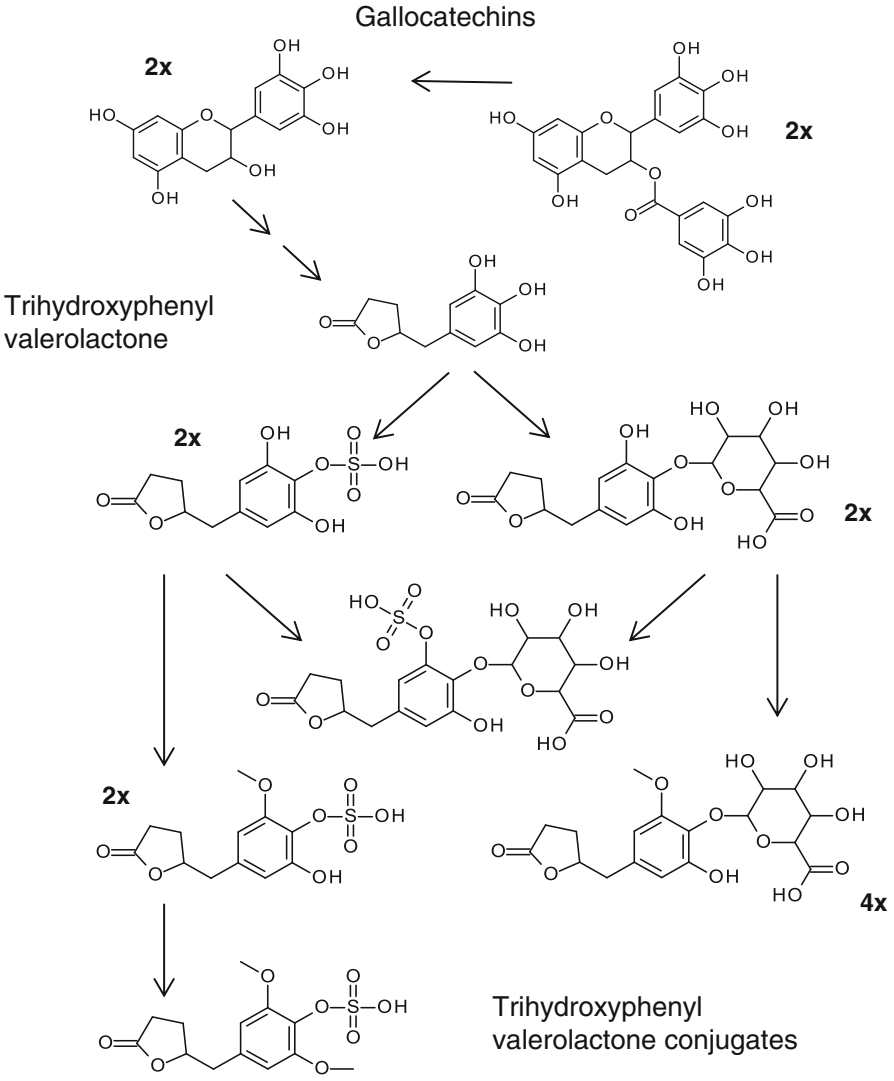


Fig. 6.2 Overview of the in silico metabolite generation process, including the example of gallocatechin. For each type of conjugate, only one positional isomer is shown. In the urine samples, often multiple stereo- or positional isomers are detected. These different isomers can usually not be uniquely assigned on the basis van MS fragmentation

described in the previous section. As an example, Fig. 6.3 shows the automatic substructure annotation of the spectral data obtained with 3′,4′-dihydroxyphenyl-valerolactone-O-glucuronide. The fragment annotation confirms that the structure is a dihydroxyphenyl-valerolactone that is O-glucuronidated. Ninety percent of the expert assignments that had been made prior to the analysis, for over 60 conjugated (poly)phenolic components in urine, were reproduced by this automated in silico

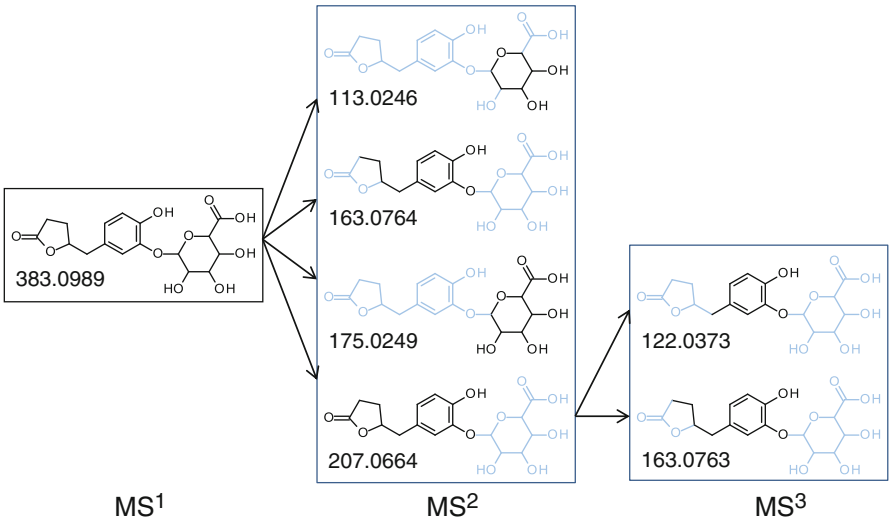


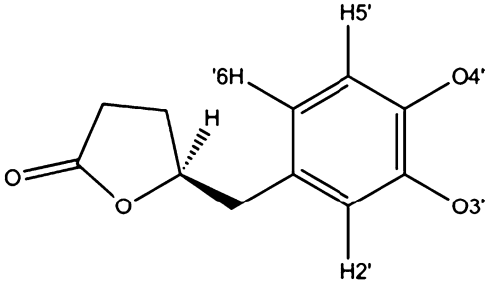
Fig. 6.3 MSn fragmentation of dihydroxyphenyl-valerolactone-glucuronide

workflow. Furthermore, the in silico analysis helped to make several additional annotations. It illustrates how MAGMa facilitates annotation of the LC-MSn data at sufficient chemical detail to provide a general biochemical interpretation. However, the precise chemical rearrangement of the structure, e.g. which positional isomer, cannot be determined from the MS fragmentation data. Additional structural information from nuclear magnetic resonance spectroscopy (NMR) is then needed to elucidate the complete structure [20].

^{1}H-NMR structure confirmation can be done based on predicted ^{1}H-NMR spectra. These spectra provide good starting values for querying the public databases of known compounds or performing structure verification by iterative fitting of the experimental spectrum. A crucial step in the ^{1}H-NMR spectra prediction is the generation of a reliable 3D structure of the molecule. We have developed an algorithm for 3D structure generation of flavonoids and their glycosylated secondary metabolites. Briefly the procedure is as follows. The glycosyl, glucuronide, sulphate or methoxyl fragments are detected in the molecule together with all hydroxyl groups. Then a set of compounds is generated representing all possible combinations for positioning these fragments in the molecule. In case of glucopyranosyl and glucuronyl fragments, the structures are generated with the correct stereochemistry and chair conformation with as many as possible hydroxyl groups at equatorial position using the CORINA software (http://www.molecular-networks.com). Then the 3D structure is used for ^{1}H-NMR spectra predictions in a specified deuterated solvent using the PERCH NMR Software (www.perchsolutions.com) following a similar procedure as described by [31]. The structures are optimised in three-dimensional space; a statistical set of conformers is generated using Monte-Carlo/molecular dynamic analysis. Solvent effects are taken into account by a dielectric continuum

Table 6.1 NMR shifts (in ppm) of aromatic protons of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and the 3′- and 4′- O-glucuronides. The induced chemical shift of the glucuronide fragment is given in *parenthesis*

ID	OH	*O*-Glc	H2′	H5′	H6′
1	3′,4′		6.75	6.76	6.69
2	3′,4′	3′	7.07 (0.32)	6.80 (0.04)	6.84 (0.15)
3	3′,4′	4′	6.81 (0.06)	7.01 (0.25)	6.72 (0.03)



model. By taking into account the stereochemistry, intramolecular interactions and solvent effects, chemical shifts and couplings are predicted accurately. The root mean square error (RMSE) of prediction of the chemical shifts of the sp3 and the aromatic protons was lower than 0.14 ppm, and those of the geminal couplings ($^2J_{H,H}$) and the vicinal couplings ($^3J_{H,H}$) were lower than 0.84 and 0.30 Hz, respectively. The predicted chemical shifts can be used for querying databases of NMR spectra of known compounds, and when experimental spectra with good signal-to-noise ratio are available, these can be fitted using the predicted spectrum as a starting point. It is not always possible to obtain clean ^{1}H-NMR spectra that allow iterative fitting. In such cases, it might still be possible to extract the chemical shifts and compare these to the predicted ones. For example, the aromatic region of compounds matching the exact mass of 3′,4′-dihydroxyphenyl)-γ-valerolactone glucuronides contained additional signals which made the fit of the spectra difficult. The MS/MS analysis limited possible structures to four: two positions for the glucuronyl moiety and R and S chirality of the C4 atom (see the scheme in Table 6.1). It is not possible to resolve the stereochemistry of the C4 atom on the basis of ^{1}H-NMR spectra only. Therefore, in the analysis, only the R isomer was included which reduced the problem to resolving the position of the glucuronyl fragment. When one of the hydroxyl groups is replaced by a glucuronyl moiety, major changes in the ^{1}H-NMR spectra are observed for the protons adjacent to the glucuronyl moiety while retaining the splitting pattern. In Table 6.1 the extracted experimental chemical shifts of the aromatic protons of the aglycone (compound 1) and the two possible glucuronide conjugates are listed. The three aromatic protons have very distinct splitting patterns: H2′ and H5′ are doublets with coupling constants of 2.1 and 8.1 Hz, respectively, whereas H6′ is a double doublet coupled to H2′ and H5′. In the 3′-O-glucuronide, the H2′ position was increased by 0.32 ppm when compared to the aglycone. In the 4′-O-glucuronide, the adjacent H5′ proton was the most affected

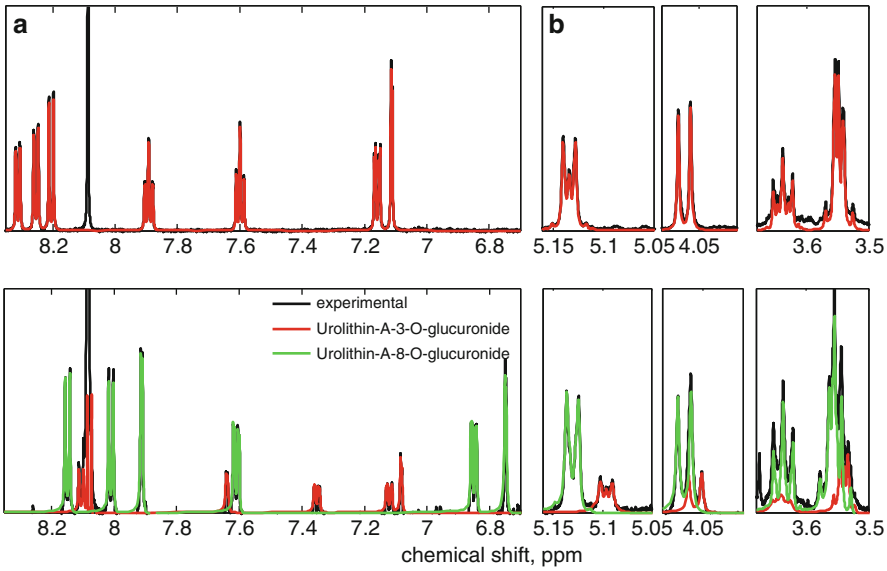


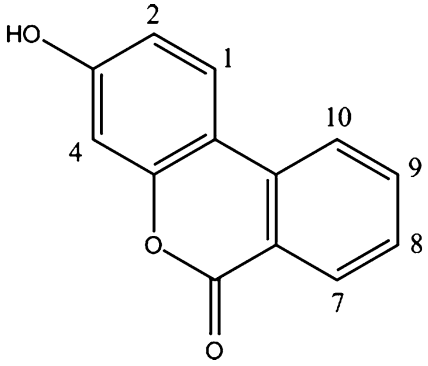
Fig. 6.4 Experimental (*black*) and fitted (*red* and *green*) spectra of urolithin-B-3-O-glucuronide (*upper*) and a mixture of urolithin-A-3-O-glucuronide (in *red*) and 8-O-glucuronide (in *green*) (*lower*). The aromatic region is shown on the *left* and the signals of the glucuronide moiety on the *right*. The strong signal at 8.081 ppm is residual formic acid which was included in the fit in the mixture of urolithin-A-3-O-glucuronides so that the overlapping signals could be resolved

and the chemical shift was increased by 0.25 ppm. The predicted chemical shifts for the aromatic protons of the correctly assigned isomer were within 0.15 ppm from the extracted experimental values. The prediction error was clearly much smaller than the induced change in the chemical shift which allowed the assignment of the correct structure. Such specific differences predicted for the 3′-O-glucuronide and 4′-O-glucuronide isomers resulted in more reliable identification of these structures from their experimental NMR spectra.

Another example of efficient structure identification is the identification/distinction of the different forms of conjugated urolithins. Urolithins are known to be microbial breakdown products of ellagic acid, a compound that has been reported to be present in strawberries, raspberries, pomegranates, walnuts and tea [32]. We were able to obtain good quality ^{1}H-NMR spectra of glucuronidated conjugates (Fig. 6.4) of urolithin-A and urolithin-B, purified from urine samples. There were two urolithin-A glucuronides that co-eluted and showed similar fragmentation patterns. The extracted chemical shifts of the aromatic protons are shown in Table 6.2 together with the literature data of the aglycones [33, 34]. The structure of urolithin (see scheme Table 6.2) can be considered as built of a chromene (H1–H4 protons) and a benzyl (H7–H10 protons) fragments. The identification of urolithin-B-3-O-glucuronide was straightforward based on the observed changes in the chemical shifts when compared to the aglycone as the largest change in the chemical shifts was observed for

Table 6.2 NMR shifts (in ppm) of the aromatic protons of urolithin-B (*ID 1*) and urolithin-A (*ID 3*) and their glucuronide conjugates. The induced chemical shift of the glucuronide fragment is given in *parenthesis*

ID	OH	*O*-Glc	H1	H2	H4	H7	H8	H9	H10
1	3		8.15	6.85	6.75	8.25	7.57	7.88	8.18
2		3	8.204 (0.054)	7.158 (0.308)	7.113 (0.363)	8.31 (0.060)	7.598 (0.011)	7.891 (0.072)	8.252
3	3,8		7.98	6.86	6.76	7.64		7.36	8.06
4	8	3	8.104 (0.124)	7.12 (0.260)	7.081 (0.321)	7.638 (–0.002)		7.351 (–0.004)	8.077 (0.017)
5	3	8	8.008 (0.028)	6.849 (–0.011)	6.748 (–0.012)	7.91 (0.270)		7.608 (0.248)	8.147 (0.087)



the H2 and H4 protons. In urolithin-A there is an additional hydroxyl group in the benzene fragment. Two isomers are known in the literature [9, 32] in which the hydroxyl group is at position 8 (urolithin-A) and position 9 (isourolithin-A). These two isomers have the same splitting pattern of the benzene fragment. We have studied all four possible positions for the hydroxyl group, namely, hydroxyl at position 3 in the chromen fragment and positions 8 and 9 in the benzyl fragment. The best agreement between the predicted and experimental chemical shifts was found for the urolithin-A-3-O-glucuronide and urolithin-A-8-3-O-glucuronide. Although the two urolithin-A glucuronides were co-eluting, the aromatic signals were well resolved in the ^{1}H NMR spectra, whereas the signals of the glucuronide moiety were heavily overlapped (see Fig. 6.4). The samples obtained by SPE extraction always contain residual amounts of formic acid which gives a singlet at 8.081 ppm. This singlet overlapped with the two doublets (H1 and H10) of urolithin-A-3-O-glucuronide. The final fit was performed using the predicted spectra of the two urolithin-A glucuronides and formic acid. In the iterative procedure, the concentrations were optimised together with the chemical shifts, couplings and line widths.

Intramolecular interactions can induce changes in the chemical shift positions of the substituent protons and sometimes also second-order effects which result in modified splitting pattern. We observed these effects in the spectra of the glucuronyl fragment in all three urolithin conjugates. The strong overlap of the H2′ and H3′

protons of the glucuronyl fragment (difference in chemical shifts of 0.006, 0.009 and 0.015 ppm for the urolithin-A-3-O-glucuronide, urolithin-B-3-O-glucuronide and urolithin-A-9-O-glucuronide, respectively) results in a complex splitting pattern of the H1′ and the H4′ protons (see Fig. 6.4). Despite this heavy overlap, we were able to extract the chemical shifts for the H2′ and H3′ protons for all urolithin glucuronides which would not be possible by manual analysis of ^{1}H-NMR data only. These examples illustrate that predicted changes of the chemical shift of the protons adjacent to the substituents when compared to the aglycone can guide the assignment of the position of the substituent.

6.5 Discussion

There is increasing awareness of the importance of the microbiota in the intestinal tract for the digestion of food and for the health of the host organism. Furthermore, evidence accumulates that functional differences exist in the microbiotic composition between individuals, which may have consequences in terms of effects of diet on various aspects of human health. To obtain a more detailed understanding, metabolomics is an essential tool, with the prerequisite that the large range of the metabolites involved in digestion still need be elucidated at the biochemical detail. Such large-scale metabolite profiling experiments will be crucial to increase our knowledge of the bioactivities of specific components of the human diet, of which flavonoids are currently the most well-known example, as well as their bioactive metabolites upon conversion by the microbiota and human biotransformation enzymes. Insight into differences in digestion between individuals is needed at a biochemical level to identify reliable biomarkers for the actual intake of bioactive dietary constituents and for the personal consequences of the diet.

Advancement in analytical mass spectrometry instrumentation (UPLC coupled to Orbitrap, TOF-based or FT mass spectrometry systems) has boosted the output obtained in metabolomics studies, yielding up to terabytes of information for a single scientific study. Data analysis of many mass directed metabolomics studies relies on an untargeted analysis of the large datasets obtained with subsequent or concomitant statistical analysis hoping to find biomarkers which are often not clearly defined nature. It is evident that a big leap forward can be obtained in these studies if components observed in the mass directed metabolomics studies could be annotated in an automated fashion, enabling a more holistic approach of the subject(s) under study. Several software approaches have been tried in the recent past to facilitate a better annotation of mass spectrometry studies by automatically retrieving candidate molecules with matching monoisotopic mass (which can be converted into elemental formula) from chemical databases and subsequent assessment of the possible structures of the observed fragments. We recently described a new extended algorithm, MAGMA, which enables to apply such approach to multistage MSn data [27]. Since the automated approaches rely on matching candidate molecules, methods are needed to extend chemical databases

with new structures covering potential metabolites not yet observed or described. One possibility is to use in silico reaction rules to predict relevant molecules. The value of such an approach was demonstrated in a workflow for the automatic annotation of metabolites of polyphenols present in urine data after consumption of tea (in press). To support the identification of unknown metabolites with NMR, MetiDB (www.metidb.org) was recently created, which contains 6,000 phenolic molecules and their calculated NMR spectra. This database will be extended with phase 2 biotransformation molecules of these phenolic molecules, which is of great importance for the quick and reliable identification of the components purified from urine. The tight integration of in silico generation of biochemically relevant molecules and their annotation and identification in LS/MS- and NMR-based metabolite profiling data have great potential to accelerate the profiling of novel metabolites and assess their potential biochemical source.

6.6 Conclusions

Computational approaches are essential for the efficient annotation and identification of the large range of metabolites of dietary constituents, produced by microbiota in the human intestinal tract and by human biotransformation upon absorption. Systematic identification will help to understand the role of the microbiota in digestion and human health.

References

1. Harborne J. Encyclopedia of plant physiology. Berlin/Heidelberg/New York: Springer; 1980.
2. Moco S, Ross A. Can we use metabolomics to understand changes to gut microbiota. In: Martin F-PJ, Kochhar S, editors. Metabolomics and gut microbiota in nutrition and disease. Springer; 2014.
3. Rietjens IMCM, Sotoca AM, Vervoort J, Louisse J. Mechanisms underlying the dualistic mode of action of major soy isoflavones in relation to cell proliferation and cancer risks. Mol Nutr Food Res. 2013;57(1):100–13.
4. van der Woude H, Alink GM, van Rossum BEJ, Walle K, van Steeg H, Walle T, et al. Formation of transient covalent protein and DNA adducts by quercetin in cells with and without oxidative enzyme activity. Chem Res Toxicol. 2005;18(12):1907–16.
5. Mihaleva VV, te Beek TAH, van Zimmeren F, Moco S, Laatikainen R, Niemitz M, et al. MetIDB: a publicly accessible database of predicted and experimental 1H NMR spectra of flavonoids. Anal Chem. 2013;85:8700.
6. Arita M, Suwa K. Search extension transforms Wiki into a relational system: a case for flavonoid metabolite database. Bio Data Min. 2008;1(1):7.
7. de Boer VCJ, Dihal AA, van der Woude H, Arts ICW, Wolffram S, Alink GM, et al. Tissue distribution of quercetin in rats and pigs. J Nutr. 2005;135(7):1718–25.
8. van der Hooft JJJ, de Vos RCH, Mihaleva V, Bino RJ, Ridder L, de Roo N, et al. Structural elucidation and quantification of phenolic conjugates present in human urine after tea intake. Anal Chem. 2012;84(16):7263–71.

9. Aura A-M. Microbial metabolism of dietary phenolic compounds in the colon. Phytochem Rev. 2008;7(3):407–29.
10. van Velzen EJJ, Westerhuis JA, van Duynhoven JPM, van Dorsten FA, GruM€n CH, Jacobs DM, et al. Phenotyping tea consumers by nutrikinetic analysis of polyphenolic end-metabolites. J Proteome Res. 2009;8(7):3317–30.
11. Hodek P, Trefil P, Stiborová M. Flavonoids-potent and versatile biologically active compounds interacting with cytochromes P450. Chem Biol Interact. 2002;139(1):1–21.
12. Lampe JW. Interindividual differences in response to plant-based diets: implications for cancer risk. Am J Clin Nutr. 2009;89(5):1553S–7.
13. Kelly GE, Nelson C, Waring MA, Joannou GE, Reeder AY. Metabolites of dietary (soya) isoflavones in human urine. Clin Chim Acta. 1993;223(1–2):9–22.
14. Chang Y, Nair M, Nitiss J. Metabolites of daidzein and genistein and their biological activities. J Nat Prod. 1995;58(12):1901.
15. Blaut M, Clavel T. Metabolic diversity of the intestinal microbiota: implications for health and disease. J Nutr. 2007;137(3):751S–5.
16. Rowland IR, Wiseman H, Sanders TAB, Adlercreutz H, Bowey EA. Interindividual variation in metabolism of Soy isoflavones and lignans: influence of habitual diet on equol production by the gut microflora. Nutr Cancer. 2000;36(1):27–32.
17. Zhang Y, Song TT, Cunnick JE, Murphy PA, Hendrich S. Daidzein and genistein glucuronides in vitro are weakly estrogenic and activate human natural killer cells at nutritionally relevant concentrations. J Nutr. 1999;129(2):399–405.
18. Collino S, Martin F-P, Moco S. Metabonomics in clinical practice. In: Kochhar S, Martin F-P, editors. Metabonomics and gut microbiota in nutrition and disease. London: Springer; 2014.
19. Daykin CA, Van Duynhoven JPM, Groenewegen A, Dachtler M, Van Amelsvoort JMM, Mulder TPJ. Nuclear magnetic resonance spectroscopic based studies of the metabolism of black tea polyphenols in humans. J Agric Food Chem. 2005;53(5):1428–34.
20. Van der Hooft JJJ, Mihaleva V, Bino RJ, de Vos RCH, Vervoort J. A strategy for fast structural elucidation of metabolites in small volume plant extracts using automated MS-guided LC-MS-SPE-NMR. Magn Res Chem. 2011;49(S1):S55–60.
21. van der Hooft JJJ, Akermi M, Unlu FY, Mihaleva V, Roldan VG, Bino RJ, et al. Structural annotation and elucidation of conjugated phenolic compounds in black, green, and white tea extracts. J Agric Food Chem. 2012;60(36):8841–50.
22. Hill AW, Mortishire-Smith RJ. Automated assignment of high-resolution collisionally activated dissociation mass spectra using a systematic bond disconnection approach. Rapid Commun Mass Spectrom. 2005;19(21):3111–8.
23. Hill DW, Kertesz TM, Fontaine D, Friedman R, Grant DF. Mass spectral metabonomics beyond elemental formula: chemical database querying by matching experimental with computational fragmentation spectra. Anal Chem. 2008;80(14):5574–82.
24. Heinonen M, Rantanen A, Mielikäinen T, Kokkonen J, Kiuru J, Ketola RA, et al. FiD: a software for ab initio structural identification of product ions from tandem mass spectrometric data. Rapid Commun Mass Spectrom. 2008;22(19):3043–52.
25. Wolf S, Schmidt S, Muller-Hannemann M, Neumann S. In silico fragmentation for computer assisted identification of metabolite mass spectra. BMC Bioinforma. 2010;11(1):148.
26. Bonn B, Leandersson C, Fontaine F, Zamora I. Enhanced metabolite identification with MSE and a semi-automated software for structural elucidation. Rapid Commun Mass Spectrom. 2010;24(21):3127–38.
27. Ridder L, van der Hooft JJJ, Verhoeven S, de Vos RCH, van Schaik R, Vervoort J. Substructure-based annotation of high-resolution multistage MSn spectral trees. Rapid Commun Mass Spectrom. 2012;26(20):2461–71.
28. Ridder L, van der Hooft JJJ, Verhoeven S, de Vos RCH, Bino RJ, Vervoort J. Automatic chemical structure annotation of an LC–MSn based metabolic profile from green tea. Anal Chem. 2013;85(12):6033–40.

29. Ridder L, Wang H, de Vlieg J, Wagener M. Revisiting the rule of five on the basis of pharmacokinetic data from rat. ChemMedChem. 2011;6(11):1967–70.
30. Ridder L, Wagener M. SyGMa: combining expert knowledge and empirical scoring in the prediction of metabolites. ChemMedChem. 2008;3(5):821–32.
31. Lehtivarjo J, Hassinen T, Korhonen SP, Perakyla M, Laatikainen R. 4D prediction of protein H-1 chemical shifts. J Biomol NMR. 2009;45(4):413–26.
32. Gonzalez-Barrio R, Truchado P, Ito H, Espin JC, Tomas-Barberan FA. UV and MS identification of urolithins and nasutins, the bioavailable metabolites of ellagitannins and ellagic acid in different mammals. J Agric Food Chem. 2011;59(4):1152–62.
33. Ito H, Iguchi A, Hatano T. Identification of urinary and intestinal bacterial metabolites of ellagitannin geraniin in rats. J Agric Food Chem. 2007;56(2):393–400.
34. Nealmongkol P, Tangdenpaisal K, Sitthimonchai S, Ruchirawat S, Thasana N. Cu(I)-mediated lactone formation in subcritical water: a benign synthesis of benzopyranones and urolithins A-C. Tetrahedron. 2013;69:9277–83.

Chapter 7
Metabonomics in Neonatal and Paediatric Research: Studying and Modulating Gut Functional Ecology for Optimal Growth and Development

Vassilios Fanos and Laura Cuzzolin

Abstract Gut microbiota play a fundamental role in human health by promoting intestinal homeostasis, stimulating development of the immune system, and providing protection against pathogens. Relatively little is known about the acquisition and development of this complex microbial community during infancy. However, emerging 'omics' technologies are now being applied to the study of the gut microbial ecology, generating new opportunities to deepen the functions of the gut microbiota in human health. All the published literature on paediatric and neonatal nutrimetabonomics is presented in a synthetic way, including studies on maternal milk and formula. The role of the disruption of the gut microbiota in various gastrointestinal diseases is considered, focusing the metabonomics approach in gut ischemia, chronic inflammatory diseases, cystic fibrosis, diabetes, and obesity. As a general rule, the best biofluid to study nutrition or to identify food-specific biomarkers is urine. In food consumption monitoring, the same concepts behind drug testing and drug monitoring can be used. In the opinion of the authors, in the near future, improved tools for the analysis of the metabolic profile (simplified like 'dipsticks' for urine) and its integration with the other 'omics' data will move metabonomics beside the child, from top research to bedside.

Keywords Child • Development • Growth • Metabonomics • Microbiome • Newborn • Gut • Ecology

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7.1 Introduction

The human host-microbe symbiosis is initiated in early life and its establishment is an important biological process.

Gut microbiota enhances the host's metabolic capacity to process nutrients and drugs and modulate the activities of multiple pathways in different organ systems [1]. So, gut microbiota play a fundamental role in human health by promoting intestinal homeostasis, stimulating development of the immune system, and providing protection against pathogens [2, 3]. The disruption of the gut microbiota has been linked to an increasing number of diseases such as necrotizing enterocolitis, inflammatory bowel disease, obesity, cancer, diabetes, and allergies [3].

Despite this evidence, relatively little is known about the acquisition and development of this complex microbial community during infancy [4]. However, emerging 'omics' technologies are now being applied to the study of the gut microbial ecology, generating new opportunities to deepen the functions of the gut microbiota in human health.

7.2 Microbiota Composition and Activity

The human intestinal tract harbours a complex microbial ecosystem of 100 trillion bacteria [5], consisting of many more types of bacteria than originally thought [6], that undergoes dynamic changes during development.

Infants are born with an essentially sterile gut, but colonization starts immediately during and after delivery: the initial inoculation of the intestinal microbiota is a key step, because this initial phase will probably influence the composition of the human microbiota throughout life [5, 7]. In general, the first settlers that initially colonize infants are facultative anaerobic bacteria (mainly *E. coli* and *Streptococci* spp.), followed by *Staphylococcus* spp., *Enterococcus* spp., and *Lactobacillus* spp. that contribute to provide a favourable condition for anaerobic bacteria [8, 9]. After the first week of life, *Bifidobacterium* spp., *Bacteroides* spp., and *Clostridium* spp. are present and *Bifidobacterium* spp. species become predominant in human milk-fed infants [10].

This dynamic microbial ecosystem stabilizes during the first 2–3 years [5] and reaches the highest complexity in the human adult [11].

7.3 Factors Affecting Intestinal Microbiota Composition and Activity

The connection between both hereditary and environmental factors plays an important role in every stage, from conception to the early postnatal period [12] (Fig. 7.1). The human microbiota is established after birth and starts out as a dynamic

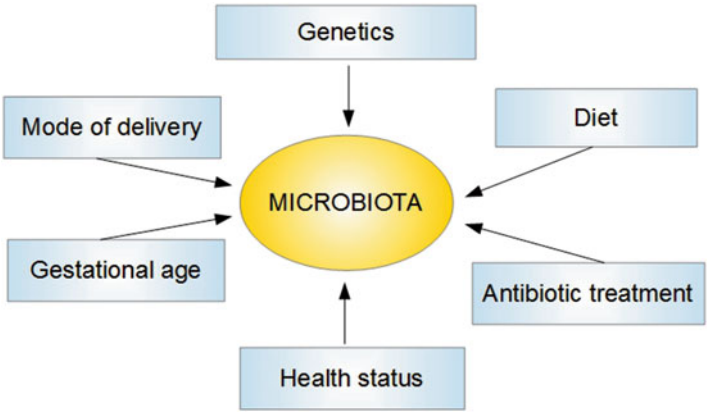

Fig 7.1 Factors influencing the development and composition of microbiota in the newborn and child

ecosystem, dominated by bifidobacteria, that stabilizes during the first 2–3 days [5, 13], reaching a homeostatic climax composition that remains relatively stable during most of adult's life [14]. Influenced by a variety of early-life exposures, the infant gut microbiota plays a crucial role in life-long health. The composition and activity of the microbiota is affected by the genetic background of the host and by the diet. Moreover, the mode of delivery (natural birth, caesarean section) and the gestational age (preterm, late preterm, and term newborns) together with health status and antibiotic treatments could affect human microbiota composition.

7.3.1 Genetic Background

Host genotype is among the factors that influence the composition of gut microbiota [15]. A single gene difference in the host can affect the population structure of gut microbiota. The genotype of the host may influence its microbiota composition either directly (secretions into the gut, control of gut motility, modification of epithelial cell surfaces) or indirectly, through food and lifestyle preferences [16]. Some authors [17] studied the degree of similarity in the predominant faecal microflora of identical twin pairs, fraternal twin pairs, and unrelated controls: the highest levels of similarity were found in genetically identical twins.

7.3.2 Mode of Delivery

The type of delivery strongly affects the composition of the microbiota. In the case of caesarean section (CS) delivery, instead of faecal bacteria derived from the mother, other environmental bacteria could affect the microbiota composition.

In particular, the microbiota of CS newborns is similar to the skin communities of the mothers and thus dominated by *Staphylococcus* spp., *Corynebacterium* spp., and *Propionibacterium* spp. [18]. Instead, vaginally delivered infants acquire bacteria resembling the vaginal microbiota of their mothers dominated by *Lactobacillus* spp., *Prevotella* spp., and *Sneathia* spp. [18]. Moreover, CS newborns are colonized later and less frequently by bifidobacteria [19]: this delay in the bifidobacteria colonization has been shown to sustain until the first month of life, while in vaginally delivered infants, it occurs at 10 days [20]. These differences could be due not only to the CS itself but also to the prophylactic use of antibiotics frequently applied during a CS [21].

As regard other bacterial groups, it has been reported that newborns born by CS are more frequently colonized by *Clostridium* spp. and less by *Bacteroides* spp. [19, 22], even if these data are more controversial. The observed differences can still remain months after birth and perhaps even longer [20, 22].

Some authors [23] studied the composition of the intestinal microflora in 46 term infants, 23 CS delivered and 23 spontaneously delivered using two PCR different techniques: during the first 3 days of life, intestinal bacteria resulted strongly influenced by mode of delivery, with marked differences among the two groups. PCR-denaturating gradient gel electrophoresis (DGGE) analysis carried out with *Bifidobacterium*-specific primers revealed the presence of this genus in 13 of 23 (56.5 %) faecal samples obtained from vaginally delivered newborns, while this genus was absent in all samples obtained from subjects delivered by CS. Moreover, some differences were found as regard *E. coli*, found in 9 of 23 (39.1 %) spontaneously delivered newborns and in only 2 of 23 (8.7 %) CS-delivered infants. PCR-temperature gradient gel electrophoresis (TGGE) analysis showed greater variations among the two groups. Finally, in all infants enrolled, *Ruminococcus* spp. were absent and *Bacteroides* spp. were found only in 8.7 % of vaginally delivered newborns.

Other authors [24] examined the intestinal microflora of term infants and found a high variability in the profiles of faecal microbiota among the studied subjects, according to previous reports [25–27]. Compared with vaginally delivered newborns, infants born by CS had particularly low bacterial richness and diversity, with significantly lower abundance of *Escherichia* spp.-*Shigella* spp. and an absence of *Bacteroides* spp. These results are consistent with a previous work that reported a delayed colonization by *E. coli* and the phylum Bacteroidetes undetectable in infants born by CS [19].

7.3.3 Gestational Age

A consequence of premature birth appears to be a delayed colonization of the gut with a limited number of bacterial species [28, 29]. These differences in timing and diversity are mainly due to the aseptic neonatal intensive care environment and the extensive use of antibiotics shortly after birth [30].

The observed bacterial diversity has been associated with recurrent *C. difficile* infections and other disease states [31]. In fact, compared to term infants, the colonization dynamics is different in preterm newborns, often colonized by potentially pathogenic species such as *Klebsiella* spp., *Enterobacter* spp., and *Clostridium* spp., instead of normal commensal microbiota such as *Bifidobacterium* spp. and *Lactobacillus* spp. [32, 33].

Some authors [30] showed a positive correlation between diversity of intestinal microbiota and digestive tolerance and weight gain. Moreover, the delayed colonization and reduced diversity observed in preterm newborns render these subjects more susceptible to bacterial disturbances and therefore at higher risk of necrotizing enterocolitis and sepsis [28, 34]. Recently, stools of 32 preterm infants exposed to current NICU practices were analyzed for assessment of the total and viable bacterial communities in the gut. Among the studied population, 7 and 13 preterm infants developed, respectively, NEC and sepsis. Total bacterial profiles of infants with NEC and total and viable profiles of infants with sepsis significantly differed from those of healthy infants, supporting a role for bacterial colonization in the pathophysiology of these diseases. Importantly, *Sphingomonas* spp. colonization was significantly associated with NEC [34].

7.3.4 Infant Diet

The composition of the intestinal microbiota can be modulated as a result of dietary exposure (human milk, formulas) as well as of intentional diet supplementations (prebiotics or probiotics).

Human milk is normally the first dietary exposure in infancy and is considered the best nutrition for growth and healthy development of the newborn, containing a wide range of health-promoting constituents [5, 35]. In particular, oligosaccharides present in human milk have prebiotic effects, fermenting in the colon and stimulating the growth of specific bacteria [36]. The metabolic profile of infants receiving human milk is characterized by a relatively high presence of acetate, a lower content of propionate, and the absence of butyrate [37]. Human milk has also been shown to a be a source of live bacteria, including staphylococci, streptococci, and bifidobacteria [38]. In particular, breast-fed infants contain a high abundance of *Bifidobacterium breve* [39]. The origin of bacteria present in human milk remains controversial, even if it is generally accepted that the newborn acquires the mother's microbiota during delivery and then transfers these bacteria to the breast skin [5] or to the mammary gland through an endogenous route [40].

Bovine milk, the most common base for infant formulas, contains insignificant levels of prebiotic oligosaccharides and this partly explains the differences observed between human milk-fed and formula-fed babies. Some authors [24] detected significant effects of diet on the several bacterial taxa, characterized by lower bacterial richness and diversity in infants who were breastfed. In detail, infants receiving formula had bacterial communities with significantly higher

abundance of the families Peptostreptococcaceae and Verrucomicrobiaceae and a significantly higher prevalence of *C. difficile*, a pathogen associated with enteric and atopic diseases [22].

The supplementation of infant formulas with prebiotics has been recently reviewed [41] and a mixture of short chain galacto-oligosaccharides and long chain fructo-oligosaccharides has been shown to selectively stimulate the growth of bifidobacteria and lactobacilli, generating metabolic profiles similar to those observed in human milk-fed infants [37, 42]. An alternative approach regards the oral administration of viable bacteria (probiotics) [43]: a few probiotics supplemented to formula-fed infants may be associated with some clinical benefits, such as a reduction in the risk of non-specific gastrointestinal infections [44] and necrotizing enterocolitis [45]. An updated meta-analysis of all relevant controlled trials performed to assess the benefits of probiotic supplementation for preterm newborns underlined a significant decreased risk of necrotizing enterocolitis and mortality, while no difference in the risk of sepsis was observed [45]. The effects of live bacteria and combinations of prebiotics and probiotics are gaining interest, but need further exploration particularly as regard long-term effects.

7.3.5 Antibiotic Treatments

The early exposure to antibiotics has significant immediate effects and probably also sustainable effects on the gut microbiota composition. Variables associated to antibiotic use (dose, length of treatment, route of administration) make difficult to draw strong conclusions on the exact impact on the microbiota. However, an antibiotic treatment surely causes disturbances in the early colonization by *Bifidobacterium* species, predisposing to an overgrowth of *Enterococcus* and *Enterobacteriaceae* species [46–48].

Recently, some authors underlined that antibiotic administration not only alters the total counts of *Bifidobacterium* but can also has an impact at the species level, with a reduction of *Bifidobacterium bifidum* and *Bifidobacterium adolescentis* [47, 48]. Despite the conviction that an early antibiotic treatment does not seem to have major long-term effects on the faecal intestinal microbiota, a complete recovery of initial bacterial community composition is rarely achieved [49]. Therefore, it has been hypothesized that exposure to antibiotic in early life may trigger the subsequent development of immune disorders, such as asthma, wheezing, and other allergic manifestations [50, 51].

7.4 Nutrimetabonomics: A Revolutionary Tool in Neonatology and Paediatrics

The metabonome is the sum of all endogenous and exogenous metabolites. The intrinsic factors are body composition, tissue turnover, resting metabolic rate, age, genotype, health status, reproductive status, and diurnal cycle, and the extrinsic

factors are diet (nutrients and nonnutrients), drugs, physical activity, colonic flora, pollution, lifestyle, and stress [52]. All these factors contribute to the metabolic genotype-phenotype relationship [53].

Metabotype is the whole set of metabolites which can be detected in body fluids and which characterizes the metabolic phenotype of an individual.

Gone are the days when one is constrained to study only a single gene or gene product in a biologically out-of-context situation'.

The term nutrimetabonomics illustrates the mutual link among nutrition and metabonomics. More practically, it describes how a biological system varies following a nutritional stimulus. The research in this field studies the effects of specific ingredients and food components elucidating the effects of specific ingredients behind individual responses. Thus, the emerging faces of nutrition is to achieve the ambitious goal of optimizing an individual's health via nutritional intervention [54].

It has been underlined that metabonomics is the logical approach to assess dysfunction and metabolic imbalances caused by dietary components [55]. This new approach will surely influence public health practice in the future [56].

Many years ago, Thomas Edison predicted that doctors of the future would no longer treat the human frame with drugs, but rather would cure and prevent diseases with nutrition.

Now, deciphering the complex interactions between nutrients and the human organism constitutes a considerable challenge for the twenty first century [57, 58]. We can add the microbiome to the previous sentence.

However, despite this background, while the number of systems biology publications has risen rapidly in the last decade, the percentage of these related to nutritional sciences research has remained constant at 3–4 % of the total [59].

Very recently, a review described recent applications of metabonomics in preclinical and clinical fields anticipating novel therapeutic and nutrition advances in paediatric research [60].

The diet has a key role in the gut microbiota modulation and shaping and in a metabolic signalling network construction. Metabolic profiling has a wide potential for the following goals: (a) understanding the complex interactions between components of the gut microbiota, (b) elucidating the cause/effect relationships associated with specific nutritional choices, and (c) evaluating the related shifts in the microbiota composition. The symbiosis between mammalians and the microbial system can play a role in the aetiology and development of several diseases, e.g. insulin resistance, Crohn's disease, irritable bowel syndrome (IBS), food allergies, gastritis and peptic ulcers, obesity, cardiovascular disease, and gastrointestinal tract. In particular gut, microbiome, and nutrients strongly interact with the host genetic elements to determine the metabotype. The knowledge of these complex interactions can provide personalized plans of treatment and prevention [61].

This is true also for long-term outcomes. In fact, since 'physiological' ageing is associated with a number of significant changes in gastrointestinal function, the development and progression of chronic diseases could be prevented, minimized, or better managed monitoring patient response on an individual basis [62].

In healthy humans, urine represented a sensitive metabolic profile that reflected acute dietary intake, whereas plasma and saliva did not [63].

As a general rule, the best biofluid to study nutrition or to identify food-specific biomarkers is urine. Urine is essentially the body's liquid repository and any nutrient or nonnutrient that is not needed or present in excess in the body will find its way in urine food-specific biomarkers that are present in the blood or urine for 5–10 h, with some persisting as long as 48 h. Thus, in food consumption monitoring, the same concepts behind drug testing and drug monitoring can be used [64].

This assumption has been recently confirmed: although both plasma and urine can be analyzed for the presence of food-derived biomarkers, with the present state of development of nutritional metabonomics, urine appears to be the preferred biofluid because (1) a greater diversity of metabolites derived from the food metabonome is observed in the urine, (2) collecting urine samples is relatively easy and noninvasive, and (3) spot urine samples collected at particular times in relation to meals and sleep period ('behavioural phase' urines) can be informative, and collection of 24 h urine samples may not be required [65, 66].

For example, it was possible to identify urinary metabolite profiles that discriminate between high and low intake of dietary protein during a dietary intervention [67]. The proposed dietary biomarkers have been recently reviewed [68].

In paediatrics, a special problem is represented that age influences metabonomics results. Age-related metabolic changes in children aged 12 years and below were investigated by Gu et al. [69] using ^{1}H NMR-based metabonomic analysis of urine. Unsupervised PCA analysis showed a distinct age-dependent clustering, indicating the effect of age on the urinary metabolite profile. Further statistical analysis led to the identification of age-related metabolic profiles. Among the metabolites that were found to correlate with age, creatinine increased with age, while creatine, glycine, betaine/TMAO, citrate, succinate, and acetone decreased. This investigation has shown that metabonomic approach has the potential to be useful in assessing the biological age of young humans as well as in providing more information about the confounding factors in the clinical application of metabonomics [69].

In neonatology, metabonomics has been extensively studied. The latest papers are related to intrauterine growth-restricted and small for gestational age neonates, prematurity, mode of delivery, hypoxic-ischemic encephalopathy, persistent ductus arteriosus, respiratory syndrome and surfactant therapy, cytomegalovirus infection, nephrouropathy, inborn errors of metabolism, pharmacometabonomics, and nutrimetabonomics (including study of maternal milk and formula). Also numerous papers have been presented in experimental neonatology. In particular, the fluids most frequently used were as follows: urine (by far the most used fluid), cord blood plasma, but also milk and stools [70–80].

Finally, we want to stress that intestinal microbiota highly influences the colonic luminal metabonome, and a comprehensive understanding of intestinal luminal metabonome is critical for clarifying host-intestinal bacterial interactions. Thus, low-molecular-weight metabolites produced by intestinal microbiota play a direct role in health and disease. Matsumoto et al. analyzed in germ-free (GF) mice and Ex-GF mice the colonic luminal metabonome using capillary electrophoresis mass spectrometry with time of flight (CE-TOFMS). CE-TOFMS identified 179 metabolites

from the colonic luminal metabonome and 48 metabolites were present in significantly higher concentrations and/or incidence in the germ-free (GF) mice than in the Ex-GF mice ($p < 0.05$), 77 metabolites were present in significantly lower concentrations and/or incidence in the GF mice than in the Ex-GF mice ($p < 0.05$), and 56 metabolites showed no differences in the concentration or incidence between GF and Ex-GF mice [81].

7.4.1 Maternal Milk, Formula, Metabonome, and Microbiome

The studies published in this field are very few.

In an earlier study, the choline content of human breast milk in the first 3 weeks after birth was compared with bovine milk and infant formula by use of ^{1}H NMR spectroscopy [82]. The observed choline species included free choline, phosphocholine, glycerophosphocholine, phosphatidylcholine, and sphingomyelin. Holmes et al. identified that total choline content in human colostrum at birth is lower than in mature milk 7 days post-partum, which correlates well with the acceleration in growth that the neonate experiences at this time point [82]. Moreover, it was speculated that for preterm infants, the choline content available in human milk is not enough for their rapid growth, as their metabolic activity is higher compared with full-term infants [82].

Some preliminary results of the authors of this chapter provide information on the biochemical variability of preterm HBM and on the potentiality of the metabonomic approach in nutrition and health [83].

The metabolic profile of preterm human breast milk (HBM) was investigated by using a metabonomics approach. To this aim, NMR spectroscopy and GC/MS, in combination with multivariate statistical analysis, were used to analyze the water-soluble and lipid fractions extracted from human milk samples, respectively, compared with preterm formula milk (FM), commonly prescribed. HBM contains relatively higher contents of this sugar with respect to FM samples. By contrary, the commercial products were suggested to be richer in maltose. Furthermore, other bins, belonging to unidentified metabolites, not present in HBM ('aliens' metabolites) were found to be important for the sample clustering. A deep examination of the score plot showed that milk samples of term and late preterm infants were located at the opposite side with respect those expressed by mothers at the lowest GA under investigation (i.e. 26 weeks).

Considering the lipidic part, the level of oleic and linoleic acids appeared to be higher in the artificial formulas than in HBM [83]. Finally, we observed a progressive change of the metabolic profile of milk from the right to the left part of the plot over the first month of lactation, suggesting a temporal variation in the carbohydrate composition. In particular, an increase of the lactose level was observed during the lactation period, in good agreement with the literature data [84].

In another experience, complimentary animal and human studies were conducted on young piglets and premature infants (34–36 weeks). Breast milk-fed vs.

formula-fed groups were analyzed by GC/MS. Metabonomics clearly was able to identify differences between breast milk-fed and formula-fed groups in the gut environment of piglets and humans. Among the most important discriminating metabolites between breast milk-fed and formula-fed groups, the authors found sugars, amino-sugars, fatty acids (namely, unsaturated fatty acids), and sterols. Thus, metabonomics and microbiota pinpointed specific sets of metabolites associated with the dominant bacterial taxa [85].

In conclusion, according with a very recent review on this topic, milk metabonomics represents a very promising field of study and with a potential to impact primary producers, industry, and consumers. Evidence has been obtained that the milk metabolites detected by NMR-based metabonomics are of importance in relation to milk nutritional quality, technological properties, quality control, and bioactivity [86].

7.5 The Complex Partnership of Disease and Nutrition: The Role of Metabonomics

7.5.1 Metabonomics, Gut Ischemia, and Necrotizing Enterocolitis

Intestinal ischemia/reperfusion (I/R) injury initiates a systemic inflammatory response syndrome with a high associated mortality rate. Early diagnosis is essential for reducing surgical mortality, yet current clinical biomarkers are insufficient is a novel strategy for studying intestinal I/R, which metabonomics has the potential for personalized risk stratification in patients exposed to intestinal I/R and may be used as part of a systems approach for quantitatively analysing the intestinal microbiome during gut injury [87].

In a mouse model for intestinal ischemia, sera were analyzed 4 h after mesenteric artery ligation by gas chromatography–mass spectrometry for 40 small molecules as their trimethylsilyl and *O*-methyloxime derivatives. The following molecular signatures were found: three highly significantly upregulated (fold-change) serum molecules in intestinal ischemia were inorganic phosphate (2.4), probably due to exit from the gut filter; urea (4.3), likely related to a reduction in glomerular filtration rate; and threonic acid (2.9). Threonic acid, which is a vit. C metabolite and is related to oxidative stress, seems to be the most specific metabolite. Five highly significantly downregulated (fold-change) serum molecules were stearic acid (1.7), arabinose (2.7), xylose (1.6), glucose (1.4), and ribose (2.2). Lactic acid, differently from other reports, remained unchanged in intestinal ischemia. Stearic acid seems to be of nutritional origin and is a precursor of oleic acid in the liver, very reduced in intestinal ischemia. The four monosaccharides (arabinose, xylose, glucose, and ribose) are produced by the microbiome and are the prove of its involvement in intestinal ischemia.

Taken all together, these data reveal alterations of gut microbiota metabolism, intestinal absorption, and renal function, together with increased oxidative stress [88].

Matching metabonomics and necrotizing enterocolitis (NEC), no results can be found. Although diet composition has been implicated as a major factor in the aetiology of various gastrointestinal diseases, such as NEC, conclusive evidence remains elusive. What is known on this topic is that breast milk, as opposed to commercial formula, appears to confer a 'protective effect' to the 'immature gut'. Yet the mechanism by which this occurs continues to remain speculative.

7.5.2 Metabonomics in Chronic Inflammatory Diseases

Gut microbial activities can be extremely important in the aetiology and development of several chronic inflammatory disorders, including inflammatory bowel disease (IBD) or irritable bowel syndrome (IBS). This topic will be covered in Chap. 14. Here we will underline only few concepts related to this section.

Urinary metabolite profiling was carried out on a mouse model of Crohn's disease suggesting alterations of tryptophan metabolism, fucosylation and fatty acid metabolism in Crohn's disease fucose, and xanthurenic acid could be useful markers of gut inflammation [89].

A mouse model of inflammatory bowel disease (IBD) was used to investigate urinary metabolites using NMR; it was found that there was an increase in trimethylamine (TMA) and fucose compared to controls. The increase in TMA was parallel to the progression of IBD [90].

Metabonomic analysis of faecal extracts of patients with both inflammatory diseases showed reduced levels of butyrate, acetate, methylamine, and TMA compared to control [91].

Urinary metabolites have also been used to distinguish CD and UC in humans. Hippurate was lowest in CD and differed significantly between CD, UC, and controls. Hippurate has been shown to be modulated according to gut microbes and this difference is likely to reflect changes in intestinal microbes [92].

By a practical point of view, urine and stools can be used as useful monitoring tools [93]. Attempt was made to study IBS with a combination of microbial and metabonomic outcomes using stool analysis. Using a GC–MS methodology, the authors highlighted higher levels of specific amino acids (alanine and pyroglutamate) and phenolic compounds (hydroxyphenylacetate and hydroxyphenylpropionate) with IBS, alterations possibly associated with specific gut microbial populations, including the abundance of lactobacilli and *Clostridium* [58, 94].

Celiac disease (CeD) is a unique autoimmune multifactorial gastrointestinal disorder in which the genetic factors (DQ2/DQ8) and the environmental trigger (gluten) are known and necessary but not sufficient for its development. Bertini et al. [95] highlighted changes in gut microbial cometabolites may be associated to aberrant microbiota in the small bowel of patients with CeD [96].

Sellitto et al. [97] characterized the longitudinal changes in the microbial communities that colonize infants from birth to 24 months and the impact of two patterns of gluten introduction (early vs. late) on the gut microbiota and metabonome, and the switch from gluten tolerance to immune response, including onset of CeD autoimmunity. CD is probably associated with intestinal and faecal dysbiosis, which is related to certain bacterial species. As shown by, the gluten-free diet lasting at least 2 years did not completely restore the microbiota and, consequently, the metabonome of CD children.

Some molecules (e.g. ethyl-acetate and octyl-acetate, some short chain fatty acids and free amino acids, and glutamine) together with microbial indices (e.g. ratio between faecal cell density of lactic acid bacteria-Bifidobacterium vs. Bacteroides-Enterobacteria) seem to be metabolic signatures of CD patients [98]. These data confirm results published in previous papers [95].

7.5.3 Cystic Fibrosis

In cystic fibrosis (CF), airway inflammation leads to an increased production of reactive oxygen species, resulting in the degradation of cell membranes and the generation of volatile organic compounds (VOCs). The study by Wolak et al. [99] demonstrated that metabonomic analysis of bronchoalveolar fluid can differentiate between different degrees of inflammation in children affected by cystic fibrosis and has the potential to identify new biomarkers of inflammation.

A more recent study was carried out with the aim of investigating whether mass spectrometry-based metabonomic analysis of volatile organic compounds (VOCs) in exhaled breath was able to discriminate between CF subjects and controls and between CF subjects with and without Pseudomonas colonization. Samples from 48 children with CF and 57 controls were examined. Analysis revealed that 1099 VOCs exhibited a prevalence of at least 7 %. A 100 % correct identification of CF subjects and controls was possible by using 22 VOCs. Therefore, metabonomic analysis of VOCs in exhaled breath appears to be a reproducible technique and is able to discriminate not only between CF subjects and controls but also between CF subjects with or without Pseudomonas colonization [100].

7.5.4 Diabetes

This part will be covered by Chap. 12. Different studies suggest that branched-chain amino acids (BCAAs) related to metabolic signature is a robust metabolic readout of insulin resistance (IR) [101]: high levels of BCAAs were detected in the obese and IR phenotype [102–104].

In particular, five branched-chain and aromatic amino acids were indeed associated with IR, namely, isoleucine, leucine, valine, tyrosine, and phenylalanine, and a

combination of three amino acids (isoleucine, phenylalanine, tyrosine) could predict future diabetes (>fivefold higher risk for individuals in top quartile) [105].

During the last decade, the rapidly growing research field of metabonomics has introduced new insights into the pathology of diabetes as well as methods to predict disease onset and has revealed new biomarkers [106].

In the adult, Wang–Sattler identified three metabolites (glycine, lysophosphatidylcholine (LPC) (18:2), and acetylcarnitine) that had significantly altered levels. The most important results of this study are that metabonomics is enough and it is not necessary to perform other 'omics' and that those who have that three metabolites altered will develop surely diabetes [107].

A recent review summarizes the current findings of metabolic research regarding diabetes in animal models and human investigations [106].

Finally, very recently it has been stressed that systems biology methodologies can identify disease biomarkers and uncover potential therapeutic targets from a combination of 'omics' datasets. Relevant examples are diabetes and obesity [107].

7.5.5 Obesity

Metabonomics has been widely studied in adult obese [108].

For example, a recent study on obese Japanese subjects reported a physiological inference between insulin resistance (IR), plasma levels of alanine, glycine, glutamate, tryptophan, tyrosine and BCAAs, and visceral fat metabolism [109]. Again, a complex relationship between dyslipidemia and IR development has been described [110].

Differently from the adults, only few studies have been dedicated to childhood obesity. In a study by Walsh et al., serum metabolite concentration profiles of obese children could be distinguished from those of normal-weight children. The identified metabolite markers are indicative of oxidative stress and of changes in sphingomyelin metabolism, in β-oxidation, and in pathways associated with energy expenditure. The altered metabolites might be considered as potential biomarkers in the generation of new hypotheses on the biological mechanisms behind obesity [111]. Mihalic et al. compared acylcarnitine (AcylCN) species, common amino acid and fat oxidation (FOX) by-products, and plasma amino acids in normal weight (NW; $n=39$), obese (OB; $n=64$), and type 2 diabetic ($n=17$) adolescents. The observations of the authors are consistent with early adaptive metabolic plasticity in youth, which over time—with continued obesity and ageing—may become dysfunctional, as observed in adults [112]. An experimental paper has been published on piglets by He et al. [113]. All the papers on paediatric obesity are presented in Table 7.1 [114].

What is becoming increasingly important is the role of perinatal programming in the development of paediatric and adult obesity. This could involve either newborns with 'not enough' or newborns with 'too much' birth weight. The common sign of hypoglycemia at birth and common pathways such us some metabolites like myo-inositol can be responsible for later appearance of metabolic syndrome and obesity later in life [79, 115–118].

Table 7.1 Metabonomics studies that evaluated the metabolic condition in infant obesity

Author	Year	Type of patient	Sample	Metabonomic analysis	Metabolites results
He et al.	2012	Newborn piglet	Serum	NMR-based metabonomic technology	HDL, VLDL, lipids, unsaturated lipids, glycoprotein, myo-inositol, pyruvate, threonine, tyrosine and creatine > in obese than in lean pigs ($p<0.05$). Serum glucose and urea < in obese pigs ($p<0.05$)
Wahl et al.	2012	Human children	Serum	Mass spectrometry-based metabonomics approach	14 metabolites (glutamine, methionine, proline, nine phospholipids, and two acylcarnitines, $p<3.8\times10^{-4}$) and 69 metabolite ratios ($p<6.0\times10^{-6}$) to be significantly altered in obese children
Mihalik et al.	2012	Human adolescents	Plasma	Tandem mass spectrometry	Fasting lipolysis and fat oxidation were higher in obese and type 2 diabetes compared with normal weight. Insulin sensitivity was lower in obese and type 2 diabetes

From Ref. [114] with permission

7.6 Conclusions

The human host-microbe symbiosis is initiated in early life and it is an important biological process.

Gut microbiota enhances the host's metabolic capacity to process nutrients and drugs and modulate the activities of multiple pathways in different organ systems. The disruption of the gut microbiota has been linked to an increasing number of diseases such as necrotizing enterocolitis, inflammatory bowel disease, obesity, cancer, diabetes, and allergies.

Now, deciphering the complex interactions between nutrients and the human organism constitutes a considerable challenge for the twenty fist century. The role of microbiome in this setting is of increasing importance. Metabolic profiling has a wide potential in deciphering for the following goals: (a) understanding the complex interactions between components of the gut microbiota, (b) elucidating the cause/effect relationships associated with specific nutritional choices, and (c) evaluating the related shifts in the microbiota composition.

As a general rule, the best biofluid to study nutrition or to identify food-specific biomarkers is urine. In food consumption monitoring, the same concepts behind drug testing and drug monitoring can be used.

The way to clinical implementation of metabonomics is still hampered by many of the problems that had to be solved for genomics and proteomics in the past, as well as new ones that require the creation of new analytic, computational, and interpretative techniques.

Some problems are similar with those observed for pharmacometabonomics: significant individual variability, issues surrounding methods for metabolite detection (NMR, MS); extremely complex datasets; possible over-interpretation of data; necessity for skilled and experienced technicians and well-trained practitioners, time-consuming processing and analysis of patient samples, resulting in delayed treatment; and high cost of the processing and analytical platforms [119, 120].

In the opinion of the authors, in the near future, improved tools for the analysis of the metabolic profile (simplified like 'dipsticks' for urine) and its integration with the other 'omics' data will move metabonomics beside the child [121].

The greatest challenge, however, will be the integration of information from different 'omics', for example, in the form of new, superior 'meta-markers' [122].

References

1. Claus SP, Ellero SL, Berger B, et al. Colonization-induced host-gut microbial metabolic interaction. MBio. 2011;2(2):e00271–10. doi:10.1128/mBio.00271-10.
2. Maynard CL, Elson CO, Hatton RD, Weaver CT. Reciprocal interactions of the intestinal microbiota and immune system. Nature. 2012;489:231–41. doi:10.1038/nature11551.
3. Young VB. The intestinal microbiota in health and disease. Curr Opin Gastroenterol. 2012;28(1):63–9. doi:10.1097/MOG.0b013e32834d61e9.

4. Fouhy F, Ross RP, Fitzgerald G, Stanton C, Cotter PD. Composition of the early intestinal microbiota: knowledge, gaps and the use of high-throughput sequencing to address these gaps. Gut Microbes. 2012;3:203–20. doi:10.4161/gmic.20169.
5. Scholtens PA, Oozeer R, Martin R, Amor KB, Knol J. The early settlers: intestinal microbiology in early life. Annu Rev Food Sci Technol. 2012;3:425–47. doi:10.1146/annurev-food-022811-101120.
6. Zoetendal EG, Rajilic-Stojanovic M, de Vos WM. High-throughput diversity and functionality analysis of the gastrointestinal tract microbiota. Gut. 2008;57:1605–15. doi:10.1136/gut.2007.133603.
7. Faa G, Gerosa C, Fanni D, Nemolato S, Fanos V. Factors influencing the development of a personal tailored microbiota in the neonate, with particular emphasis on antibiotic therapy. J Matern Fetal Neonatal Med. 2013;26:35–43.
8. Fanaro S, Chierici R, Guerrini P, Vigi V. Intestinal microflora in early infancy: composition and development. Acta Paediatr. 2003;91(441):48–55.
9. Orrhage K, Nord CE. Factors controlling the bacterial colonization of the intestine in breast fed infants. Acta Paediatr. 1999;88(430):47–57.
10. Yoshioka H, Iseki K, Fujita K. Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. Pediatrics. 1983;72:317–21.
11. Rajilic-Stojanovic M, Heilig HG, Molenaar D, et al. Development and application of the human intestinal tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. Environ Microbiol. 2009;11:1736–51. doi:10.1111/j.1462-2920.2009.01900.x.
12. Sanz Y. Gut microbiota and probiotics in maternal and infant health. Am J Clin Nutr. 2011;94:2000S–5. doi:10.3945/ajcn.110.001172.
13. Koenig JE, Spor A, Scalfone N, et al. Succession of microbial consortia in the developing infant gut microbiome. Proc Natl Acad Sci U S A. 2011;108 suppl 1:4578–85. doi:10.1073/pnas.1000081107.
14. Ottman N, Smidt H, de Vos WM, Belzer C. The function of our microbiota: who is out there and what do they do? Front Cell Infect Microbiol. 2012;2:104. doi:10.3389/fci.
15. Zhao L, Wang G, Siegel P, et al. Quantitative genetic background of the host influences gut microbiomes in chickens. Sci Rep. 2013;3:1163. doi:10.1038/srep01163mb.2012.00104.
16. Khachatryan ZA, Ktsoyan ZA, Manukyan GP, Kelly D, Ghazaryan KA, Aminov RI. predominant role of host genetics in controlling the composition of gut microbiota. PLoS One. 2008;3(8):e3064. doi:10.1371/journal.pone.0003064.
17. Stewart JA, Chadwick VS, Murray A. Investigations into the influence of host genetics on the predominant eubacteria in the faecal microflora of children. J Med Microbiol. 2005;54:1239–42. doi:10.1099/jmm. 0.46189-0.
18. Dominguez-Bello MG, Costello EK, Contreras M, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A. 2010;107(26):11971–5. doi:10.1073/pnas.1002601107.
19. Adlerberth I, Strachan DP, Matricardi PM, et al. Gut microbiota and development of atopic eczema in European birth cohorts. J Allergy Clin Immunol. 2007;120:343–50. doi:10.1016/j.jaci.2007.05.018.
20. Gronlund MM, Lehtonen OP, Eerola E, Kero P. Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after caesarean delivery. J Pediatr Gastroenterol Nutr. 1999;28:19–25.
21. Chen J, Cai W, Feng Y. Development of intestinal bifidobacteria and lactobacilli in breast-fed neonates. Clin Nutr. 2007;26:559–66. doi:10.1016/j.clnu.2007.03.003.
22. Penders J, Thijs C, Vink C, et al. Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics. 2006;118:511–21. doi:10.1542/peds.2005-2824.
23. Biasucci G, Rubini M, Riboni S, Morelli L, Bessi E, Retetangos C. Mode of delivery affects the bacterial community in the newborn gut. Early Hum Dev. 2010;86:S13–5. doi:10.1016/j.earlhumdev.2010.01.004.

24. Azad MB, Konya T, Maughan H, et al. Gut microbiota of healthy Canadian infants: profiles by mode of delivery and infant diet at 4 months. CMAJ. 2013. doi:10.1503/cmaj.121189 (Epub ahead of print).
25. Hong PY, Lee BW, Aw M, et al. Comparative analysis of fecal microbiota in infants with and without eczema. PLoS One. 2010;5:e9964. doi:10.1371/journal.pone.0009964.
26. Roger LC, Costabile A, Holland DT, Hoyles L, McCartney AL. Examination of faecal *Bifidobacterium* populations in breast- and formula-fed infants during the first 18 months of life. Microbiology. 2010;156:3329–41. doi:10.1099/mic.0.043224-0.
27. Abrahamsson TR, Jakobsson HF, Andersson AF, Björkstén B, Engstrand L, Jenmalm MC. Low diversity of the gut microbiota in infants with atopic eczema. J Allergy Clin Immunol. 2012;129:434–40. doi:10.1016/j.jaci.2011.10.025.
28. de la Cochetiere M, Piloquet H, Des Robert C, Darmaun D, Galmiche J, Roze J. Early intestinal bacterial colonization and necrotizing enterocolitis in premature infants: the putative role of Clostridium. Paediatr Res. 2004;56:366–70. doi:10.1203/01.PDR.0000134251.45878.D5.
29. Rougè C, Goldenberg O, Ferraris L, et al. Investigation of the intestinal microbiota in preterm infants using different methods. Anaerobe. 2010;16(4):362–70. doi:10.1016/ j.anaerobe.2010.06.002.
30. Jacquot A, Neveu D, Aujoulat F, et al. Dynamics and clinical evolution of bacterial gut microflora in extremely premature patients. J Pediatr. 2011;158:390–6. doi:10.1016/j.jpeds.2010.09.
31. Chang JY, Antonopoulos DA, Kaira A, et al. Decreased diversity of the fecal microbiome in recurrent *Clostridium difficile*-associated diarrhea. J Infect Dis. 2008;197(3):435–8. doi:10.1086/525047.
32. Butel MJ, Suau A, Campeotto F, et al. Conditions of bifidobacterial colonization in preterm infants: a prospective analysis. J Pediatr Gastroenterol Nutr. 2007;44:577–82. doi:10.1097/MPG.Ob013e3180406b20.
33. Magne F, Abely M, Boyer F, Morville P, Pochart P, Suau A. Low species diversity and high interindividual variability in faeces of preterm infants as revealed by sequences of 16S rRNA genes and PCR-temporal temperature gradient gel electrophoresis profiles. FEMS Microbiol Ecol. 2006;57:128–38. doi:10.1111/j.1574-6941.2006.00097.x.
34. Stewart CJ, Nelson A, Scribbins D, et al. Bacterial and fungal viability in the preterm gut: NEC and sepsis. Arch Dis Child Fetal Neonatal Ed. 2013. doi:10.1136/archdischild-2012-302119 (Epub ahead of print).
35. Walker A. Breast milk as the gold standard for protective nutrients. J Pediatr. 2010;156:3–7. doi:10.1016/j.jpeds.2009.11.021.
36. Thurl S, Munzert M, Henker J, et al. Variation of human milk oligosaccharides in relation to milk groups and lactational periods. Br J Nutr. 2011;104:1261–71. doi:10.1017/S0007114510002072.
37. Knol J, Scholtens P, Kafka C, et al. Colon microflora in infants fed formula with galacto- and fructo-oligosaccharides: more like breast-fed infants. J Pediatr Gastroenterol Nutr. 2005;40(1):36–42.
38. Collado MC, Delgado S, Maldonado A, Rodriguez JM. Assessment of the bacterial diversity of breast milk of healthy women by quantitative real-time PCR. Lett Appl Microbiol. 2009;48:523–8. doi:10.1111/j.1472-765X.2009.02567.x.
39. Boesten R, Schuren F, Ben Amor K, Haarman M, Knol J, de Vos WM. Bifidobacterium population analysis in the infant gut by direct mapping of genomic hybridization patterns: potential for monitoring temporal development and effects of dietary regimens. Microb Biotechnol. 2011;4:417–27. doi:10.1111/j.1751-7915.2010.00216.x.
40. Martin R, Langa S, Reviriego C, et al. The commensal microflora of human milk: new perspectives for food bacteriotherapy and probiotics. Trends Food Sci Technol. 2004;15:121–7.
41. Rao S, Srinivasjois R, Patole S. Prebiotic supplementation in full-term neonates: a systematic review of randomized controlled trials. Arch Pediatr Adolesc Med. 2009;163:755–64. doi:10.1001/archpediatrics.2009.94.

42. Scholtens PA, Alliet P, Raes M, et al. Fecal secretory immunoglobulin A is increased in healthy infants who receive a formula with short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides. J Nutr. 2008;138(6):1141–7.
43. Parracho H, McCartney A, Gibson G. Probiotics and prebiotics in infant nutrition. Proc Nutr Soc. 2007;66:405–11. doi:10.1017/S0029665107005678.
44. Braegger C, Chmielewska A, Decsi T, et al. Supplementation of infant formula with probiotics and/or prebiotics: a systematic review and comment by the ESPGHAN committee on nutrition. J Pediatr Gastroenterol Nutr. 2011;52:238–50. doi:10.1097/MPG.Ob013e3181fb9e80.
45. Wang Q, Dong J, Zhu Y. Probiotic supplement reduces risk of necrotizing enterocolitis and mortality in preterm very low-birth-weight infants: an updated meta-analysis of 20 randomized, controlled trials. J Pediatr Surg. 2012;47:241–8. doi:10.1016/j.jpedsurg.2011.09.064.
46. Fallani M, Young D, Scott J, et al. Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. J Pediatr Gastroenterol Nutr. 2010;51:77–84. doi:10.1097/MPG.0b013e3181d1b11e.
47. Hussey S, Wall R, Gruffman E, et al. Parenteral antibiotics reduce bifidobacteria colonization and diversity in neonates. Int J Microbiol. 2011;2011:130574. doi:10.1155/2011/130574.
48. Mangin I, Suau A, Gotteland M, Brunser O, Pochart P. Amoxicillin treatment modifies the composition of *Bifidobacterium* species in infant intestinal microbiota. Anaerobe. 2010;16:433–8. doi:10.1016/j.anaerobe.2010.06.005.
49. Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc Natl Acad Sci U S A. 2011;108 suppl 1:4554–61. doi:10.1073/pnas.1000087107.
50. Kummeling I, Stelma FF, Dagnelie PC, et al. Early life exposure to antibiotics and the subsequent development of eczema, wheeze, and allergic sensitization in the first 2 years of life: the KOALA Birth Cohort Study. Pediatrics. 2007;119:e225–31. doi:10.1542/peds.2006-0896.
51. Marra F, Lynd L, Coombes M, et al. Does antibiotic exposure during infancy lead to development of asthma? A systematic review and metaanalysis. Chest. 2009;136:e30.
52. Gibney MJ, Walsh M, Brennan L, Roche HM, German B, van Ommen B. Metabolomics in human nutrition: opportunities and challenges. Am J Clin Nutr. 2005;82(3):497–503.
53. Go VL, Nguyen CT, Harris DM, Lee WN. Nutrient-gene interaction: metabolic genotype-phenotype relationship. J Nutr. 2005;135(12 Suppl):3016S–20.
54. Mutch DM, Wahli W, Williamson G. Nutrigenomics and nutrigenetics: the emerging faces of nutrition. FASEB J. 2005;19(12):1602–16.
55. Astle J, Ferguson JT, German JB, Harrigan GG, Kelleher NL, Kodadek T, Parks BA, Roth MJ, Singletary KW, Wenger CD, Mahady GB. Characterization of proteomic and metabolomic responses to dietary factors and supplements. J Nutr. 2007;137(12):2787–93.
56. Zeisel SH. Nutrigenomics and metabolomics will change clinical nutrition and public health practice: insights from studies on dietary requirements for choline. Am J Clin Nutr. 2007;86(3):542–8.
57. Scalbert A, Brennan L, Fiehn O, Hankemeier T, Kristal BS, van Ommen B, Pujos-Guillot E, Verheij E, Wishart D, Wopereis S. Mass-spectrometry-based metabolomics: limitations and recommendations for future progress with particular focus on nutrition research. Metabolomics. 2009;5(4):435–58. Epub 2009 Jun 12.
58. Martin FP, Collino S, Rezzi S, Kochhar S. Metabolomic applications to decipher gut microbial metabolic influence in health and disease. Front Physiol. 2012;3:113. doi:10.3389/fphys.2012.00113. Epub 2012 Apr 26.
59. Moore JB, Weeks ME. Proteomics and systems biology: current and future applications in the nutritional sciences. Adv Nutr. 2011;2(4):355–64.
60. Moco S, Collino S, Rezzi S, Martin FP. Metabolomics perspectives in pediatric research. Pediatr Res. 2013;73:570–6.
61. Vernocchi P, Vannini L, Gottardi D, Del Chierico F, Serrazanetti DI, Ndagijimana M, Guerzoni ME. Integration of datasets from different analytical techniques to assess the impact of nutrition on human metabolome. Front Cell Infect Microbiol. 2012;2:156.

62. Parker BA, Chapman IM. Food intake and ageing – the role of the gut. Mech Ageing Dev. 2004;125:859–66.
63. Walsh MC, Brennan L, Malthouse JP, Roche HM, Gibney MJ. Effect of acute dietary standardization on the urinary, plasma, and salivary metabolomic profiles of healthy humans. Am J Clin Nutr. 2006;84(3):531–9.
64. Wishart DS. Metabolomics application to food science and nutrition research. Trends Food Sci Technol. 2008;19:482–93.
65. Primrose S, Draper J, Elsom R, Kirkpatrick V, Mathers JC, Seal C, Beckmann M, Haldar S, Beattie JH, Lodge JK, Jenab M, Keun H, Scalbert A. Metabolomics and human nutrition. Br J Nutr. 2011;105(8):1277–83. doi:10.1017/S0007114510004812. Epub 2011 Jan 24.
66. Beckmann M, Lloyd AJ, Haldar S, Favé G, Seal CJ, Brandt K, Mathers JC, Draper J. Dietary exposure biomarker-lead discovery based on metabolomics analysis of urine samples. Proc Nutr Soc. 2013;72(3):352–61.
67. Rasmussen LG, Winning H, Savorani F, Toft H, Larsen TM, Dragsted LO, Astrup A, Engelsen SB. Assessment of the effect of high or low protein diet on the human urine metabolome as measured by NMR. Nutrients. 2012;4(2):112–31. doi:10.3390/nu4020112. Epub 2012 Feb 20.
68. Hedrick VE, Dietrich AM, Estabrooks PA, Savla J, Serrano E, Davy BM. Dietary biomarkers: advances, limitations and future directions. Nutr J. 2012;11:109. doi:10.1186/1475-2891-11-10.
69. Gu H, Pan Z, Xi B, Hainline BE, Shanaiah N, Asiago V, Gowda GA, Raftery D. 1H NMR metabolomics study of age profiling in children. NMR Biomed. 2009;22(8):826–33.
70. Fanos V, Chevalier R, Faa G, Cataldi L. Developmental nephrology: from embryology to metabolomics, vol 10. In: Quartu S Elena (Cagliari), editors. Hygeia Press; 2011. p. 149–215 (available also as ebook)
71. Fanos V, Barberini L, Antonucci R, Atzori L. Pharma-metabolomics in neonatology: is it a dream or a fact? Curr Pharm Des. 2012;18:2996–3006.
72. Fanos V, Antonucci R, Barberini L, Atzori L. Urinary metabolomics in the newborn and infants. Adv Clin Chem. 2012;58:193–223.
73. Fanos V, Antonucci R, Barberini L, Noto A, Atzori L. Clinical application of metabolomics in neonatology. J Matern Fetal Neonatal Med. 2012;25 Suppl 1:104–9.
74. Fanos V, Mussap M, Noto A, Atzori L. Metabolomics in perinatology: where are we now? Acta Med Port. 2012;25(S2):117–20.
75. Fanos V, van den Anker J, Noto A, et al. Metabolomics in neonatology: fact or fiction? Semin Fetal Neonatal Med. 2013;18:3–12.
76. Fanos V, Locci E, Noto A, et al. Urinary metabolomics in newborns infected by cytomegalovirus: a preliminary investigation. Early Hum Dev. 2013;89:S58–61.
77. Fanos V, Fanni C, Ottonello G, et al. Metabolomics in adult and pediatric nephrology. Molecules. 2013;18:4844–5.
78. Fanos V, Iacovidou N, Puddu M, et al. Metabolomics in neonatal life. Early Hum Dev. 2013;89:S7–S10C.
79. Fanos V, Atzori L, Makarenko K, Melis GB, Ferrazzi E. Metabolomics application in maternal-fetal medicine. Biomed Res Int. 2013;2013:720514. doi:10.1155/2013/720514. Epub 2013 Jun 9. Review.
80. Fanos V, Antonucci R, Atzori L. Metabolomics in the developing infant. Curr Opin Pediatr. 2013;25:604–11.
81. Matsumoto M, Kibe R, Ooga T, Aiba Y, Kurihara S, Sawaki E, Koga Y, Benno Y. Impact of intestinal microbiota on intestinal luminal metabolome. Sci Rep. 2012;2:233. doi:10.1038/srep00233.
82. Holmes HC, Snodgrass I, Iles RA. Changes in the choline content of human breast milk in the first 3 weeks after birth. Eur J Pediatr. 2000;159:198–204.
83. Marincola FC, Noto A, Caboni P, Reali A, Barberini L, Lussu M, Murgia F, Santoru ML, Atzori L, Fanos V. A metabolomic study of preterm human and formula milk by high resolution NMR and GC/MS analysis: preliminary results. J Matern Fetal Neonatal Med. 2012;25 Suppl 5:62–7.

84. Miller JB, Bull S, Miller J, McVeagh P. The oligosaccharide composition of human milk: temporal and individual variations in monosaccharide components. J Pediatr Gastroenterol Nutr. 1994;19:371–6.
85. Poroyko V, Morowitz M, Bell T, et al. Diet creates metabolic niches in the "immature gut" that shape microbial communities. Nutr Hosp. 2011;26:1283–95.
86. Sundekilde UK, Larsen LB, Bertram HC. NMR-based milk metabolomics. Metabolites. 2013;3:204–22. doi:10.3390/metabo3020204.
87. Kinross J, Warren O, Basson S, Holmes E, Silk D, Darzi A, Nicholson JK. Intestinal ischemia/reperfusion injury: defining the role of the gut microbiome. Biomark Med. 2009;3(2):175–92. doi:10.2217/bmm.09.11.
88. Fahrner R, Beyoğlu D, Beldi G, Idle JR. Metabolomic markers for intestinal ischemia in a mouse model. J Surg Res. 2012;178(2):879–87. doi:10.1016/j.jss.2012.08.011. Epub 2012 Aug 25.
89. Lin HM, Edmunds SJ, Helsby NA, Ferguson LR, Rowan DD. Nontargeted urinary metabolite profiling of a mouse model of Crohn's disease. J Proteome Res. 2009;8(4):2045–57.
90. Murdoch TB, Fu H, MacFarlane S, Sydora BC, Fedorak RN, Slupsky CM. Urinary metabolic profiles of inflammatory bowel disease in interleukin-10 genedeficient mice. Anal Chem. 2008;80(14):5524–31.
91. Marchesi JR, Holmes E, Khan F, Kochhar S, Scanlan P, Shanahan F, Wilson ID, Wang Y. Rapid and noninvasive metabonomic characterization of inflammatory bowel disease. J Proteome Res. 2007;6:546–51.
92. Williams HR, Cox IJ, Walker DG, et al. Characterization of inflammatory bowel disease with urinary metabolic profiling. Am J Gastroenterol. 2009;104:1435–44.
93. Winterkamp S, Weidenhiller M, Otte P, Stolper J, Schwab D, Hahn EG, Raithel M. Urinary excretion of N-methylhistamine as a marker of disease activity in inflammatory bowel disease. Am J Gastroenterol. 2002;97:3071–7.
94. Ponnusamy K, Choi JN, Kim J, Lee SY, Lee CH. Microbial community and metabolomics comparison of irritable bowel syndrome faeces. J Med Microbiol. 2011;60(Pt 6):817–27.
95. Bertini I, Calabro A, De Carli V, Luchinat C, Nepi S, Porfirio B, Renzi D, Saccenti E, Tenori L. The metabonomic signature of celiac disease. J Proteome Res. 2009;8:170–7.
96. Tjellstrom B, Stenhammar L, Hogberg L, Falth-Magnusson K, Magnusson KE, Midtvedt T, Sundqvist T, Norin E. Gut microflora associated characteristics in children with celiac disease. Am J Gastroenterol. 2005;100:2784–8.
97. Sellitto M, Bai G, Serena G, Fricke WF, Sturgeon C, et al. Proof of concept of microbiome-metabolome analysis and delayed gluten exposure on celiac disease autoimmunity in genetically at-risk infants. PLoS One. 2012;7(3):e33387. doi:10.1371/journal.pone.0033387.
98. Di Cagno R, De Angelis M, De Pasquale I, Ndagijimana M, Vernocchi P, Ricciuti P, Gagliardi F, Laghi L, Crecchio C, Guerzoni ME, Gobbetti M, Francavilla R. Duodenal and faecal microbiota of celiac children: molecular, phenotype and metabolome characterization. BMC Microbiol. 2011;11:219. doi:10.1186/1471-2180-11-219.
99. Wolak JE, Esther Jr CR, O'Connell TM. Metabolomic analysis of bronchoalveolar lavage fluid from cystic fibrosis patients. Biomarkers. 2009;14:55–60.
100. Robroeks CM, van Berkel JJ, Dallinga JW, Jöbsis Q, Zimmermann LJ, Hendriks HJ, Wouters MF, van der Grinten CP, van de Kant KD, van Schooten FJ, Dompeling E. Metabolomics of volatile organic compounds in cystic fibrosis patients and controls. Pediatr Res. 2010;68(1):75–80.
101. Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF, Haqq AM, Shah SH, Arlotto M, Slentz CA, Rochon J, Gallup D, Ilkayeva O, Wenner BR, Yancy Jr WS, Eisenson H, Musante G, Surwit RS, Millington DS, Butler MD, Svetkey LP. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab. 2009;9:311–26.
102. Huffman KM, Shah SH, Stevens RD, Bain JR, Muehlbauer M, Slentz CA, Tanner CJ, Kuchibhatla M, Houmard JA, Newgard CB, Kraus WE. Relationships between circulating metabolic intermediates and insulin action in overweight to obese, inactive men and women. Diabetes Care. 2009;32:1678–83.

103. Fiehn O, Garvey WT, Newman JW, Lok KH, Hoppel CL, Adams SH. Plasma metabolomics profiles reflective of glucose homeostasis in non-diabetic and type 2 diabetic obese African-American women. PLoS One. 2010;5:e15234.
104. Boulange CL, Claus SP, Chou CJ, Collino S, Montoliu I, Kochhar S, Holmes E, Rezzi S, Nicholson JK, Dumas ME, Martin FP. Early metabolic adaptation in C57BL/6 mice resistant to high fat diet induced weight gain involves an activation of mitochondrial oxidative pathways. J Proteome Res. 2013;12:1956–68.
105. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, Lewis GD, Fox CS, Jacques PF, Fernandez C, O'Donnell CJ, Carr SA, Mootha VK, Florez JC, Souza A, Melander O, Clish CB, Gerszten RE. Metabolite profiles and the risk of developing diabetes. Nat Med. 2011;17:448–53.
106. Friedrich N. Metabolomics in diabetes research. J Endocrinol. 2012;215:29–42.
107. Wang-Sattler R, Yu Z, Herder C, Messias AC, Floegel A, He Y, Heim K, Campillos M, Holzapfel C, Thorand B, Grallert H, Xu T, Bader E, Huth C, Mittelstrass K, Döring A, Meisinger C, Gieger C, Prehn C, Roemisch-Margl W, Carstensen M, Xie L, Yamanaka-Okumura H, Xing G, Ceglarek U, Thiery J, Giani G, Lickert H, Lin X, Li Y, Boeing H, Joost HG, de Angelis MH, Rathmann W, Suhre K, Prokisch H, Peters A, Meitinger T, Roden M, Wichmann HE, Pischon T, Adamski J, Illig T. Novel biomarkers for pre-diabetes identified by metabolomics. Mol Syst Biol. 2012;8:615. doi:10.1038/msb.2012.43.
108. Meng Q, Mäkinen VP, Luk H, Yang X. Systems biology approaches and applications in obesity, diabetes, and cardiovascular diseases. Curr Cardiovasc Risk Rep. 2013;7(1):73–83. Epub 2012 Oct 18.
109. Yamakado M, Tanaka T, Nagao K, Ishizaka Y, Mitushima T, Tani M, Toda A, Toda E, Okada M, Miyano H, Yamamoto H. Plasma amino acid profile is associated with visceral fat accumulation in obese Japanese subjects. Clin Obes. 2012;2:29–40.
110. Rhee EP, Cheng S, Larson MG, Walford GA, Lewis GD, McCabe E, Yang E, Farrell L, Fox CS, O'Donnell CJ, Carr SA, Vasan RS, Florez JC, Clish CB, Wang TJ, Gerszten RE. Lipid profiling identifies a triacylglycerol signature of insulin resistance and improves diabetes prediction in humans. J Clin Invest. 2011;121:1402–11.
111. Wahl S, Yu Z, Kleber M, Singmann P, Holzapfel C, He Y, Mittelstrass K, Polonikov A, Prehn C, RömischMargl W, Adamski J, Suhre K, Grallert H, Illig T, Wang-Sattler R, Reinehr T. Childhood obesity is associated with changes in the serum metabolite profile. Obes Facts. 2012;5:660–70.
112. Mihalik SJ, Michaliszyn SF, de las Heras J, Bacha F, Lee S, Chace DH, DeJesus VR, Vockley J, Arslanian SA. Metabolomic profiling of fatty acid and amino acid metabolism in youth with obesity and type 2 diabetes: evidence for enhanced mitochondrial oxidation. Diabetes Care. 2012;35(3):605–11.
113. He Q, Ren P, Kong X, Wu Y, Wu G, Li P, Hao F, Tang H, Blachier F, Yin Y. Comparison of serum metabolite compositions between obese and lean growing pigs using an NMR-based metabonomic approach. J Nutr Biochem. 2012;23(2):133–9.
114. Dessì A, Fanos V. Pediatric obesity: could metabolomics be a useful tool? J Pediatr Neonatal Individ Med. 2013;2(2):e020205. doi:10.7363/020205.
115. Bassareo PP, Fanos V, Noto A, Solla P, Barberini L, Flore G, Puddu M, Mercuro G. Clinical metabolomics and hematic ADMA predict the future onset of cardiorenal syndrome in young grown-up subjects who were born preterm . Clin Biochem. 2013;47(6):423–6
116. Dessì A, Puddu M, Ottonello G, Fanos V. Metabolomics and fetal-neonatal nutrition: between "not enough" and "too much". Molecules. 2013;18(10):11724–32. doi:10.3390/molecules181011724.
117. Fanos V, Iacovidou N, Puddu M, Ottonello G, Noto A, Atzori L. Metabolomics in neonatal life. Early Hum Dev. 2013;89 Suppl 1:S7–10. doi:10.1016/S0378-3782(13)70003-3. Review.
118. Dessì A, Fanos V. Myoinositol: a new marker of intrauterine growth restriction? J Obstet Gynaecol. 2013;33(8):776–80. doi:10.3109/01443615.2013.831046.
119. Antonucci R, Pilloni MD, Atzori L, Fanos V. Pharmaceutical research and metabolomics in the newborn. J Matern Fetal Neonatal Med. 2012;25 Suppl 5:22–6.

120. Syggelou A, Iacovidou N, Atzori L, Xanthos T, Fanos V. Metabolomics in the developing human being. Pediatr Clin North Am. 2012;59(5):1039–58.
121. Mussap M, Antonucci R, Noto A, Fanos V. The role of metabolomics in neonatal and pediatric laboratory medicine. Clin Chim Acta. 2013;426:127–38.
122. Leichtea AB, Dufourb JF, Fedlera GM. Potentials and pitfalls of clinical peptidomics and metabolomics. Swiss Med Wkly. 2013;143:w13801.

Chapter 8
Metabolomics and Milk: The Development of the Microbiota in Breastfed Infants

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Abstract Metabolomics provides a valuable strategy for describing and annotating the structures, compositions, and functions of mammalian milk. Detailed analyses of the complex components of milk have revealed an unexpected diversity of glycans consisting of oligosaccharides, glycoproteins, and glycolipids, all of which help shape the intestinal environment and in particular the intestinal microbiome of breastfed babies. Using complete and partial ensembles of glycan mixtures, the holistic principles of metabolomics analytics were leveraged for microbial screening studies. The complex glycans of human milk proved to be highly selective in their ability to support the growth of only a very rare group of enteric bacteria. These studies led to the conclusion that a signature achievement of breast milk is the development of a unique milk-oriented intestinal microbiota that results from a functional overlap of stereospecific glycan biosynthesis in maternal mammary epithelia with equally stereospecific glycosidase enzymes encoded within the genome of the commensal bacteria. Clinical evidence in support of that hypothesis has now been generated by the simultaneous administration and quantitation of the entire repertoire of glycans in the milk going in and the feces coming out of human infants. These platforms of systems biology combining separation technologies coupled to highly accurate and sensitive mass spectrometry with exhaustive library development and computational tools provide a model for success in understanding biological processes. Metabolomics is now extending that understanding of the infant

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microbiota and its phenotype to the role of complex glycans in the microbiota of all ages. The relentless selective pressure on the process of lactation within the mammary epithelial cell over millennia of evolution has been to nourish, protect, and support the survival of the mother infant pair. The principles that have emerged to nourish infants provide a guiding model for diet and health of all humans. The tools of metabolomics are proving successful in revealing the mechanisms behind milk's "genius."

Keywords Milk • Glycobiology • Microbiota • Infant • Oligosaccharides • Glycosidase • Glycan • Glycomics • Evolution • Lactation

8.1 Introduction

The mother and breastfed infant dyad provides a model to understand diet in its larger context. The scientific opportunities afforded by this model are transformative. Mechanistic insights to the targets of dietary inputs can be revealed by studying milk genomics, chemical composition, biological properties, and its diversity across mammals and temporally across lactation. The clinical comparison of exclusive breastfeeding against various formulas provides a powerful framework to study structurally defined diets and monitor the consequences of those structures on health and disease. In this respect, studies on milk and the lactating mammary gland provide unique insights into the mechanisms by which diet can act in protection and prevention. The mammary epithelial cell is a bioreactor for bioengineering complex structures and activities that act upon virtually all of the infant's processes: immunity, growth and development, metabolism, physiology, neurological development, and microbiota colonization and maturation. The components of milk execute on this biological blueprint using integrative and pleiotropic mechanisms that are difficult if not impossible to identify using the reductionist strategies of traditional biological chemistry. The comprehensive nature of the omic sciences and in particular metabolomics is changing the way milk is studied. Milk is the functional output of mammary metabolism, and it's a biofluid representing maternal genetics, health status, and environments.

A striking example of the principles and technologies of metabolomics applied to breast milk is in the area of glycomics; glycomics has revealed the diversity and abundance of glycans notably the free human milk oligosaccharides (HMOs) that are relatively unique to lactation and the glycosylated proteins, peptides, and lipids. Interestingly, glycans reach the large intestine and can ultimately be excreted and measured in the stool in healthy infants. This is a paradox if milk is considered a source of digestible nutrients for the infant. The resolution of this paradox is found in the fact that in most breastfed infants, these glycans disappear from stool, coincident with the appearance of a group of bacteria capable of digesting and utilizing them as growth substrates. The value of this relationship between maternal milk and

intestinal bacteria to shape infant postnatal development is still being revealed, ranging from protection from pathogenic bacteria, viruses, and toxins, to promoting neurological and immune systems and enhancing barrier function of intestinal epithelia. The highly selective digestibility of milk glycans act to shape the intestinal microbiome orchestrating its transition from the sterile uterus through the chaotic introduction of environmental bacteria at birth to a stable milk-oriented microbiome (MOM). This convergence of an entire metabolite class, glycans with the selective metabolism of bacteria and their interaction with the human host, is an opportunity to define metabolism as a dietary variable and study the structure-function relationships between diet, metabolism, and intestinal bacteria development. These studies provide broader principles for nourishing complex microbiota throughout life. At the core, comprehensive and accurate measurement of the structures and composition of milk's glycan metabolome is required.

Analytical chemistry has only recently brought the tools needed to measure glycobiology, the free oligosaccharides and glycans bound to proteins, peptides, and lipids in milk. Instrumentation is not sufficient; mass spectrometry must be coupled to separation technologies, enzyme biotechnologies, and bioinformatics tools to assemble all of the information into computationally accessible libraries. These technological advancements have led to the discovery that glycans are a central component of all mammalian milks, are variable across lactation and among women, and provide a wide diversity of structures to diverse functions [1–6].

8.2 Metabolomics and Human Milk

The simplifying elegance of the linear encoding of protein structure from DNA, RNA to protein sequence that is so empowering to biology from evolution to function is equally enabling to scientific research. Scientists have been wonderfully successful in annotating DNA-dependent biological processes because of the simplicity of linear sequence. Metabolism does not possess this simplicity. The dizzying complexity of metabolism must now be studied the old fashioned way, by measuring it. Scientists are beginning to assemble the technologies to measure metabolites in the accuracy, sensitivity, and comprehensiveness that reflect actual biology. A broad goal of food research is to build a linear understanding from the genetics of agricultural commodities, through their metabolism and thence composition as foods to the specific actions of those components on the metabolism and ultimately health of individual consumers (Fig. 8.1). Step one is to define the genetic and phenotypic basis of food composition through commodity growth and processing. The next step is to understand the principles by which human metabolism is controlled via these exogenous dietary components. This challenge will be particularly daunting in higher organisms due to the importance of structure to function. In higher organisms metabolites are distributed according to the cells, tissues, organs, and whole bodies. This structural dimension will demand that metabolites are measured as a function of the 3-dimensional structures of their immediate environment,

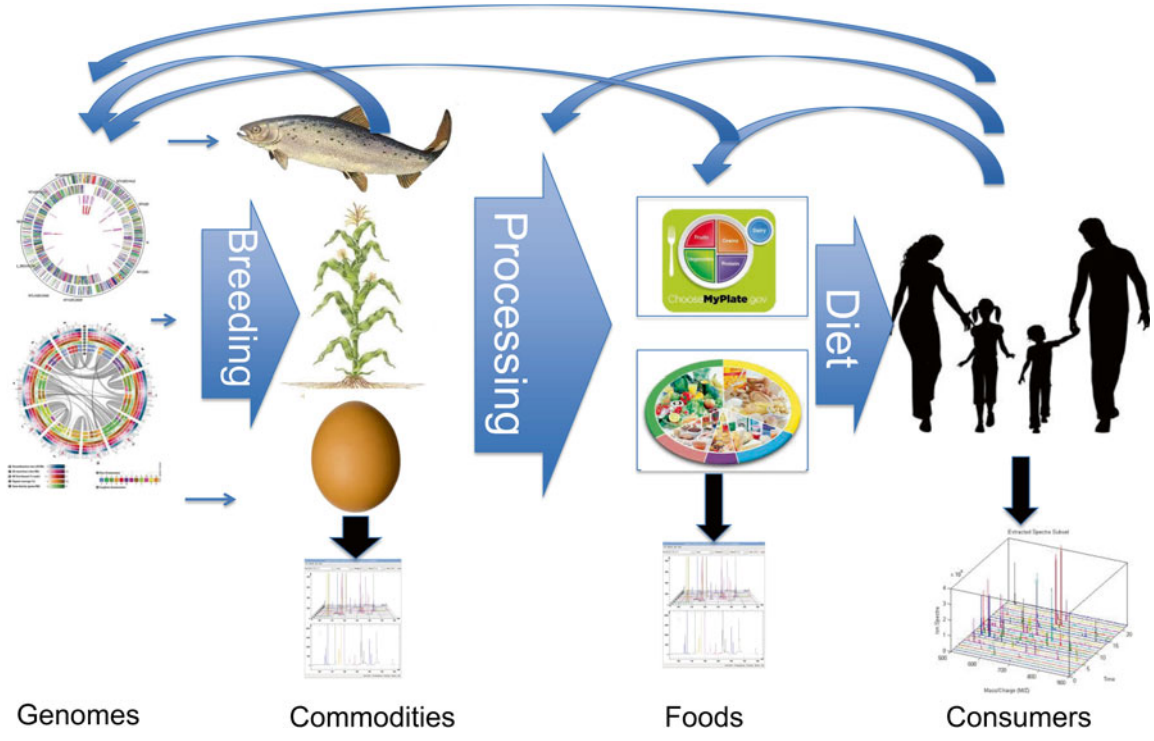

Fig. 8.1 The bidirectional flow of metabolic information through agriculture, food, and health. The genomics of agricultural commodities defines the metabolic machinery of farmed organisms which, once measured by metabolomics, can be guided by selective breeding and explicit genetic engineering. The metabolome compositions of harvested organisms that are the result of their metabolism are in turn alterable post-harvest by a wide variety of processing alternatives. The final compositions of chosen foods measured by food metabolomics define the overall diet compositions of individuals/families. Their diets influence their acute health which is in turn measureable by metabolomics of various body fluids. Departures from desired health trajectories detected by individual and population measurements then feed back into all of the agriculture and food input variables to alter diet composition and guide individuals to improved health

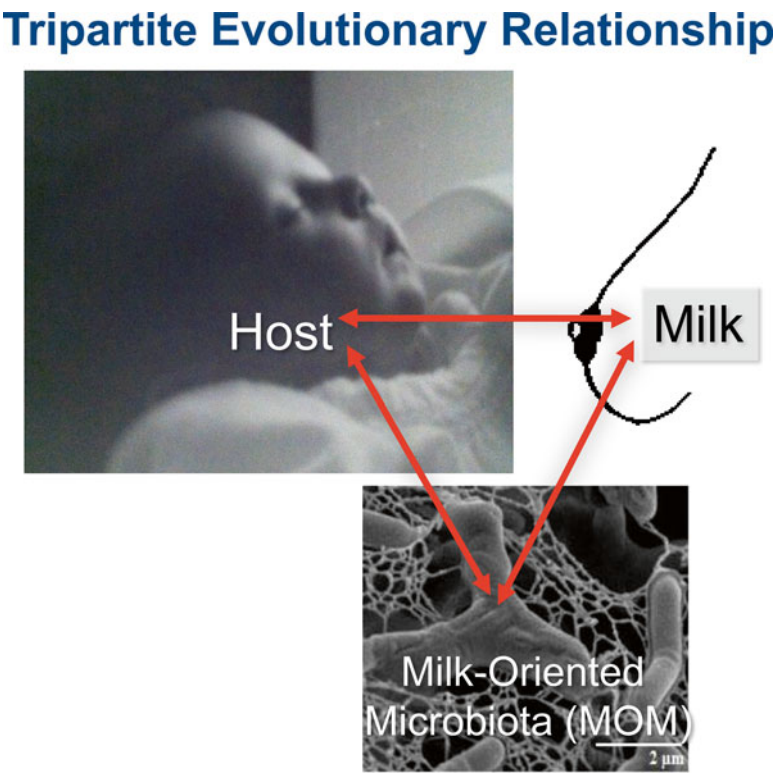

Fig. 8.2 The tripartite evolutionary relationship for mothers, infants, and their microbiota. The importance of the infant microbiota to its survival and genetic success is implied by the substantial investment of lactation in the control of this ecosystem. Understanding how and why lactation controls the infant microbiota provides scientific insights to microbial ecosystems in human intestine and beyond to all complex ecosystems in which food is a selective and discriminating input variable

techniques for which are only beginning to emerge. We have taken the approach of using the interaction between milk and microorganisms as a model for dietary metabolomics (Fig. 8.2). The principles emerging from this research provide scientists with examples of the interactions between diet and metabolism that may be instructive for higher animals.

8.3 Milk Glycomics

Glycans are the biopolymer class that has been largely ignored in spite of their abundance across the phylogenetic tree and through evolution [7]. Despite their importance in health and disease, they are not sequence encoded but rather the products of enzymatic metabolism. As a result of metabolic synthesis, the number of potential structures is massive contributing to the structural diversity seen in milk.

The complexity of a glycan is the result of a number of factors including branching, the number of different sugars, the stereospecific linkages of those sugars all leading to multiple isomers even for a single net mass to which must be added the dimension of conjugation: they are free or bound to proteins, peptides, or lipids again in a heterogeneous but stereospecific manner. Biology has apparently employed the combination of metabolism and structural diversity to leverage glycobiology into a variety of functions and most notably recognition. Perhaps not surprisingly, throughout evolution, the glycans on surfaces of cells are distinct both to "self" and to "foreign" organisms as the molecular basis for individuality. This dimension of diversity that is clearly of value to biological organisms is instead to scientists a nightmare precisely because there is no corresponding genetic template; every glycan must be explicitly analyzed to be identified. Research into the technologies and methodologies to routinely and comprehensively measure the glycans in biological and clinical samples is only now emerging, and as a result, a quantitative, metabolomics approach to glycobiology is becoming possible. Glycomics is defined as the systematic study of the total complement of sugars present in an organism in their free or protein and lipid-bound states [8, 9]. Researchers are now using glycomics to understand diet and health in the context of lactation, milk, and the role of complex glycans in various aspects of the development of the mammalian neonate.

8.3.1 Milk Oligosaccharides

The field of milk glycobiology is not new, and in fact many of the presumed roles of glycans throughout biology have been first discovered by examining milk. Nonetheless, the complexity of glycan structures has been a major hurdle to understanding specific structure – function relationships beyond binding assays. The oligosaccharides of human milk have become of particular interest in large part because they are an abundant (1–2 % w/v) and yet indigestible by the neonate. The biological challenges posed by this apparent paradox propelled a few key laboratories to pursue the analytical challenges of identifying and quantifying them. The revolution that they are bringing to analytical glycomics has been the result of innovations in separation science, enzyme biotechnology, mass spectrometry, automated library development, and computational toolsets.

8.3.2 Separation Science for Oligosaccharides

Liquid chromatography has been applied to glycan separation, yet neither normal nor reverse stationary phases provide sufficient separation power to successfully resolve glycan diversity in structure. Porous graphitized carbon (PGC) has been used as a uniquely selective stationary phase for bulk purification of glycans for

many years. Only recently has it been possible to formulate PGC as an HPLC stationary phase for the analysis of native oligosaccharides [10]. This combination of high-efficiency, high reproducibility, and stationary phase selectivity in an HPLC system has provided the first generation of separation platforms capable of the extensive separation of glycan isomers [11]. The future of oligosaccharide and glycan analyses will be extending this selectivity and efficiency to stereospecificity, quantitation, and throughput.

8.3.3 Stereospecificity of Glycan Structures

Glycans are nothing if not a forest of stereospecificity. The total number of possible structures imaginable is astronomical, yet because these glycan polymers are all the results of stereospecific enzymatic reactions, the actual number of structures found in biology is manageably finite and approachable by modern analytics and library systems. Nonetheless, precise oligosaccharide structures cannot be unequivocally identified on chiral separation phases and instead must still be determined the old fashioned way, by cleaving with stereospecific enzymes as an explicit step in the analysis [12]. The use of stereospecific enzymes will likely remain the most efficient means of assigning precise structures to oligosaccharides precisely because once a biological source is accurately described, it is not necessary to perform stereospecific analyses every subsequent analysis. The pragmatic proof of this principle has been demonstrated for the various milk oligosaccharides that have been analyzed for their stereospecificity [13].

The establishment of accurate metabolomics today cannot be achieved with online identification systems due to the complexity of the possible glycan structures relative to the separation platforms and mass spectrometry accuracy available. Instead effective methods for the structural identification of HMOs requires the construction of detailed libraries that map structures into analytical platforms taking advantage of the combinations of MS, tandem MS, and exoglycosidase digestion [12]. Neutral [14] and anionic milk oligosaccharides from humans [15] totaling 75 structural isomers have been annotated in this approach (Figs. 8.3, 8.4, and 8.5). Once begun, the library strategy has been extended to milks from other mammals to over 200 complete structures. This basic strategy is appropriate for the vast majority of applications to human milk biology since 50 structures represent 99 % of the total abundances of oligosaccharides in human milks [14–16].

Metabolomics is of relatively little utility if it only identifies structures and cannot quantify the absolute amounts of metabolites within biological samples. As a subset of the metabolome, glycan quantitation remains a major obstacle to metabolomics of glycobiology. Oligosaccharides lack discriminating chromophores for spectral detectors. As a result, oligosaccharides are often derivatized with absorbing labels including anthranilic acid (AA) or 2-aminobenzamide (AB) for quantitation [17]. To date the varying ionization efficiencies of glycans compromise the use of mass spectrometry of oligosaccharides. The alternative, using isotopically

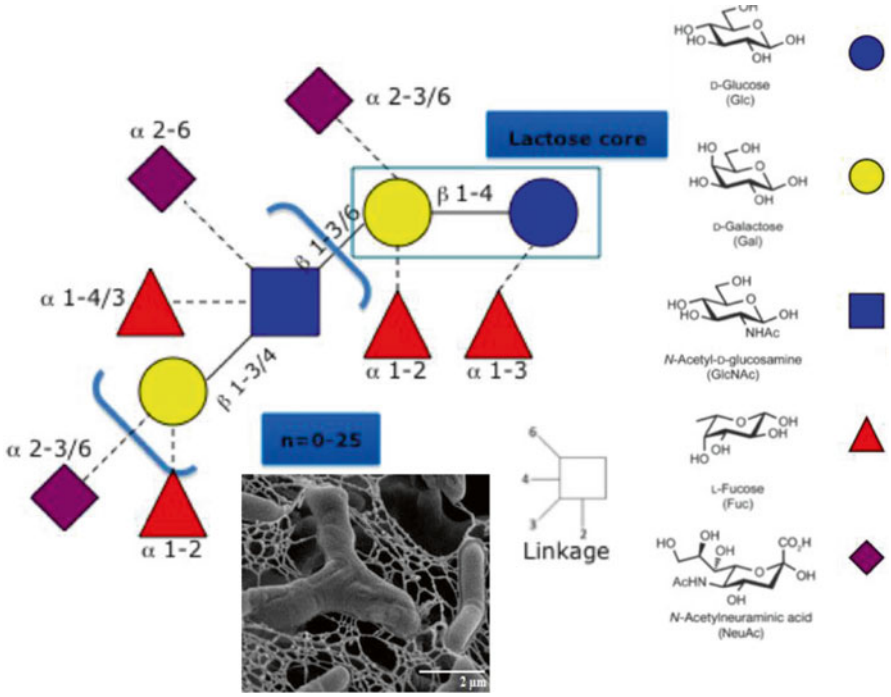

Fig. 8.3 Basic oligosaccharide structures in milk. The structural core of oligosaccharides is illustrated, the key sugar monomers that make up the oligosaccharide compositions and the possible stereospecific glycosidic bonds that are possible. *Bifidobacteria longum* subspecies *infantis* contains the genetic capability to synthesize ostensibly all of the enzymes necessary to cleave this array of complex glycans

enriched internal standards, while making MS highly accurate [18], requires the synthesis of the entire library of potential structures which is not currently available. Quantitation of metabolites remains the great challenge for the applications of metabolomics to its most relevant applications in health.

The presence of oligosaccharides has been confirmed in very early mammals and marsupials [19]. Thus, indigestible carbohydrate biopolymers provided a selective advantage throughout mammalian lactation. This advantage has apparently continued up to humans. Human milk contains greater concentration and diversity of soluble oligosaccharides than other mammalian milks [20] ranging from on average 7 g/L mature to 23 g/L in colostrum [21, 22]. These soluble oligosaccharides are composed of glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc), and sialic acid (NeuAc) monosaccharides. The basic biochemistry of oligosaccharide synthesis in the mammary gland is initiated by a lactose core of Gal and Glc catalyzed by β-galactotransferase in the presence of α-lactalbumin. The vast majority of HMO structures are based on this lactose core [23]. Lactose is then decorated by β1–3 linkage to lacto-N-biose (GlcNAc linked to Gal by β1–3 linkage) or by β1–6 linkage to N-acetyllactosamine (GlcNAc linked to Gal by β1–4 linkage).

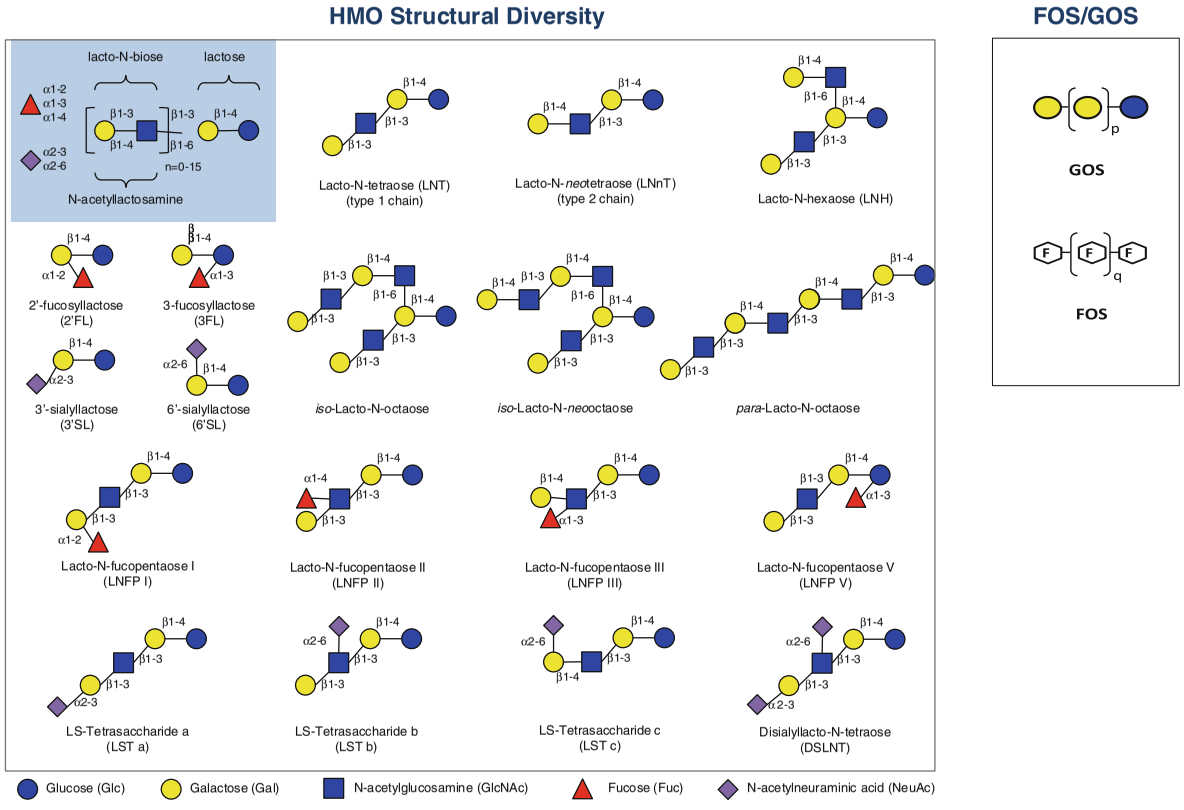

Fig. 8.4 Structural diversity of complex free glycans in human milk. The cartoon approximations of a subset of oligosaccharide structures in human milk attest to the diversity and yet specificity of this biomolecule class. Milk oligosaccharides as highly structured biomolecules are the result of multiple sugars joined by multiple stereospecific linkages

					Composition				Abundance (counts per second)		
	HMO	Structure	Mass	RT	Hex	Fuc	HexNAc	NeuAc	Milk	Feces	Urine
1	3'FL		490.190	1.31	2	1	0	0	NO	NO	NO
2	2'FL		490.190	11.69	2	1	0	0	NO	NO	NO
3	3'SL		635.227	22.330	2	0	0	1	58916	191249	370266
4	6'SL		635.227	15.231	2	0	0	1	30524	50386	NO
5	LOFT		636.248	14.470	2	2	0	0	NO	11395	11197
6	6'SLN		676.254	14.998	1	0	1	1	NO	59563	115044
7	3'SLN		676.254	23.061	1	0	1	1	NO	NO	227705
8	LNT		709.264	15.090	3	0	1	0	21650694	11349391	4096506
9	LNOT		709.264	15.200	3	0	1	0	410714	772639	83004
10	3'Sle		822.312	14.609	1	1	1	1	NO	NO	4368

Fig. 8.5 Extracted page of the table from the entire library of oligosaccharides in milks. Table illustrates the data possible to acquire with modern glycomics platforms including accurate mass, retention time, sugar subunits, and the abundance in milk and in various biofluids from infants

This growing chain structure can be further elongated with lacto-N-biose and N-acetyllactosamine by β1–3 and β1–6 linkages; Fuc connected with α1–2, α1–3, or α1–4 linkages and/or NeuAc residues attached by α2–3 or α2–6 linkages at the terminal positions (Fig. 8.3). The terminal sugars are particularly diagnostic of different mammalian milks, 60–80 % of HMOs are fucosylated, and 10–15 % of HMOs are sialylated in human milk [24].

8.4 Annotating the Functions of Human Milk Glycans

The structures of milk oligosaccharides have been selected for an unusual biological value: not to be consumed by infants. This selective pressure on lactation has been particularly intense since milk is the sole source of nourishment for mammalian infants. The genetics, synthesis, and structures of oligosaccharides in milk are unequivocally discoverable. However, the functions of oligosaccharides that were the basis for their emergence and persistence through evolution are not as easily discovered. The process of understanding their actions must first identify their actions, and then each of these actions must then be tested mechanistically as an actual valuable function in vivo. The most extensive approach to evaluating the actions and potential functions of oligosaccharides in human milk has been to establish the detailed support of the growth of specific strains of bacteria notably bifidobacteria [25, 26]. While the mechanisms and extent of microbial diversity in breastfed infants are still being actively documented, the basic observation that bifidobacterial species dominate the microbiota of breastfed infants around the world compared with formula-fed infants has been well established [27]. How an intestinal microbial ecosystem maintains a dominant and consistent bacterial population in the face of repeated and diverse inoculations with environmental microorganisms has been largely speculative until recently. Research has revealed the remarkable interaction between the stereospecific linkages defining the structures of milk oligosaccharides and the genetic repertoire of stereospecific glycosidases and solute-binding proteins that provide these bacteria a distinct competitive growth advantage within the intestine of the breastfed infant.

8.4.1 Screening Bacteria for Growth on Oligosaccharides

In an ongoing search for biological activities of these molecules to justify their abundance and diversity in milk, a prevailing hypothesis was that they are substrates for bacterial growth. However, no studies had yet documented that fact nor whether growth was selective among bacteria. Initial growth experiments in fact failed to demonstrate significant growth of bacteria when human milk oligosaccharides were the sole source of carbon in an otherwise supportive medium [25]. A series of subsequent experiments revealed that among gut-related bacteria tested (including *Lactobacillus*, *Clostridium*, *Eubacterium E. coli*, *Veillonella*, *Enterococcus* isolates), only *Bifidobacterium* and *Bacteriodes* species grew to high cell densities [28]. Growth on HMO was found in a select group of *B. bifidum* and *B. longum* subsp. *infantis* strains. In these same isolated growth conditions, even isolates of *B. longum* subsp. *longum* and *B. breve* showed poor growth, and other strains of *B. adolescentis* and *B. animales* were ostensibly unable to grow on HMO [29] (Fig. 8.6).

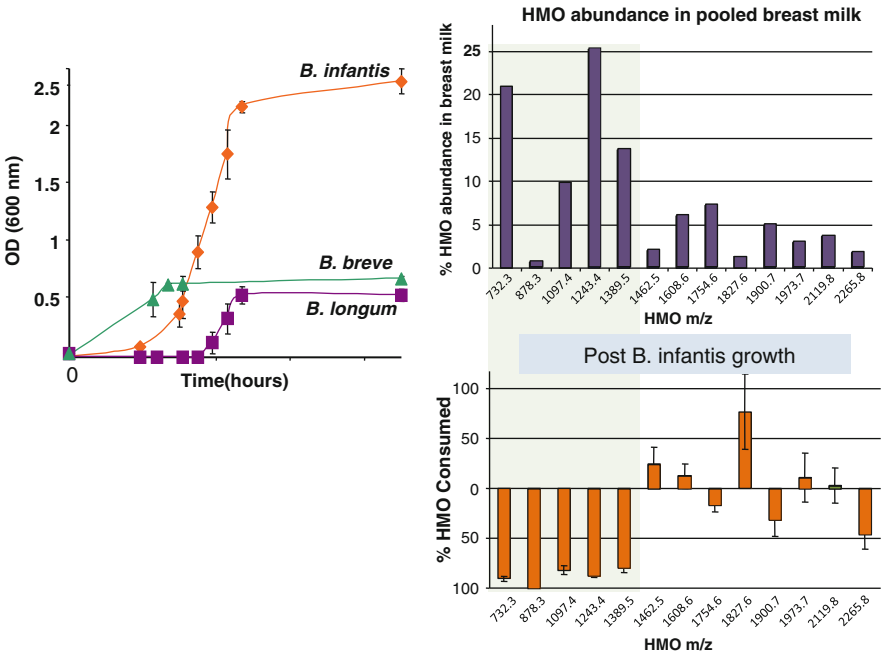

Fig. 8.6 Growth curves and human milk oligosaccharide consumption by *Bifidobacteria longum* subspecies *infantis*. The growth curves of isolated bacteria (indicated) on media containing only isolated milk oligosaccharides as carbon source of *B. longum*, *B. longum* subspecies *infantis*, and *B. breve* are shown, illustrating the conspicuously greater growth of *B. infantis*. The most abundant oligosaccharides in human milk are histogrammed in the figure on the *right* before and after consumption by *B. infantis* illustrating the complete consumption of the majority of oligosaccharides by this bacterium [25]

The complex mechanisms by which milk oligosaccharides guide bacterial growth within the ecosystem of the infant intestine have been elaborated in a series of microbial studies. Among the bifidobacteria that are able to consume HMO, different strategies are present to use HMO as a substrate. In isolated growth studies of HMO consumption, *B. longum* subsp. *infantis* ATCC15697 most efficiently consumed oligosaccharides seven sugars (DP) or below [25]. Oligosaccharides below ten sugars are the majority of species human milk [10]. Other bifidobacteria including *B. longum* subsp. *longum* DJO10A and *B. breve* ATCC15700 that grew slowly on pooled HMO were found to be consuming mostly a single, nonfucosylated/nonsialylated species, LNnT. LNnT is present in breast milk yet a small portion of the overall HMOs. *B. breve* did grow in culture on all the monomer constituents of HMO and thus if present within the gastrointestinal tract could grow on liberated monosaccharides.

The bacteria that were found to be capable of growing on HMO were analyzed for the presence of metabolic activities towards complex oligosaccharides including the key sialidase and fucosidase activities required to deconstruct complex glycan

structures. Among the strains examined, fucosidase activity was present in *B. longum* subsp. *infantis* and was only detected upon growth on HMO [25].

Distinct strategies for catalytic activity on complex biopolymer destruction are known among intestinal bacteria. The majority of intestinal bacteria secrete extracellular glycosidase enzymes that liberate free sugars that are then subsequently taken up by bacteria and metabolized. Select bifidobacteria use lacto-N-biosidase activity to break down oligosaccharides [30]. LNB is transported into *B. bifidum* via an ABC transporter and an associated LNB-specific solute-binding lipoprotein whereby it is further processed and fed into the central metabolic pathway [31].

The discovery that *B. longum* subsp. *infantis* ATCC15697 was uniquely capable of growing on human milk oligosaccharides led to an immediate project to sequence its genome. No prior experience prepared the investigators for the elegance of the genetic repertoire of this organism's sequence. *B. longum* subsp. *infantis* ATCC15697 has become the blueprint for understanding the genetic basis of glycan-specific growth and phenotype [32]. This specific strain possesses clusters of genes associated with its unique phenotype distributed in the genome into four loci. The most informative, HMO cluster 1 (Fig. 8.7), contains all the necessary glycosidases (sialidase, fucosidase, galactosidase, and hexosaminidase) and transporters necessary for importing and metabolizing HMO. Sequencing more isolates for HMO-related genomic architecture among *B. longum* subsp. *infantis* isolates provides a detailed genetic map of the mechanisms behind the vigorous growth of this clade on HMO.

The bacterial model of metabolomics illustrates the complexity of structure within metabolic pathways. Within the large HMO cluster (Fig. 8.7) are genes encoding an interesting group of extracellular solute-binding proteins (SBP; pfam 01547) demonstrated to bind oligosaccharides. These proteins provide two functions for the bacteria in their ecological niche of the breastfed infant intestine. These solute-binding proteins would tether the bacteria to glycans on the luminal side of the infant intestine and provide a net coverage of microbial binding sites thus blocking potential pathogens from the infant. Of more direct value to the bacterium, these solute-binding proteins would internalize free oligosaccharides directly infusing substrate into its endogenous metabolism. This substrate sequestering mechanism provides the *B. longum* subsp. *infantis* a unique foraging advantage in the overall microbial community. These solute-binding proteins also appear to be of singular advantage to the mammalian infant gut. A subset of these genes shows a pronounced evolutionary divergence from other SBP family 1 proteins in bifidobacteria [32]. The emergence of these genes is consistent with their functions as a mechanistic basis of symbiosis with humans through their interaction with milk oligosaccharides. The *B. longum* subsp. *infantis* genome has been shown to contain 21 family 1 SBP, more than most bifidobacteria.

The results of genomic analyses of bifidobacteria illustrate that HMO-related clusters are shared among all *B. longum* subsp. *infantis* isolates that have been examined to date, yet they are notably absent in other sequenced bifidobacteria, such as *B. longum* subsp. *longum* DJO10A [33] and *B. adolescentis* ATCC15703

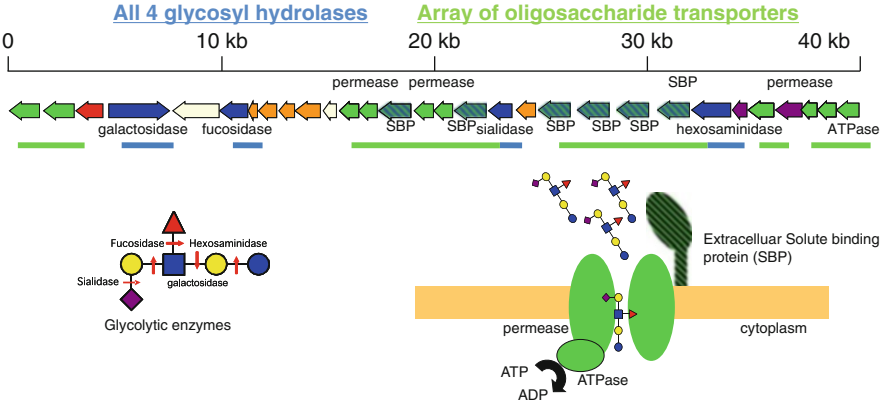

Fig. 8.7 Gene cluster 4 from the complete genome of *B. infantis* illustrating the location of the genes encoding glycosidases and oligosaccharide transporters [32]. The putative glycosidic linkages on which the glycosidic enzymes react are shown below left and the model of the cell membrane-bound solute-binding protein complex is shown *below right*

(GenBank AP009256), which grow weakly or not all (respectively) on HMO [29]. The elegance of microbial genetics is illustrated by the HMO-related gene set shared between ATCC15697 and DJO10A. This seven-gene operon is responsible for LNB metabolism further evidence of evolution selecting for metabolic substrate utilization [34]. Given that DJO10A is able to weakly grow on HMO and glycoprofiling indicated a small consumption of LNnT, it is tempting to speculate that this operon is linked to consumption of that particular HMO moiety.

While it is very hard to generalize the mechanisms of HMO catabolism across bifidobacteria because of strain heterogeneity and taxonomic confusion [35] within the genera, several important trends have emerged. The most common infant-borne bifidobacteria, *B. bifidum*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*, and *B. breve*, possess different modes for consumption of HMO (Fig. 8.6). *B. longum* subsp. *infantis* likely imports the lower molecular weight oligosaccharides via an army of dedicated ABC transporters. Once inside the cell, these oligosaccharides are catabolized by a complement of glycosidases prior to entry of the monosaccharides into central metabolic pathways. In contrast, *B. bifidum* exports fucosidases and lacto-N-biosidase to remove LNB from the HMO structure (leaving the free fucose and sialic acid behind) [48], internalize the free LNB and catabolize it intracellularly. Both *B. breve* and *B. longum* subsp. *longum* are able to consume free LNnT from an HMO pool, whereas *B. breve* can also grow on the various monomer constituents of HMO [48]. These different strategies suggest a possible mechanism for niche partitioning among the different bifidobacterial species within the developing infant gastrointestinal tract microbiota. Taken together this data provides a mechanism of action for glycan structures.

8.4.2 *Prebiotics for Infant Gut Bifidobacteria*

Breastfed infants that are colonized by protective strains of bifidobacteria benefit from the microbial activities within their developing intestine [36], which supports a valuable function in vivo for the emergence and persistence of glycans. Henry Tissier observed by microscopic analysis and culture techniques that the feces of breastfed infants were unique in containing a bacterial isolate he termed "*Bacillus bifidus communis*" [37]. For the 100 years since that initial identification, methodological techniques have wrestled to accurately type much less understand the specific bacteria within breastfed infants largely due to technical problems [38–40]. The challenges have become understandable in retrospect. Initial, culture-based studies failed to isolate significant proportions of bifidobacteria from infants, but these culture techniques failed to appreciate the oxygen sensitivity of infant bifidobacteria and were omitted. The major breakthroughs in DNA-based culture-independent methods should have identified bifidobacteria, yet unfortunately the 16s rDNA primers that are the basis of detection in these methods were not designed to effectively amplify bifidobacteria. Finally, both 16s rDNA surveys and metagenomic techniques that ostensibly sequence all DNA and again should have unequivocably identified bifidobacteria failed to appreciate the physical integrity of the double cell wall of bifidobacteria and the need to selectively handle the disruption of these barriers to DNA release for sequencing. These technical difficulties are now being resolved, and accurate measures of infant fecal microbiota are now available. With these techniques in place, studies are demonstrating very high proportions of specific strains of bifidobacteria in breastfed infants prior to transition to an adult microbiota [41]. The analyses of the breastfed intestinal track have revealed *Bifidobacterium longum* and *B. breve* with *B. bifidum* and *B. pseudocatenulatum* and *B. catenulatum* also present [42].

The basic concept that milk itself was influencing the microbial population was proposed by Gorgy and colleagues [43] on the basis of observations that *B. bifidum* (then termed *Lactobacillus bifidus*) grew on human milk fractions. The concept however implied that there was a single component responsible, the so-called *Bifidus* factor. Various studies since have demonstrated that human milk does indeed contain indigestible matter that since humans cannot break them down into digestible monomers would invariably reach the intestine [44–46]. The selectivity of growth promotion by bifidobacterial species growing on human milk oligosaccharides was first demonstrated in vitro by Ward et al. [47, 48]. A series of detailed studies have extended this initial observation demonstrating that only certain bifidobacterial species consume the majority of the stereospecific oligosaccharides of human milk [1, 25, 42]. Within specific strains, growth on oligosaccharides differed leading to the conclusion that *B. infantis* and select *B. breve* preferentially consume fucosylated and sialylated HMOs. These results indicate that bifidobacterial strains that grow well on specific glycan structures possess genetic adaptations for select growth on human milk in the infant intestine [32, 49].

The interaction between human milk oligosaccharides and bifidobacteria provides a unique opportunity to map the continuum of metabolites from a food, through the genetics of their disassembly by a "consumer" through the metabolic pathways that utilize them for growth. The genes in bifidobacteria that specifically bind and catabolize HMOs for energy have been identified, expressed, and verified for enzymatic activity [25, 28, 50–53]. The process of annotating these genes has demonstrated that different bifidobacterial species grow on HMO by distinct catalytic mechanisms. *B. infantis* possess a 43-kb gene cluster (termed HMO cluster I), encode for glycosyl hydrolases, and transport systems using a unique and highly efficient pathway to internalize and metabolize milk oligosaccharides [54, 55]. In contrast, *B. bifidum* is equipped with genes encoding a different set of catalytic activities toward HMO consumption. This strain exports fucosidases and a lacto-N-biosidase to hydrolyze lacto-N-biose from HMO structures which is in turn transported into the bacterium and metabolized [56].

The process of annotating the detailed mechanisms of the metabolism of human milk oligosaccharides by bifidobacteria has revealed consequences of that metabolism that were unanticipated. This group of metabolites causes a fundamental shift in the phenotype of the bacterium itself. Milk oligosaccharides trigger a specific HMO phenotype to *B. infantis*. In effect the bacterium shifts to a phenotypic state that is linked to its competitive success in establishing itself within the microbial ecosystem. The phenotype is also associated with interactions between the bacterium and the infant host. Chichlowski et al. [57] reported that the HMO-specific phenotype of *B. infantis* ATCC15697 on HMOs increases binding to intestinal epithelial cells in vitro. These studies suggest that the specific phenotype of bifidobacterial populations grown on human milk oligosaccharides as metabolites provides mechanisms to the organism supporting greater growth, microbiota persistence interactions with the host epithelium. This model of a metabolically distinct bacterial population induced by its "food" source is supported by in vivo administration of *B. infantis* to premature infants fed either formula or breast milk. The human breast milk-fed infants, when supplemented with *B. infantis*, had increases in fecal bifidobacteria and decreases in *γ-Proteobacteria* compared with the formula-fed group [58]. The ability of these specific bacteria to deconstruct HMOs that is encoded in their genome suggests the co-evolution of human lactation and specific commensal organisms. Thus, mothers are shaping the protective milk-oriented microbiota (MOM) of their infants through breast milk (Fig. 8.8) [53, 59]. This is one example of how milk glycans are being annotated.

8.5 A Vision for Metabolomics in the Future

The science of nutrition is faced with a daunting challenge: improving human health. The enabling principles of reductionist chemistry that were so effective in identifying essential nutrients are failing to address the more complex problems of non-communicative but diet-dependent diseases that are epidemic around the world.

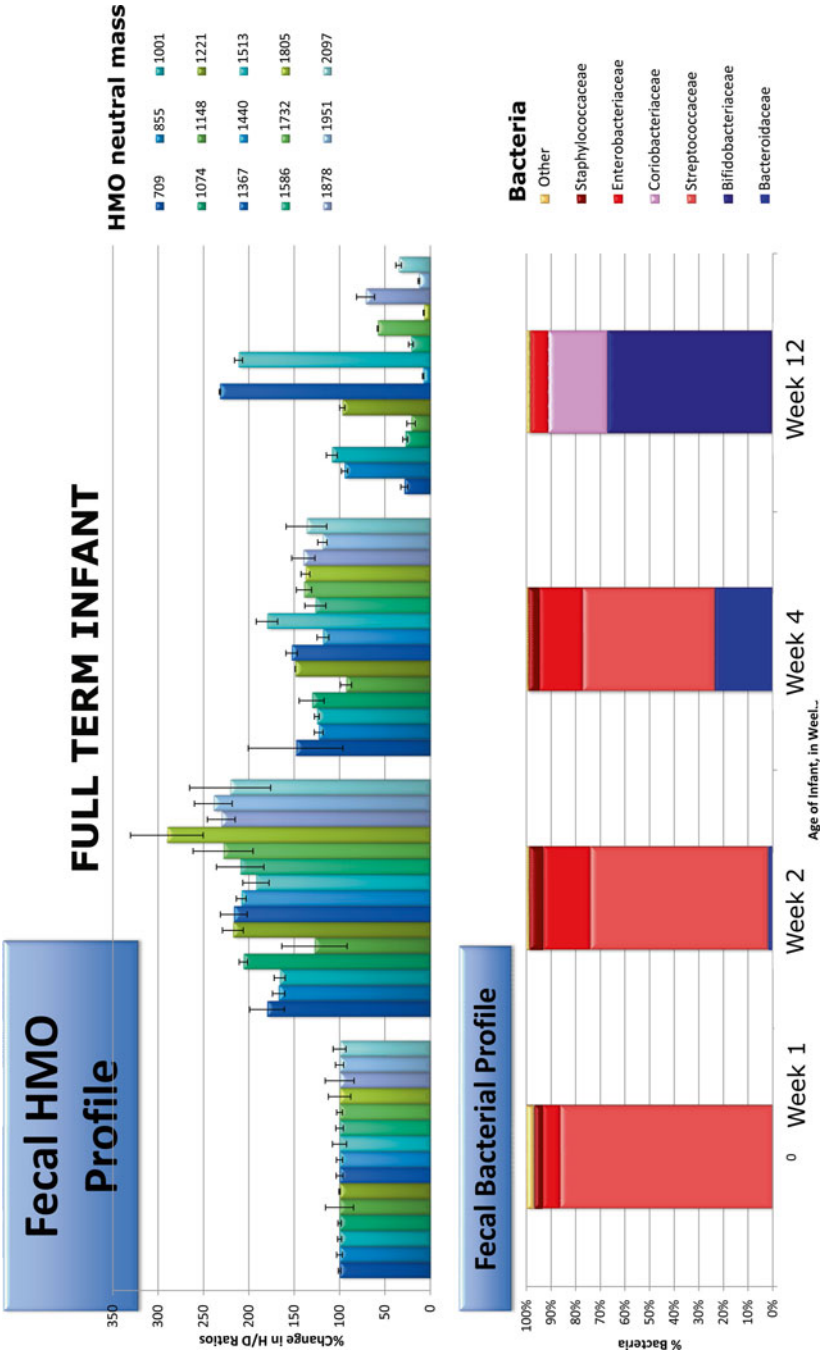

Fig. 8.8 Fecal analyses of a single infant at several time points through the first 3 months of life. *Above* are shown the oligosaccharides from human milk in the infant feces, normalized to week 1 in which all oligosaccharides from milk appeared unmodified in the feces. Successive weeks later time points illustrate the oligosaccharide abundances in the feces relative to week 1. *Below* is shown the bacteria profile of the same fecal samples through the first 3 months of life. (modified from de Leoz [60] unpublished)

Single molecules delivered to everyone in the population will not solve these problems. Nutrition as a field must lead the world into more integrative, biology-driven strategies that are not only quantitatively precise and mechanistically complete but mapped to actual foods and deployable as individual solutions. The first proofs of principle of such strategies are emerging from the, admittedly more narrowly defined, nourishment of breastfed infants. The toolsets of systems biology including genomics, metabolomics, proteomics, and glycomics have shown their power to interrogate the complexity of milk and reveal how it accomplishes an astonishingly successful biological feat, the colonization and development of the infant microbiota. Evolution clearly identified this to be an important target for mammalian health. Human mothers are nourishing the bacteria within their infants almost as enthusiastically as their infants. Yet, the strategy of nourishing the infant microbiota is a lesson for all of nutrition research. Rather than a single, simple molecule, the mammary gland produces an entire metabolome that includes: a spectrum of complex oligosaccharides and glycans that evade digestion by the infant and continue through to the infant's lower intestine. The complexity of glycans provides an intense selectivity that rewards only those bacteria genetically capable digesting the glycans and accessing their sugars. The combination of glycan complexity as available substrate and genetic capability as enzymatic specificity is a model for nutrition's microbiota research going forward. The knowledge assembled to date has begun the process of mapping the detailed mechanistic understanding of the functions of different microbial ecosystems in the infant. Key questions remain: How does a particular microbiota protect infants from pathogens and what are its weaknesses? How does a particular microbiota educate immunity in the face of the bewildering array of both pathogenic insults and completely benign passersby, and what are the causes of its failures? How does the microbiota prevent the massive activation of immunity and the anticipated increase in inflammation that would be expected from dropping a naïve, ostensibly sterile infant in the "real world," and can we apply these same principles to adults? How does a particular influence whole body metabolism and ensure appropriate food intake and suitable direction of fuels to peripheral tissues, and could these same mechanisms take visceral fat out of adults and put back "baby fat"? The successes of the first generation of metabolomics research tools applied to understand the interactions between mammary-produced oligosaccharides, and the infant microbiota are a glimpse of what this new field of biology can achieve.

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References

1. Smilowitz JT, O'Sullivan A, Barile D, German JB, Lönnerdal B, Slupsky CM. The human milk metabolome reveals diverse oligosaccharide profiles. J Nutr. 2013;143(11):1709–18.
2. Molinari CE, Casadio YS, Hartmann BT, Arthur PG, Hartmann PE. Longitudinal analysis of protein glycosylation and β-casein phosphorylation in term and preterm human milk during the first 2 months of lactation. Br J Nutr. 2012;1(1):1–11.
3. Froehlich J, Dodds E, Barboza M, McJimpsey E, Seipert R, Francis J, et al. Glycoprotein expression in human milk during lactation. J Agric Food Chem. 2010;58(10):6440–8.
4. Uchiyama S-i, Sekiguchi K, Akaishi M, Anan A, Maeda T, Izumi T. Characterization and chronological changes of preterm human milk gangliosides. Nutrition. 2011;27(10): 998–1001.
5. Liao Y, Alvarado R, Phinney B, Lönnerdal B. Proteomic characterization of human milk whey proteins during a twelve-month lactation period. J Proteome Res. 2011;10(4):1746–54.
6. Ruvoën-Clouet N, Le Pendu J, Mas E, Marionneau S, Guillon P, Lombardo D. Bile-salt-stimulated lipase and mucins from milk of 'secretor' mothers inhibit the binding of Norwalk virus capsids to their carbohydrate ligands. Biochem J. 2006;393:627–34.
7. Varki A. Sialic acids in human health and disease. Trends Mol Med. 2008;14(8):351–60. Epub 2008/07/09.
8. Aoki-Kinoshita KF. An introduction to bioinformatics for glycomics research. PLoS Comput Biol. 2008;4(5):e1000075.
9. Reinhold V, Zhang H, Hanneman A, Ashline D. Toward a platform for comprehensive glycan sequencing. Mol Cell Proteomics. 2013;12(4):866–73.
10. Ninonuevo MR, Perkins PD, Francis J, Lamotte LM, LoCascio RG, Freeman SL, et al. Daily variations in oligosaccharides of human milk determined by microfluidic chips and mass spectrometry. J Agric Food Chem. 2008;56(2):618–26. Epub 2007/12/20.
11. De Leoz ML, Wu S, Strum JS, Ninonuevo MR, Gaerlan SC, Mirmiran M, et al. A quantitative and comprehensive method to analyze human milk oligosaccharide structures in the urine and feces of infants. Anal Bioanal Chem. 2013;405(12):4089–105. Epub 2013/03/08.
12. Lebrilla CB. Is high throughput glycomics possible? Mass Spectrom (Tokyo). 2013;2(Spec Iss):S0016. doi:10.5702/massspectrometry.S0016. Epub 2013 Apr 15.
13. Sela DA, Garrido D, Lerno L, Wu S, Tan K, Eom HJ, et al. Bifidobacterium longum subsp. Infantis ATCC 15697 α-fucosidases are active on fucosylated human milk oligosaccharides. Appl Environ Microbiol. 2012;78(3):795–803.
14. Wu S, Tao N, German JB, Grimm R, Lebrilla CB. Development of an annotated library of neutral human milk oligosaccharides. J Proteome Res. 2010;9(8):4138–51.
15. Wu SA, Grimm R, German JB, Lebrilla CB. Annotation and structural analysis of sialylated human milk oligosaccharides. J Proteome Res. 2011;10(2):856–68.
16. Ninonuevo M, An H, Yin H, Killeen K, Grimm R, Ward R, German B, Lebrilla C. Electrophoresis. 2005;26:3641–9.
17. Leo F, Asakuma S, Fukuda K, Senda A, Urashima T. Determination of sialyl and neutral oligosaccharide levels in transition and mature milks of Samoan women, using anthranilic derivatization followed by reverse phase high performance liquid chromatography. Biosci Biotechnol Biochem. 2010;74(2):298–303.
18. Ninonuevo MR, Ward RE, LoCascio RG, German JB, Freeman SL, Barboza M, et al. Methods for the quantitation of human milk oligosaccharides in bacterial fermentation by mass spectrometry. Anal Biochem. 2007;361(1):15–23.
19. Messer E, Trifonoff W, Stern JG, Collins JH. Bradbury structure of a marsupial-mild trisaccharide. Carbohydr Res. 1980;83:327–34.
20. Tao N, Wu S, Kim J, An HJ, Hinde K, Power ML, et al. Evolutionary glycomics: characterization of milk oligosaccharides in primates. J Proteome Res. 2011;10(4):1548–57. Epub 2011/01/11.

21. Coppa GV, Gabrielli O, Pierani P, Catassi C, Carlucci A, Giorgi PL. Changes in carbohydrate composition in human milk over 4 months of lactation. Pediatrics. 1993;91(3):637–41.
22. Gabrielli O, Zampini L, Galeazzi T, Padella L, Santoro L, Peila C, et al. Preterm milk oligosaccharides during the first month of lactation. Pediatrics. 2011;128(6):e1520–31.
23. Kunz C, Rudloff S, Baier W, Klein N, Strobel S. Oligosaccharides in human milk: structural, functional, and metabolic aspects. Annu Rev Nutr. 2000;20:699–722.
24. Ninonuevo MR, Park Y, Yin H, Zhang J, Ward RE, Clowers BH, et al. A strategy for annotating the human milk glycome. J Agric Food Chem. 2006;54(20):7471–80. Epub 2006/09/28.
25. LoCascio RG, Ninonuevo MR, Freeman SL, Sela DA, Grimm R, Lebrilla CB, et al. Glycoprofiling of bifidobacterial consumption of human milk oligosaccharides demonstrates strain specific, preferential consumption of small chain glycans secreted in early human lactation. J Agric Food Chem. 2007;55(22):8914–9. Epub 2007/10/06.
26. Sela DA, Mills DA. Nursing our microbiota: molecular linkages between bifidobacteria and milk oligosaccharides. Trends Microbiol. 2010;18(7):298–307. Epub 2010/04/23.
27. Roger LC, Costabile A, Holland DT, Hoyles L, McCartney AL. Examination of faecal Bifidobacterium populations in breast-and formula-fed infants during the first 18 months of life. Microbiology. 2010;156(11):3329–41.
28. Marcobal A, Barboza M, Sonnenburg ED, Pudlo N, Martens EC, Desai P, et al. Bacteroides in the infant gut consume milk oligosaccharides via mucus-utilization pathways. Cell Host Microbe. 2011;10(5):507–14. Epub 2011/11/01.
29. Locascio RG, Ninonuevo MR, Kronewitter SR, Freeman SL, German JB, Lebrilla CB, et al. A versatile and scalable strategy for glycoprofiling bifidobacterial consumption of human milk oligosaccharides. Microb Biotechnol. 2009;2(3):333–42. Epub 2009/05/01.
30. Wada J, et al. Bifidobacterium bifidum lacto-N-biosidase, a critical enzyme for the degradation of human milk oligosaccharides with a type 1 structure. Appl Environ Microbiol. 2008;74(13): 3996–4004.
31. Suzuki R, et al. Structural and thermodynamic analyses of solute-binding protein from Bifidobacterium longum specific for core 1 disaccharide and lacto-N-biose I. J Biol Chem. 2008;283(19):13165–73.
32. Sela D, Chapman J, Adeuya A, Kim J, Chen F, Whitehead T, et al. The genome sequence of Bifidobacterium longum subsp. infantis reveals adaptations for milk utilization within the infant microbiome. Proc Natl Acad Sci. 2008;105(48):18964.
33. Lee JH, et al. Comparative genomic analysis of the gut bacterium Bifidobacterium longum reveals loci susceptible to deletion during pure culture growth. BMC Genomics. 2008;9:247.
34. Mattarelli P, Bonaparte C, Pot B, Biavati B. Proposal to reclassify the three biotypes of Bifidobacterium longum as three subspecies: Bifidobacterium longum subsp. longum subsp. nov., Bifidobacterium longum subsp. infantis comb. nov. and Bifidobacterium longum subsp. suis comb. nov. Int J Syst Evol Microbiol. 2008;58(Pt 4):767–72.
35. Falk P, Roth KA, Boren T, Westblom TU, Gordon JI, Normark S. Proc Natl Acad Sci U S A. 1993;90:2035.
36. Du X-L, Edelstein D, Rossetti L, Fantus IG, Goldberg H, Ziyadeh F, et al. Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. Proc Natl Acad Sci. 2000;97(22):12222–6.
37. Tissier H. Recherches sur la flore intestinale des nourrissons: état normal et pathologique: G. Carré et C. Naud; 1900.
38. Sakata S, Tonooka T, Ishizeki S, Takada M, Sakamoto M, Fukuyama M, et al. Culture-independent analysis of fecal microbiota in infants, with special reference to Bifidobacterium species. FEMS Microbiol Lett. 2005;243(2):417–23.
39. Roger LC, McCartney AL. Longitudinal investigation of the faecal microbiota of healthy full-term infants using fluorescence in situ hybridization and denaturing gradient gel electrophoresis. Microbiology. 2010;156(11):3317–28.
40. Harmsen HJ, Wildeboer-Veloo AC, Raangs GC, Wagendorp AA, Klijn N, Bindels JG, et al. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. J Pediatr Gastroenterol Nutr. 2000;30(1):61–7.

41. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. Nature. 2012;486:222–7.
42. Turroni F, Bottacini F, Foroni E, Mulder I, Kim JH, Zomer A, et al. Genome analysis of Bifidobacterium bifidum PRL2010 reveals metabolic pathways for host-derived glycan foraging. Proc Natl Acad Sci U S A. 2010;107(45):19514–9. Epub 2010/10/27.
43. György P, Norris RF, Rose CS. Bifidus factor. I. A variant of *Lactobacillus bifidus* requiring a special growth factor. Arch Biochem Biophys. 1954;48(1):193–201.
44. Coppa G, Pierani P, Zampini L, Bruni S, Carloni I, Gabrielli O. Characterization of oligosaccharides in milk and feces of breast-fed infants by high-performance anion-exchange chromatography. Bioactive components of human milk. Springer; 2001. p. 307–14. ISBN 978-1-4613-5521-2.
45. Chaturvedi P, Warren CD, Buescher CR, Pickering LK, Newburg DS. Survival of human milk oligosaccharides in the intestine of infants. Bioactive components of human milk. Springer; 2001. p. 315–23. ISBN 978-1-4613-5521-2.
46. Engfer MB, Stahl B, Finke B, Sawatzki G, Daniel H. Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. Am J Clin Nutr. 2000;71(6):1589–96.
47. Ward RE, Ninonuevo M, Mills DA, Lebrilla CB, German JB. In vitro fermentation of breast milk oligosaccharides by Bifidobacterium infantis and Lactobacillus gasseri. Appl Environ Microbiol. 2006;72(6):4497–9.
48. Ward RE, Ninonuevo M, Mills DA, Lebrilla CB, German JB. In vitro fermentability of human milk oligosaccharides by several strains of bifidobacteria. Mol Nutr Food Res. 2007;51(11):1398–405.
49. Ruiz-Moyano S, Totten SM, Garrido DA, Smilowitz JT, German JB, Lebrilla CB, et al. Variation in consumption of human milk oligosaccharides by infant gut-associated strains of bifidobacterium breve. Appl Environ Microbiol. 2013;79(19):6040–9. Epub 2013/07/31.
50. Garrido D, Nwosu C, Ruiz-Moyano S, Aldredge D, German JB, Lebrilla CB, et al. Endo-beta-N-acetylglucosaminidases from infant gut-associated bifidobacteria release complex N-glycans from human milk glycoproteins. Mol Cell Proteomics. 2012;11(9):775–85. Epub 2012/06/30.
51. Garrido D, Ruiz-Moyano S, Jimenez-Espinoza R, Eom HJ, Block DE, Mills DA. Utilization of galactooligosaccharides by Bifidobacterium longum subsp. infantis isolates. Food Microbiol. 2013;33(2):262–70. Epub 2012/12/04.
52. Garrido D, Ruiz-Moyano S, Mills DA. Release and utilization of N-acetyl-D-glucosamine from human milk oligosaccharides by Bifidobacterium longum subsp. infantis. Anaerobe. 2012;18(4):430–5. Epub 2012/05/15.
53. Zivkovic AM, Lewis ZT, German JB, Mills DA. Establishment of a milk-oriented microbiota (MOM) in early life: how babies meet their MOMs. Funct Food Rev. 2013;5:3–12.
54. Garrido D, Dallas DC, Mills DA. Consumption of human milk glycoconjugates by infant-associated bifidobacteria: mechanisms and implications. Microbiology. 2013;159(Pt 4):649–64. Epub 2013/03/06.
55. Garrido D, Kim JH, German JB, Raybould HE, Mills DA. Oligosaccharide binding proteins from Bifidobacterium longum subsp. infantis reveal a preference for host glycans. PLoS One. 2011;6(3):e17315.
56. Kitaoka M. Bifidobacterial enzymes involved in the metabolism of human milk oligosaccharides. Adv Nutr: Int Rev J. 2012;3(3):422S–9S.
57. Chichlowski M, De Lartigue G, German JB, Raybould HE, Mills DA. Bifidobacteria isolated from infants and cultured on human milk oligosaccharides affect intestinal epithelial function. J Pediatr Gastroenterol Nutr. 2012;55(3):321–7. Epub 2012/03/03.
58. Underwood MA, Kalanetra KM, Bokulich NA, Lewis ZT, Mirmiran M, Tancredi DJ, et al. A comparison of two probiotic strains of bifidobacteria in premature infants. J Pediatr. 2013;163(6):1585–91.e9. doi:10.1016/j.jpeds.2013.07.017. Epub 2013 Aug 29.
59. Bode L, Jantscher-Krenn E. Structure-function relationships of human milk oligosaccharides. Adv Nutr: Int Rev J. 2012;3(3):383S–91.
60. De Leoz ML, Kalanetra K, Bokulich N, Strum J, Underwood M, German J, Mills D, Lebrilla C. Determination of human milk glycomics and gut microbial genomics in infant feces shows correlation between lactation and development of gut microbiota. (2014 unpublished)

Chapter 9
Metabonomics and Gut Microbial Paradigm in Healthy Aging

Elena Biagi, Marco Candela, François-Pierre Martin, Sebastiano Collino, Claudio Franceschi, and Patrizia Brigidi

Abstract Metabonomics, aiming to characterize the pattern of low molecular weight metabolites participating in metabolic pathways, is a valuable tool to detect changes in metabolic regulation and subsequently link them to the health outcome, emerging as a powerful technology to capture the complexity of human aging. The growing interest of the research community in this field is demonstrated by a large number of recently published researches, here summarized, that aim to associate complex metabolic regulations with age-related biological processes. The involvement of the gut microbiota, our "forgotten organ" with its impressive metabolic capability, is a natural extension of this interest, even if still largely unexplored. In this chapter, we aim to summarize the potential of metabonomics in exploring the impact of the combined metabolism of human host and gut microbiota on aging, as well as on the health outcome of age-related processes and the probability to attain longevity.

Keywords Aging • Intestinal microbiota • Probiotics • Longevity • Metabonomics • Gut–brain axis • Nutrition aging • Biomarker • Short chain fatty acids

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9.1 Metabonomics in Healthy Aging and Brain Aging Research

The increase in the aging population and incidences of chronic diseases raises new challenges for global public healthcare in which preventive medicine approaches, particularly by the means of optimal nutrition, will be crucial. Aging can commonly be characterized as a progressive, generalized impairment of biological functions resulting in an increased vulnerability to environmental challenge and a higher risk of disease and death [1]. Cross talk can occur between multiple physiological systems, i.e., cognitive, metabolic, and gut systems, which influence the immune system [2]. Environmental factors such as lifestyle choices as well as genetic factors all contribute to healthy aging. Among these factors, the environment is the most easily modifiable. Understanding the physiology of aging is of tremendous importance to allow populations to grow old disease-free and with a good quality of life. In this respect, it is important to understand the natural aging process and to elucidate where lifestyle and/or dietary interventions can have an impact. Despite the enormous complexity of the aging process, a small number of basic molecular mechanisms underpin the aging process, including a set of highly conserved mechanisms. One of the key mechanisms is inflammation as a typical feature of the aging process is the development of a chronic, low-grade inflammatory status named "inflamm-aging" [3–5], and this condition has emerged as critical in the pathogenesis of major age-related chronic diseases such as atherosclerosis, type 2 diabetes, and neurodegeneration. Inflamm-aging plays a pivotal role in the most important geriatric conditions, such as sarcopenia, frailty, and disability, thus contributing to elderly mortality [5]. Interestingly, a variety of tissues (adipose tissue, muscle), organs (brain, liver), systems (immune system), and ecosystems (gut microbiota) of the body can contribute to the onset and progression of such a systemic inflammatory state [6], by increasing the production of a number of proinflammatory mediators or lowering that of the anti-inflammatory ones, thus tilting the equilibrium toward inflammation [7].

Physiological, genetic, and environmental changes can cause modifications in existing homeostatic conditions, which are ultimately reflected in the metabolic composition of the different biological compartments. Metabonomics attempts therefore to quantitatively profile small molecules endogenously and exogenously present in a complex biological system, relying on the analysis of fluids (blood, urine), tissue biopsies, or stools. Metabonomics is therefore a very powerful tool for capturing the complexity of the aging process. Mass spectrometry (MS) and nuclear magnetic resonance (^{1}H-NMR) spectroscopy are the most commonly used analytical tools to obtain profiles. Imaging techniques and flux analysis using stable isotopes are parallel technologies to obtain metabolite information. Multivariate statistical and bioinformatics techniques are ultimately used for data mining the complex metabolic profiles which encapsulate information on genetics, environmental factors, gut microbiota activity, and lifestyle and food habits. This combined strategy sustains the complex process of identifying emerging biomarkers

indicative of the individual response to specific physiological factors and/or nutritional/physical interventions.

The age-related chronic inflammation is believed to be pathogenic, especially with regard to its contribution to frailty and degenerative disorders. In particular, the frailty syndrome is increasingly being considered as a key risk indicator of adverse health outcomes [8]. Frailty is characterized by a decline in the functional reserve with several alterations in diverse physiological systems, including altered energy metabolism, skeletal muscle mass and strength (also termed sarcopenia), and hormonal and inflammatory functions [8]. A disturbance of the balance between the synthesis and breakdown of muscle proteins can lead to the loss of muscle mass. In addition, elderly may be also prone to be resistant to anabolic stimuli which is likely a key factor in the loss of skeletal muscle mass with aging. Such a perturbation of muscle metabolism with aging has been proposed to play a role in the development of sarcopenia [9], which ultimately alters walking performance and physical endurance and results in a perception of exhaustion and fatigue. It has been therefore foreseen that well-adapted exercise training and nutritional management programs should be an effective means of counteracting muscle weakness and physical frailty in elderly [10]. In particular, combining resistance exercise with essential amino acid supplementation restores the muscle protein anabolic response in older men [8]. Future research is needed to determine whether these novel interventions will be successful in preventing sarcopenia and improving muscle strength and function in older adults, and in this system, biology approaches will help not only personalize the management program but may also help understand specific requirements for nonresponders. For instance, metabolic profiling was successfully applied to blood profiling in the frame of a human exercise study focused on the effects of beverages containing glucose, galactose, or fructose taken after exercise and throughout a recovery period [11]. Others have shown novel perspectives in monitoring the metabolic significance of long-term strength and endurance training [12]. These applications showed how we could further our understanding on individual predisposition and inferences on nutritional/exercise response by monitoring energy (glucose, lipid, amino acid) and oxidative stress metabolism [12].

Very recently, a series of metabonomics studies were expanded to report for the first time the metabolic phenotype of longevity. Plasma metabolic phenotype of three long-lived murine models was characterized by NMR metabonomics. Here a panel of metabolic differences were generated for each model, 30 % dietary restricted, insulin receptor substrate 1 null (Irs1−/−), and Ames dwarf (Prop1df/df), relative to their controls, and, subsequently, the three long-lived models were compared to one another. Concentrations of mobile very low-density lipoproteins, trimethylamine, and choline were significantly decreased in the plasma of all three models. Such comparative approach suggests that the metabolic networks underlying lifespan extension are not exactly the same for each model of longevity and are consistent with multifactorial control of the aging process [13].

As centenarians well represent the model of successful and healthy aging [14], there are many important implications in revealing the underlying molecular mechanisms behind such acquired longevity. Centenarians avoid or delayed the major

inflammation-driven age-related diseases, such as cardiovascular diseases, diabetes mellitus, Alzheimer disease (AD), and cancer [4, 7]. A NMR-/MS-based metabonomics and targeted lipidomics approaches were applied in a representative aging cohort of centenarians (mean age 100.9 years), elderly (mean age 70 years, including offspring of centenarians and their age-matched controls), and young individuals (mean age 31 years) to reveal the molecular footprints of aging and longevity. With increasing age, targeted MS profiling of blood serum displayed a marked decrease in tryptophan concentration, while unique alterations in specific glycerophospholipids and sphingolipids were seen in the longevity phenotype. Untargeted metabonomics profiling of urine revealed that the longevity process is marked by changes in gut microbial metabolites, as displayed by increase urinary excretions of phenylacetylglutamine, p-cresol sulfate, and 2-hydroxybenzoate. Moreover, centenarian offsprings, who are reported to have delay in age-related diseases, have a distinct serum metabolic phenotype from siblings of non-long-living parents, with changes in amino acids (serine, phenylalanine) and lysophosphatidylcholines. Overall, these results describe different biological processes associated to aging and longevity, including differential lipid mediator networks and increased gut microbiota dysbiosis [15]. Additionally, an investigation on specific lipids associated with familial longevity in females was explored by Gonzalez-Covarrubias et al. in the plasma lipidome by measuring 128 lipid species in 1,526 offspring of nonagenarians (59 years ± 6.6) and 675 (59 years ± 7.4) controls from the Leiden Longevity Study [16]. Here in women 19 lipid species associated with familial longevity with ether phosphocholine and sphingomyelin species are identified as candidate longevity markers. While this population reflects a different cohort with plausible differences in lifestyle and dietary habits, common to the previous study, the authors postulated that lipid signatures in plasma lipidome of female individuals could suggest a better antioxidant capacity and lower lipid peroxidation capabilities with probable effects on the longevity process. The contrast between offspring of nonagenarian siblings that express the propensity for longevity and their partners was also found to be reflected by cellular characteristics in vitro. In dermal fibroblast strains from offspring, oxidative stress induced less reactive oxygen species, less senescence, more apoptosis, and slower growth speed when compared to strains from the partners of the offspring [17]. Aiming to find differences in cellular metabolism in vitro between these fibroblast strains, cell culture supernatants collected at 24 h and 5 days were analyzed using ^{1}H nuclear magnetic resonance [18]. Between 24 h and 5 days of incubation, supernatants of all fibroblast strains showed decreased levels of glucose, pyruvate, alanine–glutamine, valine, leucine, isoleucine, serine, and lysine and increased levels of glutamine, alanine, lactate, and pyroglutamic acid. The alanine–glutamine and glucose consumption were higher for fibroblast strains derived from offspring when compared to strains of their partners. Also, the production of glutamine, alanine, lactate, and pyroglutamate was found to be higher for fibroblast strains derived from offspring.

Aging is characterized by a common development of physical and mental disorders that implies metabolic decline with loss of hepatic, renal, coronary, or cardiac function, with increased risk of suffering cancer, inflammatory, cardiovascular, and

neurodegenerative diseases. Among these factors, AD is the most common dementia among all the clinically recognized dementias in the aging population. Although the cause is not known, there are profound biochemical alterations in multiple pathways in the AD brain including changes in amyloid beta-protein metabolism, tau phosphorylation, membrane lipid deregulation, and synaptic neurotransmission [19]. Lipids are key regulators of brain function and have been increasingly implicated in neurodegenerative disorders including AD. Han et al. analyzed plasma from 26 AD patients and 26 cognitively normal controls in a nontargeted approach using multidimensional mass spectrometry-based shotgun lipidomics [20]. Here a significant disruption of plasma sphingolipidome was detected in AD, with long aliphatic chains sphingomyelin species, 22 and 24 carbon atoms, significantly lower in AD, compared to controls, and ceramide species (N16:0 and N21:0) significantly higher in AD. Sato et al. [21] applied lipidomics to focus on steroid-related compounds to identify novel AD plasma biomarkers with significant correlation with mini-mental state examination scores. A systems-based approach was also employed to determine the lipidome changes in brain tissues affected by AD. Using liquid chromatography–mass spectrometry, the profile extracts from the prefrontal cortex, entorhinal cortex, and cerebellum of late-onset AD were generated by Chan et al. [22]. Here 26 different lipid subclasses including lysophospholipids, glycerophospholipids, sphingolipids, glycerolipids, and sterols were studied. While the cerebellum lacked major alterations in lipid composition, an elevation of a signaling pool of diacylglycerols as well as sphingolipids in the prefrontal cortex of AD patients was found. Furthermore, the diseased entorhinal cortex showed specific enrichment of lysobisphosphatidic acid, sphingomyelin, the ganglioside GM3, and cholesterol esters, suggesting common pathogenic mechanisms associated with endolysosomal storage disorders.

The development of systems biology approaches and the new generation of biomarker patterns will provide the opportunity to associate complex metabolic regulations with key aging biological processes. This will subsequently lead to the development of systems mechanistic hypotheses that could be targeted with new nutritional and therapeutic personalized concepts aimed at healthy aging.

9.2 The Gut Microbiota in Human Aging

The gastrointestinal tract (GIT) is one of the most essential interfaces of mammalian organism interacting with nutrients, exogenous compounds, and gut microbiota, and its condition is influenced by the complex interplay between these environmental factors and host genetic elements. Along the GIT, the gut microbiota is a key determinant of the gut functional ecology and metabolic homeostasis, through fine interactions with regulatory processes involved in the absorption, digestion, metabolism, and excretion of dietary nutrients as well as barrier integrity, motility, and mucosal immunity [23, 24]. The GIT ecosystem hosts a diverse and highly evolved microbial community composed of hundreds of different microbial species [25, 26], which can be viewed as a metabolically adaptable,

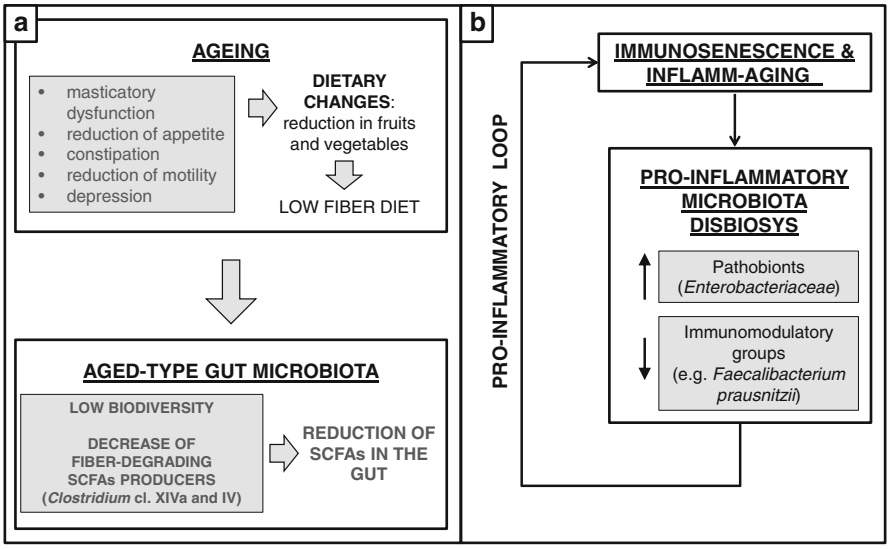

Fig. 9.1 Aging and gut microbiota. (**a**) Dietary habits usually change during the aging process, because of several age-related physiological and environmental factors; the resulting low-fiber diet is among the causes of reduction of gut microbiota diversity and changes in its phylogenetic composition and, consequently, reduction of some gut microbiota functions, i.e., production of SCFAs. (**b**) Immunosenescence and inflamm-aging nurture the overgrowth of pathobionts in the gut ecosystem to the detriment of immunomodulatory bacterial groups; the resulting gut microbiota dysbiosis promotes inflammation itself in a sort of self-sustaining loop

rapidly renewable, and metabolically flexible ecosystem varying in addition with the host's age, diet, and health status [27]. For instance, bacterial colonization of the gut by commensal bacteria has been shown to alter intestinal physiology of the host by modulation of genes and metabolic processes implicated in nutrient absorption, mucosal defenses, and xenobiotic metabolism.

Age-related physiological changes in the GIT, as well as modification in lifestyle, nutritional behavior, and functionality of the host immune system, inevitably affect the gut microbiota (Fig. 9.1a).

The most informative studies about the age-related modifications of the gut microbiota structure and composition have been conducted over the last 10 years, since when 16S rRNA gene-based molecular characterization technologies have been made available [28–30]. Briefly, studies are in general agreement in reporting a large interindividual variability, as well as a reduced biodiversity, and compromised stability of the intestinal microbiota in older subjects with respect to younger individuals [31–35]. An age-related increase in facultative anaerobes, including streptococci, staphylococci, enterococci, and enterobacteria has also been commonly reported in elderly [32, 33, 36–38].

A certain country specificity in how the aging process impacts on the intestinal microbiota, possibly related to differences in lifestyle and dietary habits, has been reported [36, 39], particularly in relation to the dominant components of the gut

microbiota, *Firmicutes* and *Bacteroidetes*. For what concerns *Firmicutes*, members of the *Clostridium* cluster XIVa were found to decrease in Japanese, Finnish, and Italian elderly and centenarians, whereas an inverse trend was found in German old adults [32, 33, 36, 40]. The species *Faecalibacterium prausnitzii* (*Clostridium* cluster IV), known for its anti-inflammatory properties and ability to produce short chain fatty acids (SCFAs), markedly decreased in Italian elderly and centenarians [32, 36], as well as in frail, hospitalized, and antibiotic- and anti-inflammatory-treated elderly [41–44]. Conversely, an age-related increase in *Bacteroidetes* was found in German, Austrian, Finnish, and Irish elderly [33, 34, 36, 44], but this was not confirmed in Italian elderly and centenarians [32, 36]. In the case of Irish elderly, *Bacteroidetes* were found to be the dominant phylum instead of *Firmicutes*, which has always been regarded as the most abundant in healthy adults [34].

Similarly to what is observed in inflammatory bowel diseases and obesity [45–47], the age-related changes in gut microbiota composition summarized above may concur to the complex process that both sustains and nurtures inflamm-aging. Indeed, several bacterial groups known to increase in the elderly (e.g., enterococci and *Enterobacteriaceae*) are often classified as "pathobionts," defined as bacteria present in the healthy gut microbiota in low concentration, which are able to thrive in inflamed conditions, promoting the inflammation itself [31].

The age-related changes in the phylogenetic architecture of the gut microbiota impact on those metabolic and physiological functions for which the human meta-organism depends on its microbial counterpart, with consequences at the functional level and possibly affecting the risk of elderly people to develop some types of diseases. For instance, the production of SCFAs, i.e., butyrate, acetate, and propionate, is an essential feature of a healthy gut ecosystem, because SCFAs, especially butyrate, have nutritive, anti-inflammatory, antineoplastic properties and exert a protective role for the intestinal epithelium, increasing its resistance [48–50]. The lower capacity to produce butyrate in the elderly gut microbiome [32, 51], correlated with decreased amounts of *F. prausnitzii*, *Eubacterium hallii*, and bacteria belonging to the *Eubacterium rectale/Roseburia* group, together with the increase in proinflammatory pathobionts (i.e., bacteria present in the healthy gut microbiota in low concentration, which are able to thrive in inflamed conditions, sustaining inflammation itself), may nurture the inflamm-aging process in the GIT (Fig. 9.1b) [31]. Moreover, the declined butyrate-producing capacity may contribute to the development of degenerative diseases and anorexia [52, 53].

SCFAs are also a fundamental component of the microbiota–host bio-network to maintain the GI epithelial integrity, by stimulating the release of mucins, the gel-forming protein component of the colonic mucus layer that contributes to the physical separation between microbiota and enterocytes [54], and enhancing transepithelial resistance [55]. The age-related microbiota depletion in SCFA producers may concur in weakening gut epithelium, allowing the passage of whole bacterial cells and their products, ending up in an abnormal inflammatory response [56].

Moreover, a proinflammatory dysbiosis, together with the decreased butyrate production in the intestine, has also been linked to an increased risk of colorectal cancer [57, 58], whose incidence is augmented in aged people [59].

Finally, changes in the gut microbiota composition may be rated as one of the age-related physiological processes that, all together, are linked to "how healthily" a human being will age. It has recently been demonstrated by Claesson et al. that the healthiest elderly, living in community setting and having a high-quality diet, possess a distinct gut microbiota from less healthy elderly living in residential care [60]. However, whether these changes are simply a biomarker of the aging process or could be counted among its driver cannot yet be decided based on the available data. Certainly, the aging gut microbiota possesses features that can actively affect the health status of old people, i.e., contributing to the onset of pathological conditions known to affect the elderly with a higher incidence than the young adults, such as *C. difficile*-associated diarrhea and colon cancer, as well as their eventual responsiveness to therapies [30]. Considering the aging human being as a metaorganism, composed by both human and bacterial cells that together answer to the changing metabolic requests of the human body, could be the right approach for promoting healthy aging and longevity.

9.3 Variation of Microbiota–Host Co-metabolism in Aging

Age-related variations in microbiota–host transgenomic metabolism can impact different ecological services provided by our symbiotic gut microbial community. Indeed, the microbiota–host co-metabolism has been reported as strategic for several host physiological functions – such as regulation of extraction and storage of food energy, epigenome modulation, and brain function – and its impairment can be detrimental for health and longevity (Table 9.1).

As an end product of the gut microbiota fermentation processes, gut butyrate shows a multifactorial role in host nutrition, and its reduction as a consequence of the age-related microbiota deterioration can provide several consequences for the host nutritional state. For instance, microbial butyrate is essential to enhance the efficiency of food energy extraction, representing the primary energy source for colonic epithelium and providing the 15 % of the total energy request [46, 61]. On the other hand, supporting insulin secretion, microbial butyrate is also involved in the regulation of the host energy storage. Finally, butyrate has been reported to be a key regulator of appetite, enhancing the production of leptin and inducing *pyy* expression [23]. Beside butyrate, also the age-related decrease of microbial propionate and acetate can concur in weakening of the host nutritional state. In particular, these metabolites have a role in the regulation of food energy storage; acetate is involved in hepatic liponeogenesis, while propionate regulates gluconeogenesis [61].

SCFAs, in particular butyrate, have also been recently shown to be involved in the modulation of the host epigenome by means of the inhibition of the histone deacetylase. Acting as regulators of the epithelial cell transcriptome, the decrease of microbial SCFA along aging can influence different physiological aspects of the host biology, such as digestion, immunity, gut–brain function, and hormone

Table 9.1 End products of microbial metabolism in the gut and their activities with respect to the human host physiology

Metabolite	Origin	Functions/activities
Butyrate	Carbohydrate fermentation by gut microorganisms	Energy source; stimulates leptin production; regulates neutrophils; inhibits inflammatory cytokines; increase TJ expression; histone deacetylase inhibitor; binding to G-protein-coupled receptors: improves of insulin secretion; induction of *Pyy* expression, supports fat deposition; antimicrobial activity; modulation of the sympathetic nervous system and gut motility
Acetate		Supports lipid synthesis; supports energy metabolisms; binding to G-protein-coupled receptors; antimicrobial activity; modulation of the sympathetic nervous system and gut motility
Propionate		Supports gluconeogenesis; binding to G-protein-coupled receptors; antimicrobial activity; modulation of the sympathetic nervous system and gut motility
Methylamine	Metabolism of choline by gut microorganisms	Altered product of choline metabolisms; has been associated with obesity, diabetes and cardiovascular disease, liver steatosis
Dimethylamine		
Trimethylamine		
Trimethylamine-N-oxide		
4-Cresyl sulfate	Tyrosine putrefaction by gut microorganisms	Association with cancer; diabetes; autism; depression
4-Cresil glucuronide		
5-Hydroxytryptamine	Bacterial metabolism of tryptophan	Influences brain and behavior
GABA	Neurotransmitters of bacterial origin	Influences behavior
Noradrenaline		
Dopamine		
Acetylcholine		
LPS	Gram – surface component	Metabolic endotoxemia

secretion [62]. Interestingly, recent findings demonstrated that neurotransmitters or neuroactive metabolites of microbial origin, such as SCFA and 5-hydroxytryptamine, are effective in the stimulation of the sympathetic nervous system and gut motility [23]. The reduction of these neuroactive microbial metabolites along aging can result in changing in brain function and behavior, supporting cognitive deficiencies and depression which are typical of the frail elderly [63]. Finally, the impairment of microbiota–host mutualism in the elderly can drive to modifications of the choline

metabolism by gut microorganism, resulting in an increase risk to develop diabetes and cardiovascular diseases [64]. Analogously, an increase of tyrosine putrefaction by gut microorganism in an aged-type microbiota can eventually result in the increased risk of developing cancer and depression.

9.4 Metabonomics Potential in Aging and Gut Microbiota Research

Increasing scientific evidence has been reported on the fundamental role of gut microbiota in both positive and negative triggers of specific metabolic states of individuals and populations [61, 65]. Therefore, it is today critical to understand the molecular foundations of the impact of the gut microbial activity on human health and nutritional status [66–68].

The current omics revolution offers an unprecedented opportunity to explore how our gut symbionts contribute to our physiology and human health. Future systems biology approaches combining state-of-the-art microbial and metabolic modeling and discovery approaches, including metagenomics and metabonomics, will help in deciphering the molecular foundations of these transgenomics interactions. Recently, an exhaustive gene catalogue has been published containing virtually all of the prevalent gut microbial genes in large human cohort, and reporting to which extent many bacterial species are shared by different individuals [69]. Such an approach could be used for global characterization of the genetic potential of ecologically complex environments [69] but also to help understand how gut microbiota specificities could be exploited to develop new therapeutic and nutritional strategies. Systems biology approaches, including metabonomics, have emerged over the last two decades as a novel way forward to provide insights into the role of mammalian gut microbial metabolic interactions in individual susceptibility to health and disease outcomes. Since it is more likely that gut microbiome required for proper functioning of the gut ecosystem in the elderly is different from that of the young subjects, knowledge and systems models will help in assessing earlier deviations that lead to unhealthy aging and development of chronic inflammatory conditions. In particular, both system-wide (i.e., whole organism) and organ-specific metabolism may have components driven by gut microbial activities [70, 71], which suggests that the dynamics of the gut microbiome could help to maintain or reestablish host metabolic homeostasis in disease and early onsets of metabolic deregulations. Gut microbial activities can be extremely specific, as for the development and maintenance of the mucosal innate and adaptive immune system [52], but also very complex, such as in the etiology and development of several chronic inflammatory disorders, including inflammatory bowel diseases (IBD), irritable bowel syndrome (IBS), and gastrointestinal cancers [72, 73]. A series of investigations in human [74] and animal models [75–77] have provided a set of reference metabolic profiles of gut intestinal biopsies that can be used to assess not only compartment structure and function but also the gut microbial impact at the tissue level [78]. Such applications will help in identifying main

metabolic processes conserved across species on which gut microbiota modulates to shape the microenvironment. For instance, the investigations illustrated how microbial-dependent variations along the upper intestine, an element often underestimated due to low bacterial populations, may affect utilization efficiency of dietary proteins and amino acids and their subsequent availability to extra-intestinal tissues. Moreover, some reference data were generated to investigate changes in gut functionality, such as gut permeability, using metabolic profiling of biofluids [79, 80], since normal ageing is associated with a number of significant changes in intestinal function, which may impact on daily energy intake [81]. Since under-nutrition is common in older people and has been implicated in the development and progression of chronic disease commonly affecting the elderly, such insights will be key for tailoring personal nutritional intervention and monitoring patient response on an individual basis. Furthermore, indirect and minimally invasive metabolic indicators of specific intestinal structural or functional states (e.g., digestion, mobility, permeability) will be crucial for patient risk assessment and monitoring. For instance, such applications seem promising in the field of chronic intestinal inflammatory conditions. Both manifestations of IBD, ulcerative colitis (UC) and Crohn's disease (CD), are mediated by common and distinct mechanisms influenced by multiple environmental factors and specific genetic predispositions, including gut microbiota. Advancing knowledge regarding the mechanisms of IBD has led to the development of different therapeutic solutions based on surgery [82], cannabinoids [83], immunosuppression [84], and alternatively probiotic supplementation [85]. Although prognostic and monitoring tools are currently lacking, metabolic profiling in combination with state-of-the-art clinical and medical readouts is foreseen to be a valuable tool to differentiate and follow-up IBD evolution and response to disease-modifying interventions. This is, for instance, exemplified through the discovery of metabolic indicators of different IBD determinants, including mucosal healing, gut permeability, absorption, digestion, or inflammatory states. As an example, Winterkamp et al. reported previously how N-methylhistamine, a key metabolite in mast cell metabolism involved in the pathogenesis of IBD, could be used as an indicator of disease activity in patients [86]. In this study, the urinary excretion of N-methylhistamine was associated with elevated histamine production and metabolism in CD and UC and could be used as a reliable diagnostic tool to monitor clinical and endoscopic disease activity in IBD. Additional proofs of concept on the feasibility to identify some metabolic indicators of early onsets of chronic inflammatory development offer also novel promising directions for patient monitoring and early patient stratification [87]. Additional applications of noninvasive profiling of stool from patients provided novel insights into the remodeling of the gut microbial communities and activities, concomitant to malabsorption and element of protein-losing enteropathy [88, 89]. Another severe GIT disorder for which the pandemic is affecting mainly the elderly is colorectal cancer (CRC) [90]. Effective screening methods have significantly improved the treatment modalities and effectiveness in the geriatric population. Nowadays, screening methods are mainly based on fecal assays for detecting blood presence or altered DNA and endoscopic and computerized radiologic techniques. Therefore, CRC early diagnosis still suffers from the absence of noninvasive or minimally invasive

diagnostics for early detection. Numerous studies reported that the general population of intestinal bacteria is associated with initiation of colon cancer via the production of carcinogens, cocarcinogens, or pro-carcinogen substances [91]. Recently, metabonomics applications identified novel lipid markers of early CRC in American and Japanese populations using MS analyses of serum samples [92, 93]. Moreover, the application of NMR metabolic profiling to fecal water extracts has interesting potential as a diagnostic tool for detecting CRC [94]. NMR metabolic profiles of fecal water extracts from CRC patients were markedly distinct from controls with lower concentrations of SCFA (acetate and butyrate) and higher levels of proline and cysteine, the latter being two major components of most colonic epithelium mucus glycoproteins. Conventional tumor markers are relatively unsuitable for detecting carcinoma at an early stage and such novel insights may become critical in the development of tailored approaches to reduce disease burden.

9.5 Conclusions

The gut microbiota influences human health status throughout the whole lifespan, from birth to aging. It is generally accepted that the disruption of the finely balanced interaction between host and microbiota can contribute to the progression of diseases and/or predispose the human host to develop age-related disorders later in life. Yet, recent advances in systems biology approaches including metabonomics and microbial ecology have shown that the contribution of the intestinal microbiota to the overall health status of the host has been so far underestimated. It is therefore of crucial importance for nutrition and health to understand and to metabolically characterize the interactive molecular processes between the host and its microbiome. By opening a direct biochemical window into the metabonome, metabonomics is a unique science perfectly suited for the identification of biomarkers capable of providing better understanding of the complex metabolic phenomenon. This makes metabonomics a very efficient approach for generation of metabolic patterns for the comprehensive characterization of metabolic health, including healthy aging, and in the generation of new insights in the understanding of the interactions between diet and metabolism. By understanding the mechanisms by which diet and lifestyle influence metabolism, also by means of influencing the microbiota composition and functionality, it will be possible to develop personalized strategies to maintain a reasonable health status during aging and possibly to attain longevity, maintaining a high quality of life as long as possible.

References

1. Kirkwood TB. Understanding the odd science of ageing. Cell. 2005;120:437–47.
2. Weinert BT, Timiras PS. Invited review: theories of ageing. J Appl Physiol. 2003;95: 1706–16.

3. Franceschi C, Bonafè M, Valensin S, et al. Inflamm-ageing. An evolutionary perspective on immunosenescence. Ann N Y Acad Sci. 2000;908:244–54.
4. Franceschi C. Inflammaging as a major characteristic of old people: can it be prevented or cured? Nutr Rev. 2007;65:S173–6.
5. Cevenini E, Monti D, Franceschi C. Inflamm-ageing. Curr Opin Clin Nutr Metab Care. 2013;16:14–20.
6. Cevenini E, Caruso C, Candore G, et al. Age-related inflammation: the contribution of different organs, tissues and systems. How to face it for therapeutic approaches. Curr Pharm Des. 2010;16:609–18.
7. Franceschi C, Capri M, Monti D, et al. Inflammaging and anti-inflammaging: a systemic perspective on ageing and longevity emerged from studies in humans. Mech Ageing Dev. 2007;128:92–105.
8. Evans WJ, Paolisso G, Abbatecola AM, et al. Frailty and muscle metabolism dysregulation in the elderly. Biogerontology. 2010;11:527–36.
9. Fry CS, Rasmussen BB. Skeletal muscle protein balance and metabolism in the elderly. Curr Ageing Sci. 2011;4:260–8.
10. Fiatarone MA, O'Neill EF, Ryan ND, et al. Exercise training and nutritional supplementation for physical frailty in very elderly people. N Engl J Med. 1994;330:1769–75.
11. Bruce SJ, Breton I, Decombaz J, et al. A plasma global metabolic profiling approach applied to an exercise study monitoring the effects of glucose, galactose and fructose drinks during post-exercise recovery. J Chromatogr B Analyt Technol Biomed Life Sci. 2010;878:3015–23.
12. Yan BAJ, Wang G, et al. Metabonomic investigation into variation of endogenous metabolites in professional athletes subject to strength-endurance training. J Appl Physiol. 2009;106:531–8.
13. Wijeyesekera A, Selman C, Barton RH, et al. Metabotyping of long-lived mice using 1H NMR spectroscopy. J Proteome Res. 2012;11:4425–35.
14. Franceschi C, Motta L, Valensin S, et al. Do men and women follow different trajectories to reach extreme longevity? Italian Multicenter Study on Centenarians (IMUSCE). Ageing (Milano). 2000;12:77–84.
15. Collino S, Montoliu I, Martin FP, et al. Metabolic signatures of extreme longevity in northern italian centenarians reveal a complex remodeling of lipids, amino acids, and gut microbiota metabolism. PLoS One. 2013;8:e56564.
16. Gonzalez-Covarrubias V, Beekman M, Uh HW, et al. Lipidomics of familial longevity. Aging Cell. 2013;12:426–34.
17. Dekker P, Maier AB, et al. Stress-induced responses of human skin fibroblasts in vitro reflect human longevity. Ageing Cell. 2009;8:595–603.
18. Dekker P, Meissner A, Dirks RW, et al. Human in vivo longevity is reflected in vitro by differential metabolism as measured by (1)H-NMR profiling of cell culture supernatants. Mol Biosyst. 2012;8:783–9.
19. Brookmeyer R, Johnson E, Ziegler-Graham K, et al. Forecasting the global burden of Alzheimer's disease. Alzheimers Dement. 2007;3:186–91.
20. Han X, Rozen S, Boyle SH, et al. Metabolomics in early Alzheimer's disease: identification of altered plasma sphingolipidome using shotgun lipidomics. PLoS One. 2011;6:e21643.
21. Sato Y, Suzuki I, Nakamura T, et al. Identification of a new plasma biomarker of Alzheimer's disease using metabonomics technology. J Lipid Res. 2012;53:567–76.
22. Chan RB, Oliveira TG, Cortes EP, et al. Comparative lipidomic analysis of mouse and human brain with Alzheimer disease. J Biol Chem. 2012;287:2678–88.
23. Tremaroli V, Bäckhed F. Functional interactions between the gut microbiota and host metabolism. Nature. 2012;489:242–9.
24. Sommer F, Backhed F. The gut microbiota – masters of host development and physiology. Nat Rev Microbiol. 2013;11:227–38.
25. Tannock GW. New perceptions of the gut microbiota: implications for future research. Gastroenterol Clin North Am. 2005;34:361–82.
26. Xu J, Mahowald MA, Ley RE, et al. Evolution of symbiotic bacteria in the distal human intestine. PLoS Biol. 2007;5:e156.

27. Jia W, Li H, Zhao L, Nicholson JK. Gut microbiota: a potential new territory for drug targeting. Nat Rev Drug Discov. 2008;7:123–9.
28. Cheng J, Palva AM, de Vos WM, et al. Contribution of the intestinal microbiota to human health: from birth to 100 years of age. Curr Top Microbiol Immunol. 2013;358:323–46.
29. Biagi E, Candela M, Fairweather-Tait S, et al. Ageing of the human metaorganism: the microbial counterpart. Age (Dordr). 2012;34:247–67.
30. Biagi E, Candela M, Turroni S, et al. Ageing and gut microbes: perspectives for health maintenance and longevity. Pharmacol Res. 2013;69:11–20.
31. Pédron T, Sansonetti P. Commensals, bacterial pathogens and intestinal inflammation: an intriguing ménage à trois. Cell Host Microbe. 2008;3:344–7.
32. Biagi E, Nylund L, Candela M, et al. Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. PLoS One. 2010;5:e10667.
33. Mäkivuokko H, Tiihonen K, Tynkkynen S, et al. The effect of age and non-steroidal anti-inflammatory drugs on human intestinal microbiota composition. Brit J Nutr. 2010;103:227–34.
34. Claesson MJ, Cusack S, O'Sullivan O, et al. Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc Natl Acad Sci U S A. 2011;108:4586–91.
35. Rea MC, O'Sullivan O, Shanahan F, et al. Clostridium difficile carriage in elderly subjects and associated changes in the intestinal microbiota. J Clin Microbiol. 2012;50:867–75.
36. Mueller S, Saunier K, Hanisch C, et al. Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. Appl Environ Microbiol. 2006;72:1027–33.
37. Mariat D, Firmesse O, Levenez F, et al. The firmicutes/bacteroidetes ratio of the human microbiota changes with age. BMC Microbiol. 2009;9:123.
38. Rajilić-Stojanović M, Heilig HGHJ, Molenaar D, et al. Development and application of the human intestinal tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. Environ Microbiol. 2009;11:1736–51.
39. Biagi E, Candela M, Franceschi C, et al. The ageing gut microbiota: new perspectives. Ageing Res Rev. 2011;10:428–9.
40. Hayashi H, Sakamoto M, Kitahara M, et al. Molecular analysis of fecal microbiota in elderly individuals using 16S rDNA library and T-RFLP. Microbiol Immunol. 2003;47:557–70.
41. Bartosch S, Fite A, Macfarlane GT, et al. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. Appl Environ Microbiol. 2004;70:3575–81.
42. Tiihonen K, Tynkkynen S, Ouwehand A, et al. The effect of ageing with and without non-steroidal anti-inflammatory drugs on gastrointestinal microbiology and immunology. Br J Nutr. 2008;100:130–7.
43. van Tongeren SP, Slaets JPJ, Harmsen HJM, et al. Fecal microbiota composition and frailty. Appl Environ Microbiol. 2005;71:6438–42.
44. Zwielehner J, Liszt K, Handschur M, et al. Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in fecal population sizes and diversity of Bacteroides, bifidobacteria and Clostridium cluster IV in institutionalized elderly. Experim Gerontol. 2009;44:440–6.
45. Cani PD, Delzenne NM. The role of the gut microbiota in energy metabolism and metabolic disease. Curr Pharm Des. 2009;15:1546–58.
46. Neish AS. Microbes in gastrointestinal health and disease. Gastroenterology. 2009;136:65–80.
47. Maslowski KM, Mackay CR. Diet, gut microbiota and immune responses. Nat Immunol. 2011;12:5–9.
48. Barcenilla A, Pryde SE, Martin JC, et al. Phylogenetic relationships of butyrate-producing bacteria from the human gut. Appl Environ Microbiol. 2000;66:1654–61.
49. Louis P, Flint HJ. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS Microbiol Lett. 2009;294:1–8.

50. Thibault R, Blachier F, Darcy-Vrillon B, et al. Butyrate utilization by the colonic mucosa in inflammatory bowel diseases: a transport deficiency. Inflamm Bowel Dis. 2010;16:684–95.
51. Hippe B, Zwielehner J, Liszt K, et al. Quantification of butyryl CoA:acetate CoA-transferase genes reveals different butyrate production capacity in individuals according to diet and age. FEMS Microbiol Lett. 2011;316:130–5.
52. Guigoz Y, Doré J, Schiffrin EJ. The inflammatory status of old age can be nurtured from the intestinal environment. Curr Opin Clin Nutr Metab Care. 2008;11:13–20.
53. Donini LM, Savina C, Cannella C. Nutritional interventions in the anorexia of ageing. J Nutr Health Ageing. 2010;14:494–6.
54. Petersson J, Schreiber O, Hansson GC, et al. Importance and regulation of the colonic mucus barrier in a mouse model of colitis. Am J Physiol Gastrointest Liver Physiol. 2011;300:G327–33.
55. Suzuki T, Yoshida S, Hara H. Physiological concentrations of short-chain fatty acids immediately suppress colonic epithelial permeability. Br J Nutr. 2008;100:297–305.
56. Macia L, Thorburn AN, Binge LC, et al. Microbial influences on epithelial integrity and immune function as a basis for inflammatory diseases. Immunol Rev. 2012;245:164–76.
57. Candela M, Guidotti M, Fabbri A, et al. Human intestinal microbiota: cross-talk with the host and its potential role in colorectal cancer. Crit Rev Microbiol. 2011;37:1–14.
58. Ou J, DeLany JP, Zhang M, et al. Association between low colonic short-chain fatty acids and high bile acids in high colon cancer risk populations. Nutr Cancer. 2012;64:34–40.
59. Boyle P, Ferlay J. Mortality and survival in breast and colorectal cancer. Nat Clin Pract Oncol. 2005;2:424–5.
60. Claesson MJ, Jeffery IB, Conde S, et al. Gut microbiota composition correlates with diet and health in the elderly. Nature. 2012;488:178–84.
61. Nicholson JK, Holmes E, Kinross J, et al. Host-gut microbiota metabolic interactions. Science. 2012;336:1262–7.
62. Kau AL, Ahern PP, Griffin NW, et al. Human nutrition, the gut microbiome and the immune system. Nature. 2011;474:327–36.
63. Collins SM, Surette M, Bercik P. The interplay between the intestinal microbiota and the brain. Nat Rev Microbiol. 2012;10:735–42.
64. Holmes E, Li JV, Marchesi JR, et al. Gut microbiota composition and activity in relation to host metabolic phenotype and disease risk. Cell Metab. 2012;16:559–64.
65. Holmes E, Kinross J, Gibson GR, et al. Therapeutic modulation of microbiota-host metabolic interactions. Sci Transl Med. 2012;4:137rv6.
66. Martin FP, Collino S, Rezzi S, et al. Metabonomic applications to decipher gut microbial metabolic influence in health and disease. Front Physiol. 2012;3:113.
67. El AS, Hooiveld G, Tremaroli V, et al. The gut microbiota and mucosal homeostasis: Colonized at birth or at adulthood, does it matter? Gut Microbes. 2013;4:118–24.
68. Moco S, Collino S, Rezzi S, et al. Metabonomics perspectives in pediatric research. Pediatr Res. 2013;73:570–6.
69. Qin J, Li R, Raes J, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464:59–65.
70. Martin FP, Sprenger N, Yap IK, et al. Panorganismal gut microbiome-host metabolic crosstalk. J Proteome Res. 2009;8:2090–105.
71. Martin FP, Sprenger N, Montoliu I, et al. Dietary modulation of gut functional ecology studied by fecal metabonomics. J Proteome Res. 2010;9:5284–95.
72. Martin FP, Collino S, Rezzi S. 1H NMR-based metabonomic applications to decipher gut microbial metabolic influence on mammalian health. Magn Reson Chem. 2011;49 Suppl 1:S47–54.
73. Collino S, Martin FP, Rezzi S. Clinical metabolomics paves the way towards future healthcare strategies. Br J Clin Pharmacol. 2013;75:619–29.
74. Wang Y, Holmes E, Comelli EM, et al. Topographical variation in metabolic signatures of human gastrointestinal biopsies revealed by high-resolution magic-angle spinning (1)H NMR spectroscopy. J Proteome Res. 2007;6:3944–51.

75. Wang Y, Tang H, Holmes E, et al. Biochemical characterization of Rat intestine development using high-resolution magic-angle-spinning 1H NMR spectroscopy and multivariate data analysis. J Proteome Res. 2005;4:1324–9.
76. Martin FP, Wang Y, Yap IK, et al. Topographical variation in murine intestinal metabolic profiles in relation to microbiome speciation and functional ecological activity. J Proteome Res. 2009;8:3464–74.
77. Martin FP, Montoliu I, Kochhar S, et al. Chemometric strategy for modeling metabolic biological space along the GIT and assessing microbial influences. Anal Chem. 2010;82:9803–11.
78. Martin FP, Wang Y, Sprenger N, et al. Effects of probiotic lactobacillus paracasei treatment on the host Gut tissue metabolic profiles probed via magic-angle-spinning NMR spectroscopy. J Proteome Res. 2007;6:1471–81.
79. Alonso C, Guilarte M, Vicario M, et al. Acute experimental stress evokes a differential gender-determined increase in human intestinal macromolecular permeability. Neurogastroenterol Motil. 2012;24:740–e349.
80. Rezzi S, Martin FP, Alonso C, et al. Metabotyping of biofluids reveals stress-based differences in gut permeability in healthy individuals. J Proteome Res. 2009;8:4799–809.
81. Parker BA, Chapman IM. Food intake and ageing–the role of the gut. Mech Ageing Dev. 2004;125:859–66.
82. Harper PH, Truelove SC, Lee EC, et al. Split ileostomy and ileocolostomy for Crohn's disease of the colon and ulcerative colitis: a 20 year survey. Gut. 1983;24:106–13.
83. Duncan M, Davison JS, Sharkey KA. Review article: endocannabinoids and their receptors in the enteric nervous system. Aliment Pharmacol Ther. 2005;22:667–83.
84. Actis GC, Pazienza P, Rosina F. Mesalamine for inflammatory bowel disease: recent reappraisals. Inflamm Allergy Drug Targets. 2008;7:1–5.
85. Reid G, Jass J, Sebulsky MT, et al. Potential uses of probiotics in clinical practice. Clin Microbiol Rev. 2003;16:658–72.
86. Winterkamp S, Weidenhiller M, Otte P, et al. Urinary excretion of N-methylhistamine as a marker of disease activity in inflammatory bowel disease. Am J Gastroenterol. 2002;97:3071–7.
87. Baur P, Martin FP, Gruber L, et al. Metabolic phenotyping of the Crohn's disease-like IBD etiopathology in the TNF(DeltaARE/WT) mouse model. J Proteome Res. 2011;10:5523–35.
88. Marchesi JR, Holmes E, Khan F, et al. Rapid and noninvasive metabonomic characterization of inflammatory bowel disease. J Proteome Res. 2007;6:546–51.
89. Le GG, Noor SO, Ridgway K, et al. Metabolomics of fecal extracts detects altered metabolic activity of gut microbiota in ulcerative colitis and irritable bowel syndrome. J Proteome Res. 2011;10:4208–18.
90. Holt PR, Kozuch P, Mewar S. Colon cancer and the elderly: from screening to treatment in management of GI disease in the elderly. Best Pract Res Clin Gastroenterol. 2009;23:889–907.
91. O'Keefe SJ. Nutrition and colonic health: the critical role of the microbiota. Curr Opin Gastroenterol. 2008;24:51–8.
92. Ritchie SA, Ahiahonu PW, Jayasinghe D, et al. Reduced levels of hydroxylated, polyunsaturated ultra long-chain fatty acids in the serum of colorectal cancer patients: implications for early screening and detection. BMC Med. 2010;8:13.
93. Ritchie SA, Heath D, Yamazaki Y, et al. Reduction of novel circulating long-chain fatty acids in colorectal cancer patients is independent of tumor burden and correlates with age. BMC Gastroenterol. 2010;10:140.
94. Monleon D, Morales JM, Barrasa A, et al. Metabolite profiling of fecal water extracts from human colorectal cancer. NMR Biomed. 2009;22:342–8.

Chapter 10
Roles of Herbal Medicine in Modulating Gut Microbiota Associated with Health and Diseases

Yulan Wang and Huiru Tang

Abstract The perturbation of gut microbiota is increasingly recognized to be associated with human health and diseases. The modulation of the gut microbiotal community as a means to alleviate disease conditions provides a unique opportunity for herbal medicine, due to the two-way interaction between gut microbiota and herbal medicine. Herbal medicine contains a range of polyphenols that require action from gut microbiota to effectively perform their biological function. The gut microbiota are subsequently stimulated through this action. In this chapter, we outline the associations between gut microbiota and disease (particularly inflammatory bowel disease (IBD), diabetes, and cancer), and the roles of herbal medicine in alleviating disease conditions through modulating gut microbiota. In addition, we discuss the functional uses and challenges of herbal medicine, which include the quality control and elucidating mechanisms of action. Finally, we describe how a metabonomics technique can provide a means for the quality control of herbal medicines and can be an efficient tool for elucidating the molecular mechanisms of different herbal treatments. Future research on herbal medicine should be focused on

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directly measuring altered gut microbiota and integrating this with a mechanistic metabonomics evaluation.

Keywords Gut microbiota • Diseases • Metabonomics • Herbal medicine

10.1 Introduction

Humans have a large number of microbiota resident in the gut. Interactions between host metabolism and gut microbiota, between gut microbiota themselves, and between food/medicine ingested and gut microbiota make the human gut a very complex system. There are approximately about 1,000 microbial species present in the mammalian gut, which codevelop with the host throughout the host's lifetime. Microbes play an important role in host nutrition and health by supplying and digesting nutrients, preventing pathogen colonization, and maintaining balanced mucosal immunity, which is vital for our health. A shift in the gut microbial composition can stimulate a specific disease-prone (dysbiosis) or disease-protective activity (probiosis). For example, *Lactobacillus reuteri* strains can produce thiamine to benefit the host [1], whereas *Bifidobacteria* may inhibit the colonization of potential pathogens by competing with the nutrients and the binding site on the mucosa [2]. The gut microbial community is very complex and dynamic [3], which can be affected by the host's genome, birth, age [4], nutrition [5], lifestyle, disease [6], and therapeutic interventions (e.g., antibiotics [7], herbal medicines [8], and surgery treatments [9]). In turn, the unbalanced microbial colonies may disturb the physiological homeostasis, leading to various diseases such as colon cancer, inflammatory bowel disease (IBD) [10], irritable bowel syndrome (IBS) [11], obesity [12–14], diabetes [15], cardiovascular disease [16], autism [17], and allergic asthma [18]. The close associations between the gut microbiotal community and disease status give a unique opportunity for treatment by using traditional herbal medicine, via restoring the balance of the gut microbiotal community. Shaping the balance of the gut microbiotal community by herbal medicine involves a two-way interaction. The active biological ingredients of the herbal material are largely polyphenolic compounds, which often cannot be absorbed directly by humans. Fortunately, however, enzymes secreted by gut microbiota can metabolize these non-bioavailable phytochemicals, facilitating their utilization. The enzyme-producing bacteria in return are selectively stimulated, thereby modifying the balance of the gut microbiota [8, 19] (Fig. 10.1).

The beneficial effects of herbal medicine have gained growing interest in herbal remedies, leading to a strong growth in consumer demand in plant-based products. The current global market for plant-based health products is estimated to hit 93.15 billion dollars by 2015 (http://www.nutraingredients-usa.com). Prominent concerns regarding the quality of phytomedicines are that they contain mixtures of many compounds, which are often derived from plants or animal origins. Traditionally, the quality of these herbs is assessed by the experiences of herbal practitioners, who sometimes

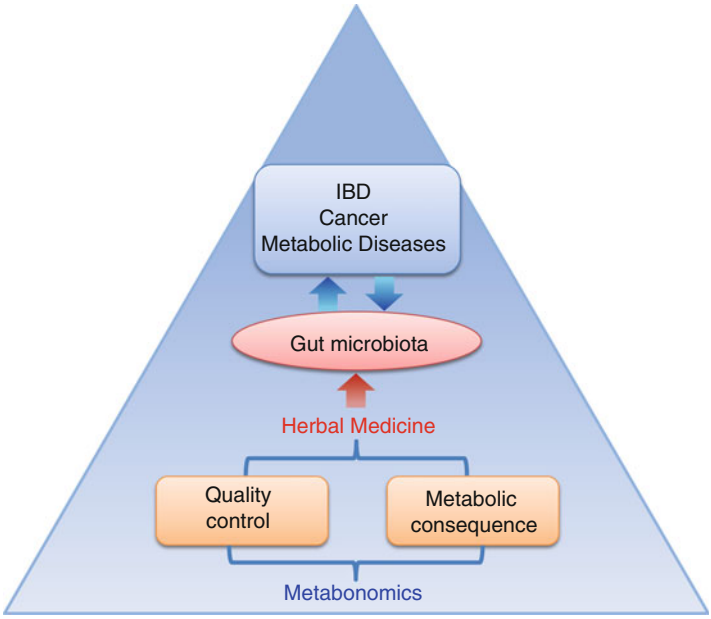

Fig. 10.1 A summary of the key roles of metabonomics in herbal medicine and the herbal medicine alleviating disease conditions via modifications of gut microbiota

refer to "active" molecules or arbitrarily chosen "marker" compounds in complex plant extracts. The lack of scientifically accepted standards for herbal medicine has long been an obstacle preventing herbal medicine from being recognized and integrated into the standard healthcare system in Western countries. Therefore, one of the challenges that phytomedicinal practitioners have encountered is the quality control of herbal medicine. As a consequence of the lack of efficient quality control tools, large variations of phytomedical products are encountered. For example, an analysis of 14 commercially available feverfew samples showed that each batch generated a unique and characteristic spectra profile, with two of the batches being markedly different from the other 12 [20]. The development of new quality control methods based on the entire biochemical composition of the preparation without reference to "active" molecules will help improve the quality and will make it more acceptable. Modern metabonomics techniques appear to be well suited for this purpose. Metabonomics involves the study of multivariate metabolic responses of complex organisms to physiological and/or pathological stressors, including the consequent disruption of systems regulation [21–24]. Metabonomics involves multivariate analysis of data from Nuclear magnetic resonance (NMR) and mass spectrometry (MS) spectroscopy. The commonly used multivariate data analysis tool includes a range of pattern recognition techniques and random forest method as detailed in Chaps. 2 and 3. Using ^{1}H NMR spectroscopy, a wide range of plant metabolites can be detected including sugars, amino acids, organic acids, and polyphenols. In such cases, all the chemical components present in a single plant extract can be viewed simultaneously as a

Table 10.1 Summary of alternations of gut microbiota associated with diseases and corresponding herbal medicine treatment

Diseases	Gut microbiota	Herbal medicine
IBD	*Bacteroides fragilis*, *enterococci*, *lactobacilli*, *bifidobacteria* [34]	Quercetin [26, 27]
		Curcumin [28]
		Prebiotics [32, 33]
		Echinacea purpurea [34]
Metabolic diseases	*Lactobacillus*, *Clostridium* [38]	*Jiangtang Xiaozhi* [39, 40]
	Enterococcus faecalis [42]	*Mimosa pudica*, *Emblica officinalis*, *Azadirachta indica* [42]
		Huangguaxiang [43]
Cancer	*H. pylori*	*Juzentaihoto* [45–48]
		Pelargonium sidoides [49, 50]
		Calophyllum brasiliense Camb. (*Clusiaceae*), *Mouriri elliptica* Martius (*Melastomataceae*), and *Hancornia speciosa* Gomez (*Mangaba*) [51–53]

"metabolic fingerprint." The holistic nature of metabonomics can overcome the drawback of considering "active components" (Table 10.1).

A single-component drug is the major characteristic of many Western medicines, and the metabolism of the drug and the molecular target are often clearly defined. However, herbal medicine consists of multiple components in nature, with mixtures of various herbs; thus, the identification of molecular mechanisms and acting targets is hindered by the complexity of the herbal medicine. Traditional drug discovery routes are unsuitable for evaluating the mechanism of action of herbal medicines. Metabonomics, on the other hand, measures the endpoint metabolic perturbations of an intervention, including the multiple components of the herb. Therefore, the metabonomics technique can also be adopted to evaluate mechanisms of a given complex herbal medicine. The advantage of utilizing such a technique is its holistic nature, which can provide important clues as to how herbal medicine really works.

In this chapter, we discuss the roles of gut microbiota in diseases and the ways in which herbal medicine could alleviate disease conditions via modification of the gut microbiotal community. In addition, the challenges encountered in herbal medicine will be discussed and examples of possible solutions are shown.

10.2 Herbal Medicine Affects the Association Between Disease State and Gut Microbiota

Many diseases are closely associated with an imbalanced gut microbiota, and herbal medicines are capable of alleviating these disease conditions by offsetting the imbalance of the microbiotal community. Inflammatory bowel disease (IBD) is

one such disease. IBD primarily includes ulcerative colitis (UC) and Crohn's disease (CD) and is a significant public health burden. The pathogenesis of IBD has not yet been identified, but it has been widely accepted that the combination and interaction of genetics, environmental influences, and immunologic abnormalities contribute to the occurrence and perpetuation of this disease [25]. Aminosalicylates, antibiotics, and a range of immunomodulation drugs are used to manage IBD conditions. Complementary improvement of IBD conditions using nondigestible food ingredients has also gained increased recognition. Quercetin is a flavonoid commonly present in food and other plant materials. Reports have shown that quercetin has antiviral, antioxidant, and anti-inflammatory properties [26]. Quercetin-containing microcapsules have been given to mice with acetic acid-induced colitis, which have demonstrated that quercetin treatment is able to decrease neutrophil recruitment, attenuate histological alterations, and reduce macroscopical damage. In addition, quercetin-containing microcapsules can also prevent the reduction of the anti-inflammatory cytokine IL-10 and provide antioxidant properties [27]. Curcumin is another component abundant in plant foods that possesses anti-inflammatory and antioxidant activity and has been demonstrated to be a therapeutic agent for IBD [28]. These phytochemicals are known to be metabolized by gut microbiota [29]; hence, despite the anti-inflammatory and antioxidant actions of these phytochemicals, the amelioration of IBD conditions via modification of gut ecosystems could be one of the underlying mechanisms. This is because the bioavailability and bioactivity of these phytochemicals depend on enzymes of the gut microbiota [30]. The gut ecosystem could in turn be modified by the phytochemical ingested. Indeed, probiotics supplementations have been suggested to be beneficial in IBD [31], and long-term ingestion of prebiotics can selectively stimulate or limit the growth and/or activity of bacteria in the colon, and thus provides a more sustainable effect on colonic bacteria, which improves host health [32]. Nondigestible dietary fibers can act as prebiotics, and it has been reported that ingesting a number of different dietary fibers seems to be beneficial in IBD [33]. They also demonstrated that children with CD who achieved remission by either complete or partial enteral feeding displayed significant modifications to their fecal microbiota. The profiles of fecal microbiota were stable over time for healthy children, which suggested that targeting enteral microbiota using phytochemicals or prebiotics can help achieve remission for IBD patients. Many herbal-based materials have shown antimicrobial effects against certain pathogenic bacteria in vitro; hence, there is an expectation for significant changes in gut microbiota in vivo. A study was performed on the effects of the dietary supplement of *Echinacea purpurea* on aerobic and anaerobic bacteria common to the human gastrointestinal tract. They found that human subjects who consumed 1,000 mg of standardized *E. purpurea* for 10 days had significantly increased total aerobic bacteria, particularly *Bacteroides fragilis*. Supplementation did not significantly alter the number of enteric bacteria, enterococci, lactobacilli, bifidobacteria, or total anaerobic bacteria [34].

Similar to IBD, type 2 diabetes is believed to be a result of complex gene-environmental interactions. Recent evidence points to the importance of gut microbiota as an environmental factor in metabolic diseases, including obesity [14, 35] and diabetes [36]. Diabetic patients showed clear dysfunction of choline

metabolism, indicating a close association between gut microbiota and diabetes [37]. It was further suggested that the presence of *Lactobacillus* species correlated positively with the levels of fasting glucose and glycosylated hemoglobin, whereas the presence of *Clostridium* species correlated negatively with the levels of fasting glucose, glycosylated hemoglobin, insulin, C peptide, and plasma triglycerides [38]. Metformin is the first line of drug in delaying the onset of diabetic condition, and the low-risk alternative herbal medicines are often used to improve glucose tolerance. The *Jiangtang Xiaozhi* capsule is a traditional Chinese herbal formulation, consisting of *radix astragali* and *rehmannia* root, *radix pseudostellariae* and *Mongolian snakegourd* root. There have been animal studies and a small clinical trial, along with studies of the effects of the individual herbs, which showed that the formula has a great potential to improve diabetic condition [39]. Recently, a large clinical trial, including 71 participants treated with *Jiangtang Xiaozhi* capsules for 16 weeks, showed a significant difference in the levels of fasting insulin between the treatment group and the placebo group. Patients taking *Jiangtang Xiaozhi* had a significant improvement in high-density lipoprotein (HDL) level compared to the placebo group [40]. Although attention has not yet been paid to scrutinizing changes of gut microbiota after treating the diabetic patients on this particular case, many of the diabetes-alleviating drugs possess strong antimicrobial properties [41]. Other studies have shown that antidiabetic herbal plants have antibacterial activity, which is not surprising given the fact that most of the phytochemicals become bioavailable after interacting with gut microbiota. Some Indian herbal medicines, which have been known for their hypoglycemic activities, were screened for four Gram-negative and three Gram-positive bacteria. The results showed that the extract of *Mimosa pudica* has a strong antibacterial activity against Gram-positive bacteria such as *Enterococcus faecalis* and *Proteus vulgaris*. Authors have also demonstrated that extracts of *Emblica officinalis* have a broad spectrum of antimicrobial activity followed by *Syzygium cumunii* and *Azadirachta indica* [42]. *Huangguaxiang* (*Matteuccia struthiopteris*) was investigated on the hypoglycemic activities of streptozotocin-induced diabetic rats. The results showed that treatment with *Huangguaxiang* for 8 weeks significantly reduced the levels of triglycerides, low-density lipoprotein, and cholesterol, while levels of bifidobacterium and lactobacillus were also altered following *Huangguaxiang* treatment [43].

The potential roles of herbal medicine in treating cancer or preventing cancer metastasis mainly lie in the possible synergic effects of phytochemicals with chemotherapies and/or the promotion of the immunologic response of the host via interactions with enteric microbiota [44]. The traditional Japanese medicine *juzentaihoto*, containing ten different herbal plants, has been widely used for the prevention of various types of cancer metastasis [45, 46]. Antifungal effects have also been shown for *juzentaihoto* in preventing Candida infection [47, 48]. The most direct evidence for herbal medicine as a treatment for cancer via targeting microbiota is their use in treating *Helicobacter pylori* (*H. pylori*)-induced gastric cancer. *H. pylori*, a Gram-negative bacterium, is commonly found in the human stomach and can cause various diseases including gastritis, peptic ulcer, and gastric cancer. In addition to antibiotics treatment against *H. pylori*, treatments using alternative

herbal medicine are common worldwide. The mode of action could be antibacterial activity, inhibition of adhesion of *H. pylori* to gastric mucus, or both in combination. The extract of *Pelargonium sidoides* roots, a South African herbal medicine, has been shown to inhibit *H. pylori* growth and has a strong adhesion to AGS cells and to intact gastric tissues from *H. pylori*-infected humans [49, 50]. A range of plants native to Brazil, including *Calophyllum brasiliense* Camb. (*Clusiaceae*), *Mouriri elliptica* Martius (*Melastomataceae*), and *Hancornia speciosa* Gomez (*Mangaba*), have also displayed anti-*H. pylori* activity among others [51–53].

Phytomedicine has been commonly used for treating many conditions, apart from the aforementioned cases, due to the fact that phytomedicines are normally comprised of many plant extracts with perhaps thousands of metabolites. In most cases, the molecular mode of action of the active ingredients of these herbal extracts is unknown [54]. In addition, the origins of the plant, time of harvest, and preparation methods will affect the efficacy of the phytomedicine. Therefore, authenticating the medicinal plant and elucidating the mechanism of action using an objective tool, such as metabonomics, would be a way forward to tackle the many research challenges in this area.

10.3 Challenges and Solutions in Herbal Medicine

10.3.1 Assessing the Quality of Phytomedicine

Currently, the quality control of phytomedicines is carried out based on the active ingredients present in a plant. A drawback of this method is that some of the unknown ingredients may have potential synergic interactions with each other and may have certain biological functions. As a consequence, the mechanism of action of these products becomes an impossible task. Thus, the quality control of both raw and final products in a holistic manner is necessary to ensure the consistency of these products and to provide a fundamental ground for further understanding the molecular mechanisms of these products.

Metabonomics that employs ^{1}H NMR spectroscopy facilitates the simultaneous detection of chemical components present in a plant extract as a "metabolic fingerprint" and can meet the requirements for the quality control of an herbal medicine in a holistic manner. Multiple-component analysis, based on the combination of high-resolution NMR spectroscopy with pattern recognition, has been employed to investigate the effects of origin on the chemical compositions of chamomile (*Matricaria recutita*). Clear differences between chamomiles from Northern Africa (Egypt) and Eastern Europe (Hungary and Slovak Republic) can be seen. Chamomiles also have distinguished profiles from Hungary and Slovak Republic based on their metabolomic compositions, despite being close in terms of geographic location. Furthermore, this method is effective for monitoring the "purity" of chamomile samples, such as the percentage of stalks mixed with flowers,

suggesting that this is an excellent method for authenticity and quality control [55]. From a processing point of view, NMR-based metabonomics methods have also been extremely powerful in distinguishing samples extracted with different methods and samples collected at different seasons and dried with different procedures [56]. Metabonomics studies have also been carried out on the extracts of *Artemisia annua* to discriminate samples from different sources and classify them according to their antiplasmodial activity, without preknowledge of this activity [57]. The use of partial least squares analysis also allows the predictions of actual values of such activities for independent samples not used in the model construction. Another study was conducted on the complex pharmaceutical preparations, such as *St. John's wort*, using multivariate analysis of full-resolution ^{1}H NMR spectral data [58]. The results showed that ten preparations from markets were compositionally diverse, and such diversity resulted from plant extract preparation rather than post-extraction processes. The combination of NMR technique and LC-DAD-MS method has been used to investigate the differences between three *Salvia miltiorrhiza* Bunge (SMB) cultivars. The study demonstrated that the combinational use of these methods was effective for plant metabonomics phenotype analysis [59]. These examples are only a reflection on the developments in this area and are by no means exhaustive. In fact, many studies have also been carried out in terms of phytomedicines and authenticity, and it is conceivable that such applications of metabonomics technology will be extended much further in the near future.

10.3.2 Elucidating the Mechanisms of Phytomedicine

Since herbal medicine is a mixture of many plant materials with many chemical components, it is almost impossible to conduct classic pharmacological assays to clearly demonstrate metabolisms of each chemical or unravel specific drug target in an herbal-based drug. These difficulties have restricted their use worldwide. Metabonomics simplifies the complexity by measuring the endpoints of an intervention or a drug effect and hence could provide an alternative strategy for the assessment of herbal medicine. The utilization of a metabonomic approach to evaluate the metabolic action of an herbal medicine has been demonstrated in the human ingestion of chamomile tea [60]. A total of 14 participants were given chamomile tea every morning, and urine samples were collected after one and a half hours after drinking the tea. The metabonomics analysis of urine samples was able to show that chamomile ingestion is a mild intervention to the human body in general, and it causes a reduction in oxidative stress and alters the state of gut microbiota, which was reflected by alternations in the levels of hippurate. The effects of chamomile ingestion on human metabolism were not completely recoverable within a successive week after ingestion. It could suggest that the recovery of gut microbiota is a long process. Metabonomics was also used to evaluate the mechanism of *Xia Yu Xue* decoction, which is a traditional Chinese medicine used for treating liver diseases. Metabolic trajectory showed the trend of renormalization of the *Xia Yu Xue*

decoction to CCl_4-induced liver dysfunction. In addition, changed metabolites indicated modulations of energy metabolism, microflora metabolism, amino acid and fatty acid metabolism, which are found to be associated with *Xia Yu Xue* decoction ingestion [61]. The traditional medical treatment targets on the balances of *yin* and *yang*. "Kidney-yang deficiency syndrome" is one of the conditions that relate to functional disorders associated with the hypothalamic-pituitary-gland axis [62]. *Epimedium* (Berberidaceae) is known in Chinese medicine as able to strengthen "yang" and is often used to treat "kidney-yang deficiency." The metabonomics technique was applied to investigate the capability of *Epimedium koreanum* to restore metabolic disorder in animals with "kidney-yang deficiency." After 15 days of orally administering *Epimedium koreanum* extract, metabolic disorders associated with "kidney-yang deficiency" returned to normal. These disordered metabolic pathways included amino acid metabolism, lipid metabolism, and energy metabolism. Importantly, *Epimedium koreanum* ingestion possessed effects on balancing gut microbiota [63]. Most herbal medicines contain a range of polyphenolic compounds, which often act as active ingredients. Gallic acid is one of these polyphenolic compounds, which has been shown to inhibit xanthine oxidase [64, 65], ribonucleotide reductase [66, 67], and histamine release in mast cells [68]. Systematically analyzing the metabolic effects of gallic acid to the metabonomes of rat plasma, liver, urine, and feces showed that gallic acid promoted oxidative stress and resulted insignificant metabolic changes involving glycogenolysis, glycolysis, tricarboxylic acid cycle (TCA), and the metabolism of amino acids, purines, and pyrimidines, together with gut microbiota functions [69].

10.4 Concluding Remarks

In this chapter, we have shown examples of the close associations between human diseases and the gut microbiota living within us, and we have exemplified how herbal medicine could treat disease and maintain health by modifying gut microbiota. In addition, we have emphasized the advantages and challenges of herbal medicine. The literature has shown that there is a potential for future research on the efficacies of herbal medicine and the requirement for international standards for herbal medicine to be established. We have shown that metabolomics can facilitate the understanding of the intrinsic quality of herbal medicine and the evaluation of the therapeutic effects of the complex herbal formulas. This technique should be equally effective for evaluating mineral treatment and acupuncture, which have not been discussed in this chapter. Understanding the mechanisms of action is a necessary step for herbal medicine to be more adoptable worldwide, which should certainly be warranted some attention. It is with no doubt that further development of the metabonomics technique, such as high-sensitivity and high-specificity detection of metabolites, as well as advances in effective molecular identification, would promote the process of evaluation and acceptance of herbal medicine. Although metabonomics can provide a great understanding of the molecular mechanisms of herbal

medicine, direct measurement of alterations in gut microbiota associated with herbal treatment using microbiological assays is still lacking. Biological assays evaluating changes in gut microbiotia should be concurrently joined with a metabonomics evaluation of the mechanisms of specific herbal formulas to provide a comprehensive view on the action of herbal medicine.

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References

1. Carroll IM, et al. Alterations in composition and diversity of the intestinal microbiota in patients with diarrhea-predominant irritable bowel syndrome. Neurogastroenterol Motil. 2012;24(6):521-+.
2. Hooper LV, Gordon JI. Commensal host-bacterial relationships in the gut. Science. 2001;292(5519):1115–8.
3. Gordon JI. Honor thy gut symbionts redux. Science. 2012;336(6086):1251–3.
4. Kurokawa K, et al. Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes. DNA Res. 2007;14(4):169–81.
5. Tremaroli V, Backhed F. Functional interactions between the gut microbiota and host metabolism. Nature. 2012;489(7415):242–9.
6. Le Gall G, et al. Metabolomics of fecal extracts detects altered metabolic activity of gut microbiota in ulcerative colitis and irritable bowel syndrome. J Proteome Res. 2011;10(9):4208–18.
7. Rea MC, et al. Effect of broad- and narrow-spectrum antimicrobials on Clostridium difficile and microbial diversity in a model of the distal colon. Proc Natl Acad Sci. 2011;108(Supplement 1):4639–44.
8. Li HK, et al. Traditional Chinese medicine: balancing the gut ecosystem. Phytother Res. 2009;23(9):1332–5.
9. Zhang HS, et al. Human gut microbiota in obesity and after gastric bypass. Proc Natl Acad Sci U S A. 2009;106(7):2365–70.
10. Nagalingam NA, Lynch SV. Role of the microbiota in inflammatory bowel diseases. Inflamm Bowel Dis. 2012;18(5):968–80.
11. Ponnusamy K, et al. Microbial community and metabolomic comparison of irritable bowel syndrome faeces. J Med Microbiol. 2011;60(6):817–27.
12. Greenblum S, Turnbaugh PJ, Borenstein E. Metagenomic systems biology of the human gut microbiome reveals topological shifts associated with obesity and inflammatory bowel disease. Proc Natl Acad Sci U S A. 2012;109(2):594–9.
13. Turnbaugh PJ, Gordon JI. The core gut microbiome, energy balance and obesity. J Physiol Lond. 2009;587(17):4153–8.
14. Turnbaugh PJ, et al. A core gut microbiome in obese and lean twins. Nature. 2009;457(7228):480–U7.
15. Mathis D, Benoist C. The influence of the microbiota on type-1 diabetes: on the threshold of a leap forward in our understanding. Immunol Rev. 2012;245:239–49.
16. Wang HY, et al. Bioactivity enhancement of herbal supplements by intestinal microbiota focusing on ginsenosides. Am J Chin Med. 2011;39(6):1103–15.
17. Topping DL, Clifton PM. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiol Rev. 2001;81(3):1031–64.

18. Russell SL, et al. Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. EMBO Rep. 2012;13(5):440–7.
19. Zhao L, et al. Targeting the human genome-microbiome axis for drug discovery: inspirations from global systems biology and traditional Chinese medicine. J Proteome Res. 2012;11(7):3509–19.
20. Bailey NJC, et al. Multi-component metabolic classification of commercial feverfew preparations via high-field H-1-NMR spectroscopy and chemometrics. Planta Med. 2002;68(8):734–8.
21. Nicholson JK, Lindon JC, Holmes E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica. 1999;29(11):1181–9.
22. Nicholson JK, et al. Metabonomics: a platform for studying drug toxicity and gene function. Nat Rev Drug Discov. 2002;1(2):153–61.
23. Tang HR, Wang YL. Metabonomics: a revolution in progress. Prog Biochem Biophys. 2006;33(5):401–17.
24. Lindon JC, Holmes E, Nicholson JK. Metabonomics: systems biology in pharmaceutical research and development. Curr Opin Mol Ther. 2004;6:265–72.
25. Hanauer SB. Inflammatory bowel disease: epidemiology, pathogenesis, and therapeutic opportunities. Inflamm Bowel Dis. 2006;12:S3–9.
26. Vasanthi HR, ShriShriMal N, Das DK. Phytochemicals from plants to combat cardiovascular disease. Curr Med Chem. 2012;19(14):2242–51.
27. Guazelli CFS, et al. Quercetin-loaded microcapsules ameliorate experimental colitis in mice by anti-inflammatory and antioxidant mechanisms. J Nat Prod. 2013;76(2):200–8.
28. Ali T, Shakir F, Morton J. Curcumin and inflammatory bowel disease: biological mechanisms and clinical implication. Digestion. 2012;85(4):249–55.
29. Atkinson C, Frankenfeld CL, Lampe JW. Gut bacterial metabolism of the soy isoflavone daidzein: exploring the relevance to human health. Exp Biol Med. 2005;230(3):155–70.
30. Karlsson FH, et al. A closer look at bacteroides: phylogenetic relationship and genomic implications of a life in the human gut. Microb Ecol. 2011;61(3):473–85.
31. Ewaschuk JB, Dieleman LA. Probiotics and prebiotics in chronic inflammatory bowel diseases. World J Gastroenterol. 2006;12(37):5941–50.
32. Lim CC, Ferguson LR, Tannock GW. Dietary fibres as "prebiotics": implications for colorectal cancer. Mol Nutr Food Res. 2005;49(6):609–19.
33. Lionetti P, et al. Enteral nutrition and microflora in pediatric Crohn's disease. J Parenter Enteral Nutr. 2005;29(4):S173–8.
34. Hill LL, et al. Echinacea purpurea supplementation stimulates select groups of human gastrointestinal tract microbiota. J Clin Pharm Ther. 2006;31(6):599–604.
35. Noble D, et al. Risk models and scores for type 2 diabetes: systematic review. Br Med J. 2011;343:1–31.
36. Qin JJ, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature. 2012;490(7418):55–60.
37. Zhang X, et al. Human serum metabonomic analysis reveals progression axes for glucose intolerance and insulin resistance statuses. J Proteome Res. 2009;8(11):5188–95.
38. Karlsson FH, et al. Gut metagenome in European women with normal, impaired and diabetic glucose control. Nature. 2013;498(7452):99-+.
39. Ge Z, Jin L, Guo Y. Experimental study of jiangtang xiaozhi tablet on decreasing the levels of blood glucose and serum lipids in transgenic mice with diabetes mellitus. Chin J Integ Tradit West Med. 2012;32(8):1095–8.
40. Grant SJ, et al. Chinese herbal medicine for impaired glucose tolerance: a randomized placebo controlled trial. BMC Complement Altern Med. 2013;13:104. p. (14 May 2013)-(14 May 2013).
41. Baharum SN, Nathan ME, Mokhtar MAA. Molecular characterization of lipase producer from local marine environment. J Pure Appl Microbiol. 2009;3(2):387–92.

42. Jyothi KS, et al. Anti-bacterial response of twelve plants of folk-lore medicine used against diabetes mellitus. J Phytol Res. 2007;20(2):161–70.
43. Hai-bo W, et al. Role of Huangguaxiang in lipid metabolism and its effect on normal intestinal bacteria of diabetic rats. Chin J Microecol. 2009;21(10):916–7.
44. Wang C-Z, Calway T, Yuan C-S. Herbal medicines as adjuvants for cancer therapeutics. Am J Chin Med. 2012;40(4):657–69.
45. Saiki I. A Kampo medicine "Juzen-taiho-to" – prevention of malignant progression and metastasis of tumor cells and the mechanism of action. Biol Pharm Bull. 2000;23(6):677–88.
46. Sakamoto S, et al. Anticancer effects of a Chinese herbal medicine, Juzen-taiho-to, in combination with or without 5-fluorouracil derivative on DNA-synthesizing enzymes in 1,2-dimethylhydrazine induced colonic-cancer in rats. Am J Chin Med. 1991;19(3–4): 233–41.
47. Abe S, et al. Protective effect of oral administration of a traditional medicine, Juzen-taiho-to, and its components on lethal Candida albicans infection in immunosuppressed mice. Immunopharmacol Immunotoxicol. 1998;20(3):421–31.
48. Akagawa G, Abe S, Tansho S. Protection of C3H/HE J mice from development of Candida albicans infection by oral administration of Juzen-taiho-to and its component, ginseng radix: possible roles of macrophages in the host defense mechanisms. Immunopharmacol Immunotoxicol. 1996;18(1):73–89.
49. Beil W, Kilian P. EPs (R) 7630, an extract from Pelargonium sidoides roots inhibits adherence of Helicobacter pylori to gastric epithelial cells. Phytomedicine. 2007;14:5–8.
50. Wittschier N, Faller G, Hensel A. An extract of Pelargonium sidoides (EPs 7630) inhibits in situ adhesion of Helicobacter pylori to human stomach. Phytomedicine. 2007;14(4):285–8.
51. Moleiro FC, et al. Mouriri elliptica: validation of gastroprotective, healing and anti-Helicobacter pylori effects. J Ethnopharmacol. 2009;123(3):359–68.
52. Moraes TM, et al. Hancornia speciosa: Indications of gastroprotective, healing and anti-Helicobacter pylori actions. J Ethnopharmacol. 2008;120(2):161–8.
53. Souza MC, et al. In vitro and in vivo anti-Helicobacter pylori activity of Calophyllum brasiliense Camb. J Ethnopharmacol. 2009;123(3):452–8.
54. Vitor JMB, Vale FF. Alternative therapies for Helicobacter pylori: probiotics and phytomedicine. FEMS Immunol Med Microbiol. 2011;63(2):153–64.
55. Wang YL, et al. Metabolomic strategy for the classification and quality control of phytomedicine: a case study of chamomile flower (Matricaria recutita L.). Planta Med. 2004;70(3): 250–5.
56. Xiao C, et al. Revealing the metabonomic variation of rosemary extracts using H-1 NMR spectroscopy and multivariate data analysis. J Agric Food Chem. 2008;56(21):10142–53.
57. Bailey NJC, et al. Prediction of anti-plasmodial activity of Artemisia annua extracts: application of H-1 NMR spectroscopy and chernometrics. J Pharm Biomed Anal. 2004;35(1):117–26.
58. Rasmussen B, et al. Multivariate analysis of integrated and full-resolution H-1-NMR spectral data from complex pharmaceutical preparations: St. John's wort. Planta Med. 2006;72(6): 556–63.
59. Dai H, et al. Combined NMR and LC-DAD-MS analysis reveals comprehensive metabonomic variations for three phenotypic cultivars of Salvia Miltiorrhiza Bunge. J Proteome Res. 2010;9(3):1565–78.
60. Wang YL, et al. A metabonomic strategy for the detection of the metabolic effects of chamomile (Matricaria recutita L.) ingestion. J Agric Food Chem. 2005;53(2):191–6.
61. Gou X, et al. Urinary metabonomics characterization of liver fibrosis induced by CCl4 in rats and intervention effects of Xia Yu Xue Decoction. J Pharm Biomed Anal. 2013;74:62–5.
62. Yang J, et al. The total flavones from Semen cuscutae reverse the reduction of testosterone level and the expression of androgen receptor gene in kidney-yang deficient mice. J Ethnopharmacol. 2008;119(1):166–71.

63. Huang D, et al. An integrated plasma and urinary metabonomic study using UHPLC-MS: intervention effects of Epimedium koreanum on 'Kidney-Yang Deficiency syndrome' rats. J Pharm Biomed Anal. 2013;76:200–6.
64. Lespade L, Bercion S. Theoretical study of the mechanism of inhibition of xanthine oxidase by flavonoids and gallic acid derivatives. J Phys Chem B. 2010;114(2):921–8.
65. Masuoka N, Nihei K-i, Kubo I. Xanthine oxidase inhibitory activity of alkyl gallates. Mol Nutr Food Res. 2006;50(8):725–31.
66. De Bona KS, et al. Syzygium cumini extract decrease adenosine deaminase, 5′ nucleotidase activities and oxidative damage in platelets of diabetic patients. Cell Physiol Biochem. 2010;26(4–5):729–38.
67. Madlener S, et al. Gallic acid inhibits ribonucleotide reductase and cyclooxygenases in human HL-60 promyelocytic leukemia cells. Cancer Lett. 2007;245(1–2):156–62.
68. Kim SH, et al. Gallic acid inhibits histamine release and pro-inflammatory cytokine production in mast cells. Toxicol Sci. 2006;91(1):123–31.
69. Shi X, et al. Gallic acid intake induces alterations to systems metabolism in rats. J Proteome Res. 2013;12(2):991–1006.

Chapter 11
Gut Microbiota and Metabolic Diseases: From Pathogenesis to Therapeutic Perspective

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Abstract Intestinal microbiota is now considered as a "new organ" which, over and above their genetic origin, de-orphans the pandemic development of metabolic diseases. The trillions of bacteria and their corresponding million genes which inhabit our gut provide a unique source of molecular hypotheses to explain the wide diversity of metabolic diseases and hence form a basis to reach the important objective of personalized medicine. The gut and more recently the tissue microbiome could be the source of: (1) new biomarkers predicting and classifying metabolic diseases to help the clinician to propose the best therapeutic strategy, and (2) new pharmacological and nutritional strategies to treat the cause rather than the consequence of diabetes and obesity. The field of immunometabolism should be extended to microbio-immunometabolism, thus reconciling the role of the environment, the genetic background, and individual diversity in relation to the onset and development of metabolic diseases.

Keywords Diabetes • Bacterial translocation • Immune system • Incretins • Inflammation • Immunometabolism • Intestine • Metabolic diseases • Metabolic inflammation • Metabolic infection • Microbiome • Obesity • Tissue microbiota

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11.1 Introduction

Evidence now shows that the pandemic progression of diabetes and obesity, as well as their numerous complications (cardiovascular, hepatic, renal, neuronal), is caused by lifestyle factors including sedentarity and fat-enriched diet which has replaced fiber-enriched diet (Fig. 11.1). A dramatic outcome of this epidemic is the increasing number of cardiovascular events leading to mortality [1–4] – the mechanisms at play need to be delineated in order to define new therapeutic strategies. Over the last decade lethal cardiovascular events associated with diabetes have progressed by 62 % [5, 6]. This is much higher than the risk linked to cholesterol levels or hypertension. The incidence of type 2 diabetes is 4–5 % in Europe, 8–10 % in the USA, and higher in South Asia [7]. These numbers have more than doubled over the last 20 years. Therefore, one can suggest that even if genetic analyses provide the basis for such an epidemic, changes in our genome cannot be solely responsible. One interpretation is that our genome is no longer adapted to environmental factors. Numerous environmental hypotheses have been proposed. First, epigenetic non-coded functions that are independent of genomic factors could have an impact. Second, perhaps more realistically, the impact of changes in feeding habits and

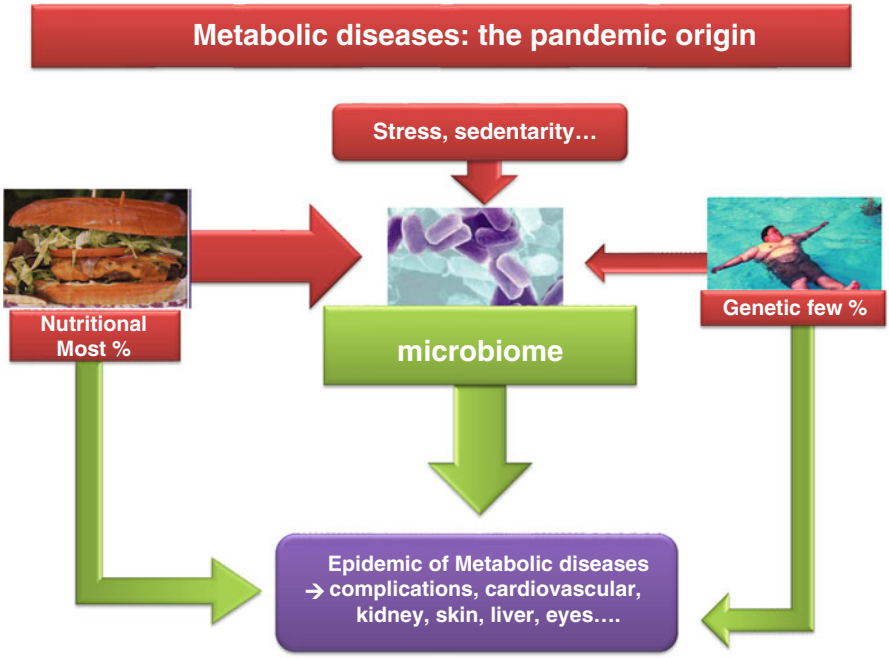

Fig. 11.1 The pandemic origin of metabolic diseases. The impact of genetics, diet, and other factors on the incidence and the development of metabolic disease pandemic could have a change in gut microbiota as an origin. Gut microbiota would also favor the development of complications of metabolic diseases such as cardiovascular, liver, eye, and skin complications

social behavior is likely to be an important cause of the growing incidence of metabolic diseases. This phenomenon could be linked to the microbiota, our "second genome" harboring almost ten times more prokaryotic cells than eukaryotic cells in our body [8]. All mammalian organisms are born sterile, without any microbiota which is inherited at birth. During the first hours, days, and weeks of life, microbiota from the mother and the environment colonize the body of the newborn in a specific order [9]. The microbiota colonizes the intestine during the first 3–5 years of life and is species-, age-, and sex-dependent [10]. The initial infant gut microbiota is a simple structure usually dominated by bifidobacteria, and through a series of successions and replacements, it shifts to a more complex adult pattern [11–15]. The microbiota also undergoes substantial changes at the extremes of life, in infants and older people, the ramifications of which are still being explored [16]. Then, throughout life the bacterial ecology tends to vary mostly according to the environment [17]. Each individual has at least 160 shared species and a number of well-balanced host-microbial molecular relationships that define groups of individuals [18, 19]. This second genome is the metagenome. The importance of this metagenome resides in its gene repertoire, 100 times superior to the eukaryotic nuclear genome [18, 19], thus providing a huge genetic diversity susceptible to convey a plethora of functions [18, 19]. Indeed, the tremendous efforts that have been made in bioinformatic analyses have allowed the encoding and the deciphering of all sequences. Humans host different metagenomes from multiple locations such as the skin, lungs, vagina, and the mouth in addition to the intestine [8, 20]. The human gut hosts 100 trillion microorganisms, encompassing up to thousands of species at an average concentration of 10^{14} per ml and weighing in average 1.5 kg [21]. A major observation is that the metagenomic diversity is extremely large and represents a signature of each individual. Its plasticity is significant and depends on numerous environmental and genetic factors that can evolve over time and could explain the rapid development of metabolic diseases. The adult intestinal microbiota has been shown to be relatively stable over time [22] and is sufficiently similar between individuals to allow identification of a core microbiome comprising 66 dominant operational taxonomic units (OTUs) that correspond to 38 % of sequence reads from 17 individuals [18, 19]. The core microbiota changes to become distinct in elderly subjects from that observed for younger adults with a greater proportion of *Bacteroides* spp. and typical abundance patterns of *Clostridium* groups. Interestingly, the onset of metabolic diseases increases with age and is associated with a change in intestinal microbiota as observed during aging [16, 23, 24]. Similarly, it was shown that changes in gut microbiota characterize obesity and diabetes [18, 19]. This suggests that each member can interact in a perfect mutual symbiosis defining a steady microbiota [8, 20, 25]. Hence, a new concept has emerged with an important influence on our understanding of these pathologies. Major advances have been made over the course of the last decade, thanks to the development of high-throughput sequencing of the microbiota and to the use of germ-free mice. These have allowed the demonstration of the causality of the microbiota from the gut [26], from the oral cavity, and more recently from the tissue [27] on the development of metabolic diseases. The following decades will be dedicated to the identification of the

molecular crosstalk between the microbiota and the host to understanding mechanisms controlling diabetes and obesity. The intestinal immune system appears to be a major player in the field since at birth bacterial colonization of the intestine represents an outbreak of antigens that can educate the immune system, as well other major functions such as the vascular and the nervous systems. The latter through its connection with the brain could participate in the maturation of the central nervous system over the course of a lifetime [28]. Consequently, beyond the key physiological role of the intestinal microbiota in normal development, numerous pathological issues could be the consequence of an impaired microbiota. In addition to the first described role of the microbiota on the development of intestinal bowel diseases, there is now evidence that a change in the intestinal bacterial ecology could affect metabolic, vascular, liver, heart, oral, and neurodegenerative diseases. More evidence is required to confirm a role in arthritis and skin immunological diseases. The recent increase in the use of bariatric surgery for the treatment of massive obesity and incidental diabetes has generated further evidence reinforcing the role of intestinal microbiota in the control of metabolic diseases.

New clinical approaches for prevention and therapy are now being planned. Functional food and pharmaceutical strategies, based on the targeting of the microbiota to host interactions, can be initiated. In the face of the large diversity of metabolic phenotypes, i.e., a large spectrum of fasted and postprandial glycemia and different fat mass distribution, "intelligent and directed" food complements can be proposed. They should not prevent or treat massively the populations but will focus on subgroups of patients with similar microbiota-related diseases. The relevance of treating the impact of gut microbiota on the diseases requires the development of companion biomarkers. They should first define subgroups of patients with similar microbiota profiles and should be able to follow up the efficacy of the microbiota change in correlation with the metabolic phenotype treated. Hence, they will drive the therapeutic approach toward appropriate subgroups of patients to improve the efficacy of the treatment and reduced secondary effects. Pharmaceutical approaches targeting a mechanism central to the molecular crosstalk between microbiota and the host, such as metabolic inflammation, have currently been developed by means of fecal transplant and immunomodulation. Therefore, it is now time to consider the intestinal microbiota as a new organ controlling metabolism. This organ is characterized by a high level of plasticity so that it can adapt to a change in the host behavior in a reciprocal manner for the control of broad host physiological functions. The understanding of its molecular components will lead to a totally new way of interpreting physiological, clinical, and therapeutic data. Therefore, the limit between nutritional and pharmacological strategies is vanishing. Intelligent food supplements and cause-based pharmacological approaches will be the treatments of tomorrow that still have to be identified.

Pharmaceutical strategies will benefit from the gain of knowledge generated based on the intestinal microbiota to host relationship. The corresponding molecular crosstalk is currently being assessed and will be reviewed below. It includes mechanisms controlling immunomodulation, bile acid conjugation, the intestinal barrier, energy harvesting, and entero-endocrine hormone secretion. The bacterial

factors, i.e., the metafactors controlling these eukaryotic functions, are of inflammatory origin such as the lipopolysaccharides, peptidoglycan fragments, flagellin, or derived from fermentation products. Hence, pharmacological strategies should emerge from these concepts and target subgroups of patients with these new drugs acting on the host to microbiota relationship.

An important matter relates to the identification of subgroups of patients sensitive to these new approaches, most likely characterized by a specific dysbiosis and impaired gut and tissue microbiota. Hence, microbiota-based biomarkers precisely identifying these subgroups will help the clinician to treat the patients with the appropriate pharmaceutical strategy.

11.2 From the Main Features of the Pathophysiology of Metabolic Diseases to Microbiota

Metabolic diseases are all characterized by alterations in energy balance which explains, at least in part, the occurrence of obesity. The disease is the consequence of either an increased energy uptake or a reduced energy expenditure demonstrating that different mechanisms are responsible for increased body weight. Furthermore, the accumulation of fat in the body is compartmentalized, i.e., abdominal, subcutaneous. or intratissular such as in the liver, the heart, or the pancreatic islets with different consequences on overall health. The incidence of diabetes is much increased in patients with normal body weight but with abdominal fat accumulation which is considered the most deleterious for health [29]. Similarly, the accumulation of triglycerides within the Langerhans islets impairs insulin secretion [30] that can be restored by leptin treatment [31] activating uncoupling proteins such as UCP2 [32]. At the onset of obesity, the storage of energy is associated with hypertrophy of the adipocytes and their hyperplasia to ensure a sufficient number of cells [33–35]. The signals favoring adipose depot development could be linked to increased adipocyte metabolism leading to a local hypoxia [36] and the recruitment of adipocyte precursors [37, 38]. The maintenance of the adipose depot architecture is ensured by a concomitant proliferation of endothelial precursors [39, 40], increasing the adipose tissue capillaries and hence blood and energy supply [40]. Hyperphagia, supplying large amounts of energy, and hyperinsulinemia, although associated with normal blood glucose profiles, also suggest a neuroendocrine origin of the disease notably, the gut to brain axis which is recruited in response to an oral glucose load [41] or food intake [42, 43]. A key enteroendocrine factor, glucagon-like peptide 1 (GLP-1), triggers the gut–brain axis [42, 44, 45] which is impaired during high-fat-diet-induced metabolic diseases [46]. The brain is also sensitive to hormones from the periphery such as insulin and leptin which no longer efficiently control food intake and energy partitioning. Altogether, impaired gut and peripheral nutrients and hormone sensing systems, as well as signaling effectors toward the adipose depot via the brain, are impaired during obesity. The increase of brain to adipose depot signals then enhances the capacity of the adipose depot to store

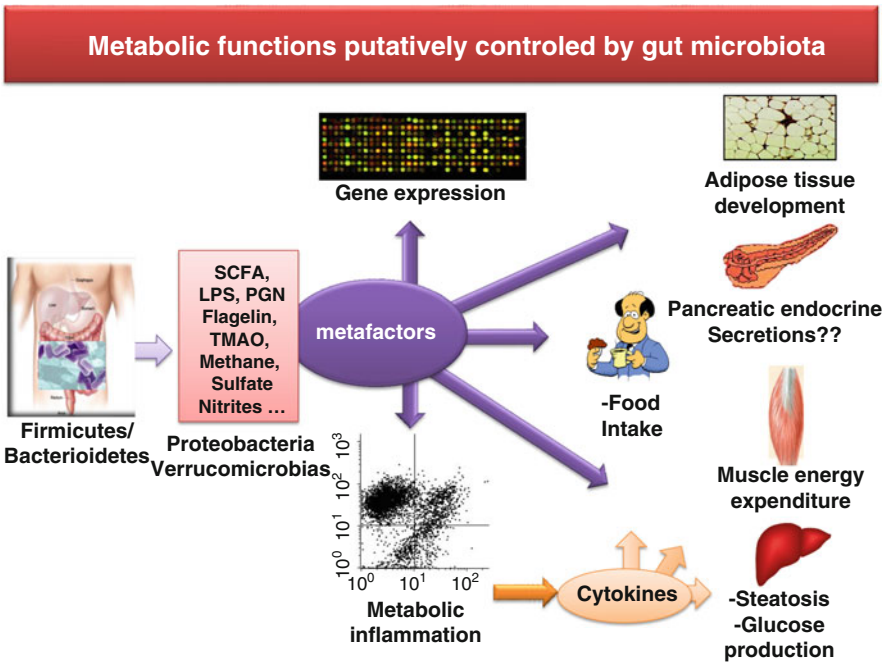

Fig. 11.2 Metabolic functions putatively controlled by gut microbiota. Metafactors, i.e., bacterial compounds such as short-chain fatty acids, acetate, propionate, butyrate (SCFA), lipopolysaccharides (LPS), peptidoglycan (PGN), flagellin, and trimethylamine oxide (TMAO), could be involved in the control of metabolic functions, by means of gene expression and by installing a metabolic inflammation leading to cytokine production which could then impair all metabolic functions

energy (Fig. 11.1). However, it should be noted that increasing food intake and activating the storage of energy in fat depots cannot be considered as a pathological mechanism since it is a normal behavior of the body following a fasting period to replenish the fat stores. The pathology starts when this process is no longer down-regulated. Different levels of deregulation occur involving either early control of food intake and energy distribution or processes that control each step of the obesity process. The intestinal microbiota has been proposed to interfere with hyperphagia and to some extent with energy storage – this will be reviewed below. The emerging role of gut microbiota in the gut to brain axis for the control of neural development, behavior, and food intake will certainly generate new molecular hypotheses regarding the development of obesity [28, 47–49].

Type 2 diabetes arises due to impaired secretion and action of insulin which evolve either simultaneously or independently according to numerous influencing factors (Fig. 11.2). An increased glucose intolerance with glycemic profiles higher than controls is a common feature at the onset of the disease [50, 51]. This is associated with hepatic insulin resistance where insulin does not efficiently reduce hepatic glucose production following a meal [50–52]. Liver insulin resistance is hence involved in glucose intolerance and type 2 diabetes and explains fasting glycemia

[53–56]. Simultaneously, insulin-stimulated glucose uptake is reduced which is most likely a consequence of an impaired cellular lipid metabolism [57]. Numerous hypotheses could explain insulin resistance and among those the role played by a low-grade inflammation, called metabolic inflammation since it is chronic and not related to infection or cancer [58]. Briefly, metabolic inflammation is characterized by an increased infiltration of immune cells within organs involved in the glycemic control such as the adipose tissue, the liver, and muscles [59, 60]. Cells from the innate and adaptive immune systems secrete cytokines such as TNFa and IL1-b that impair insulin signaling, thus affecting both glucose and lipid metabolism (Fig. 11.2) [59, 60]. Inflammation is increasingly regarded as a key process underlying metabolic diseases [59–64]. In the adipose tissue of individuals with metabolic diseases, this mechanism includes features characteristic of active local inflammation [63, 65, 66]. The cytokines released impair insulin signaling [67–69], thus leading to cytokine-mediated insulin resistance [70, 71]. Macrophage infiltration of adipose tissue has been described in both mice and humans [64–66, 72–74]. It is suggested that these cells express TNF-alpha and iNOS that are characteristic of M1-type macrophages which are responsible for almost all adipose tissue TNF-alpha expression and significant amounts of iNOS and IL-6 expression [73, 75, 76]. In addition, using functional analyses and microarray technology, it has been demonstrated that adipocyte progenitors and macrophages are characterized by a closed genome and phenotypome [77, 78]. Based on measurements of phagocytic activity and gene profiling analysis of different progenitor cells, we revealed that the origin of inflammation could also be attributed to cells initially present in adipose fat pads such as preadipocytes [77, 78]. Therefore, both infiltrating and resident cells are most likely involved in the processes characterizing adipose tissue inflammation. Similarly, lymphocytes are associated with adipose tissue inflammation [64, 79, 80]. CD8 T cells are present in obese mouse adipose tissue even before the infiltration of the tissue by macrophages. The results also showed that the immunological and genetic depletion of $CD8^+$ T cells lowered macrophage infiltration and adipose tissue inflammation and improved systemic insulin resistance. Conversely, adaptive transfer of $CD8^+$ T cells to CD8-deficient mice aggravated adipose inflammation. Co-culture and other in vitro experiments revealed a cycle of interactions between $CD8^+$ T cells, macrophages, and adipose tissue. Moreover, $CD4^+$ regulatory T lymphocytes (Treg) ($CD4^+FoxP3^+$), inhibitory cells of the immune system, decrease in obese adipose tissue [80]. Increases in Treg by antibody treatment (IL-2/anti-IL-2-induced Treg proliferation) improve HFD-induced insulin resistance [80]. Furthermore, transfer of $CD4^+$ T lymphocytes with anti-inflammatory properties decreases HFD-induced glucose intolerance and insulin resistance [79]. The reasons for adipose tissue T lymphocyte and macrophage infiltration are unknown, but it was suggested that the corresponding antigens could be related to intestinal microbiota [6, 27, 81].

Altogether, little is known about the sequence of events which lead to an increased number of macrophages and lymphocytes in metabolic tissues. The origin of this cascade of events could be related to a change in intestinal microbiota and, as detailed below, to a change in tissue microbiota [27]. This is linked to a

translocation of bacteria and bacterial components from the intestine to tissues establishing a tissue microbiota leading to metabolic inflammation. Hence, the role of gut microbiota could be related to its direct role on molecular targets controlling insulin secretion and action, hepatic glucose production, adipose tissue development, and therefore, the incidence of diabetes. These hypotheses will be detailed below.

Although more than 150 genetic loci are associated with the monogenic or multifactorial forms of obesity and type 2 diabetes, their impact on the incidence of the disease is rather low (5–10 % and 2 %, respectively) [82]. In genetically identical twins the incidence of type 2 diabetes is 20–60 % [83, 84]. Even in type 1 diabetes, genetically identical twins develop the disease with an incidence of no more than 50 %. A major impact of the environment has recently been attributed to the role of intestinal microbiota where genes involved in the recognition of bacterial patterns were involved [85, 86]. The innate immunity to microbiota relationship was causally implied. It was shown that the commensal microbial community alters sex hormone levels and regulates autoimmune disease fate in individuals with high genetic risk [87]. On the other hand, environmental factors such as stress, a sedentary lifestyle, and nutritional habit could explain the pandemic progression of metabolic impairment. Humans could be considered as "super-organisms" as a result of their symbiotic association with the gut microbiota [88].

11.3 Lessons from the Gut Microbiota to Metabolic Diseases Relationship

Complex microbial ecosystems occupy the skin, mucosa, and alimentary tract of all mammals, including humans [8]. The species that make up these communities vary between hosts as a result of restricted migration of microorganisms between weak and strong ecological interactions within hosts. Furthermore, diet, genotype, and colonization history also influence this ecology [20]. Hence, a mutual relationship characterizes the host to microbiota crosstalk by which each partner has its own interest and informs the other of the environmental and metabolic situation. The microbiota is now considered as a symbiont that shares with its host the influence of the environment, diet, stress, and the physiological state. Specific communities inhabit the different epithelia according to the physical and biochemical characteristics of each location. With regard to the gastrointestinal tract, it is colonized by a vast community of symbionts and commensals that have important effects on immune function, nutrient processing, and a broad range of other host activities [89].

The precise role of intestinal microbiota on the control of metabolic diseases emerged in 2004 with the discovery that germ-free mice resist high-fat-diet-induced obesity [90]. It was shown that germ-free mice colonized with microbiota harvested from the cecum of a healthy mouse gain 60 % body fat content and became insulin resistant within 14 days despite reduced food intake. The mechanism was due to an increased production of monosaccharides. These molecules are generated from the

metabolism of polysaccharides by the cecal microbiota, thus providing an additional source of carbohydrate to the body. However, a rate-limiting factor was that a large part of the microbiota cannot be identified since it is highly difficult to culture. This major problem has been overcome by the use of very-high-throughput sequencing techniques coupled with new bioinformatics approaches. This strategy allowed the identification of the taxons within the microbiota from human and animal intestinal content [91, 92] as well as the overall catalog of bacterial genes [18, 19]. This has allowed the correlation of metabolic diseases to specific bacteria or groups of bacterial genes [93]. An increased *Firmicutes*-to-*Bacteroidetes* ratio seems to be a signature of metabolic diseases influencing processes related to energy harvesting, intestinal permeability, bile acid metabolism, brain functions related to metabolism, and immunomodulation.

11.3.1 The Gut Microbiota as a Signature of Metabolic Diseases

An altered gut microbiota has been linked to metabolic diseases including obesity [26, 94], diabetes [19], and cardiovascular diseases [95]. A core microbiome can be found at the gene level, despite large variations in community membership, and that variations from the core are associated with obesity [25, 94, 96–98]. Using a protocol for a metagenome-wide association study (MGWAS) based on deep shotgun sequencing of the gut microbial DNA from 345 Chinese individuals, approximately 60,000 type 2 diabetes-associated markers have been identified and validated, thus establishing the concept of a metagenomic linkage group [19]. This MGWAS analysis showed that patients with type 2 diabetes were characterized by a moderate degree of gut microbial dysbiosis, a decrease in the abundance of some universal butyrate-producing bacteria, and an increase in various opportunistic pathogens, as well as an enrichment of other microbial functions conferring sulfate reduction and oxidative stress resistance. Three enterotypes could be identified from the Chinese samples which were primarily made up of several highly abundant genera, including *Bacteroides*, *Prevotella*, *Bifidobacterium*, and *Ruminococcus*. However, no significant relationship between enterotype and type 2 diabetes status was found. However, when using the gene reference (KEGG orthologue genes and eggNOG group profiles) rather than the phylogenic profiles, a total of 1,345 biomarkers were identified. Type 2 diabetes-associated biomarkers were mostly involved in membrane transport systems. By contrast, control-enriched markers were frequently involved in cell motility and metabolism of cofactors and vitamins. When studying pathway levels, the gut microbiota of type 2 diabetic patients showed enrichment in membrane transport of sugars, branched-chain amino acid (BCAA) transport, methane metabolism, xenobiotics degradation and metabolism, and sulfate reduction. By contrast, there was a decrease in the level of bacterial chemotaxis, flagellar assembly, butyrate biosynthesis, and metabolism of cofactors and vitamins. Seven of the markers were also related to oxidative stress resistance suggesting that the gut

environment of type 2 diabetic patients stimulates bacterial defense mechanisms against oxidative stress [19]. A further specific mathematical model for biomarker identification has been developed and showed compositional and functional alterations in the metagenomes of a specific cohort of 145 women with type 2 diabetes [99]. Type 2 diabetic women were characterized by an increased abundance of four *Lactobacillus* species and decreases in the abundance of five *Clostridium* species. The total *Lactobacillus* species correlated positively with fasting glucose and HbA1c (glycosylated hemoglobin), whereas the *Clostridium* species correlated negatively with fasting glucose, HbA1c, insulin, C-peptide, and plasma triglycerides. Importantly, impaired glucose tolerance could be identified on the basis of this mathematical modeling. The impact of medication and hyperglycemia on the metagenome was not considered as major confounding factors. Comparisons with a Chinese cohort demonstrated that the biomarkers identifying type 2 diabetes were different from the European population suggesting that the metagenome analysis to predict type 2 diabetes should be specific for the age and geographical location of the populations studied. It should be further noted that the mechanisms at the origin of this observation are numerous and hence will most likely depend upon each individual.

11.3.1.1 Impacts of Diet on Microbiota-Related Metabolic Diseases

Socio-demographic and environmental factors have a great influence on the incidence of metabolic [100] and cardiovascular diseases [101], introducing the possibility of identifying functional metagenomic factors under the control of environmental factors such as stress, food habits, and sedentarity. Diet and nutritional status are among the most important modifiable determinants of human health, and gut bacteria feed on the nutrients absorbed during a meal leading to changes in metabolism, the overall intestinal ecology, and the way bacteria interact with the host. The first analyses of metagenomic sequencing have been performed on obese patients followed up during 1 year of a restricted calorie diet [97, 98, 102]. A clear metagenomic signature was identified in obese patients characterized by a reduction in the relative abundance of the *Bacteroidetes*-to-*Firmicutes* ratio which represents more than 80 % of the overall bacterial population in feces [97, 98] (Fig. 11.3). This ratio evolved toward that of lean patients during weight loss showing that the microbiota can evolve according to the environmental factors. It is clear that dietary manipulation, including HF feeding, profoundly alters the profile of the gut microbiota [27, 81, 103–107]. An enrichment in gram-negative to gram-positive bacteria appeared to be associated with the early onset of high-fat-diet-induced diabetes [81]. An elegant validation of the role of diet on human microbiota has been performed in germ-free mice colonized with human microbiota and fed with a fat-enriched diet [104]. A single day of fat-enriched diet was sufficient to change the overall ecological homeostasis within the gut microbiome, the corresponding metabolic pathways, and hence the microbiome gene expression. The influence of high-fat diet on gut microbiota has been validated in other species such as the pig [108, 109]

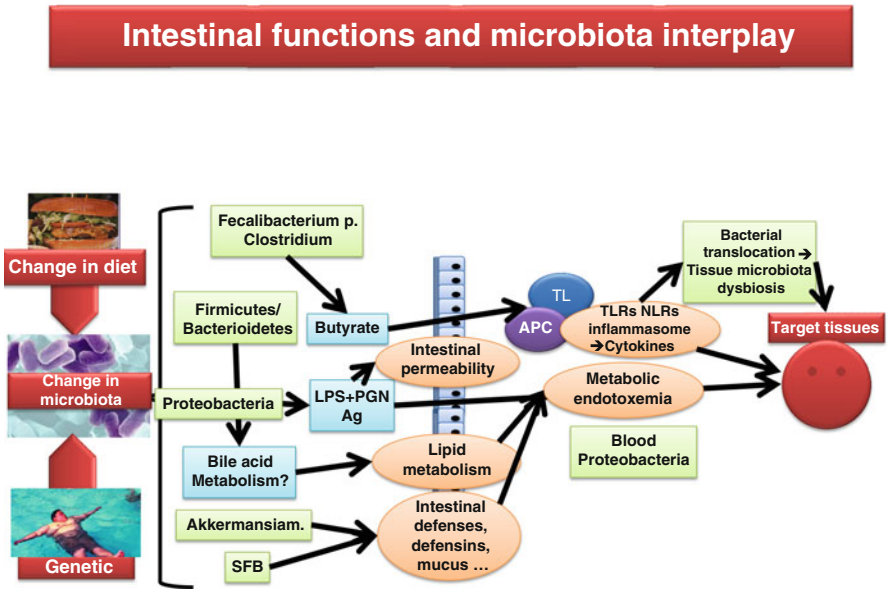

Fig. 11.3 Intestinal functions and microbiota interplay. Upon a change in diet or a specific genetic background, the gut microbiota changes. A modification of the *Firmicutes*-to-*Bacteroidetes* ratio leading to a change in polysaccharide fermentation, bile acid metabolism, an accumulation of *Proteobacteria* (gram negative; releasing LPS, peptidoglycans, and other antigens), a reduced in *Faecalibacterium*- and *Clostridium*-producing butyrate, a reduced in *Akkermansia muciniphila*, and segmented filamentous bacteria (at least in rodents, SFB) would alter intestinal functions. Intestinal permeability, defenses (mucin and defensin production), innate (*APC*, antigen-presenting cells) and adaptive (*T lymphocytes*, TL) immunity, and lipid metabolism could lead to bacterial translocation and tissue microbiota dysbiosis, metabolic endotoxemia, and metabolic inflammation of the targeted tissues. Altogether, the tissue biology would be impaired and be considered as a risk factor for the development of cardiometabolic diseases

which is highly influenced by pre- and probiotics [110] and sucrose [111]. Defined lipids such as oleic-acid-derived molecules and a combination of n-3 fatty acids markedly increased total bacterial density and restored the proportions of clostridial cluster, *Enterobacteriales* and *Bifidobacterium* that were changed during HFD feeding [112] along with the metabolic status. Conversely saturated fat increased the *Firmicutes*-to-*Bacteroidetes* ratio [113, 114]. In addition to the tremendous impact of dietary fibers, other molecules such as polyphenols influence gut microbiota and further control metabolism [115, 116]. Type 2 diabetes has also its own metagenomic signature, and type 2 diabetes-associated markers have been associated with the disease and can be useful to classify the different subgroups of type 2 diabetic patients [19]. Among these *Akkermansia muciniphila* seems to be a good candidate with a recently identified role in the control of the intestinal barrier [107]. A reduction of *Akkermansia muciniphila*, which is involved in mucus degradation, has also been observed [107]. These bacteria, when used as a probiotic, control body weight gain [107] and its prevalence increased in response to prebiotics [117]

accompanying metabolic control through a mechanism that could involve gut peptide secretion [118]. Insulin action also has a metagenomic signature in humans [119]. Furthermore, hypotheses regarding the functional role of gut microbiota can be generated by studying the genes characterizing the microbiome. A decrease in the abundance of some universal butyrate-producing bacteria and an increase in various opportunistic pathogens, as well as an enrichment of other microbial functions conferring sulfate reduction and oxidative stress resistance, were also characterized. A change in intestinal microbiota has also been characterized in non-alcoholic fatty liver diseases (NAFLD) where the incidence is between 16 % and 30 % of the general population [120] and further rises toward 80 % in patients with obesity and type 2 diabetes [121]. Some biomarkers have been identified such as a reduction of *Faecalibacterium* and *Anaerosporobacter* and a higher abundance of *Parabacteroides*, *Allisonella*, certain *Lactobacillus* species, and selected members of the phylum *Firmicutes* (*Lachnospiraceae*; genera, *Dorea*, *Robinsoniella*, and *Roseburia*) [122]. The causal role of gut microbiota on NAFLD has been shown by microbiota transfer in the mice. The colonization of germ-free mice with the gut microbiota from a high-fat-diet-induced NAFLD mouse induced the disease. Sequencing of the 16S ribosomal RNA revealed differences at the phylum, genera, and species levels [123]. Some mechanisms have been proposed that implicate intestinal permeability, low-grade inflammation and immune balance in the development of hepatosteatosis [124], the modulation of dietary choline and bile acid metabolism, and the production of endogenous ethanol [125] that will be discussed below. Another important feature of the change in intestinal microbiota is that in addition to the change of phylum ratio obesity is associated with phylum-level changes in the microbiota and reduced bacterial diversity [97, 98, 126].

11.3.1.2 Impact of the Host Genome

In addition to the role of the environment on the shaping of gut microbiota during metabolic diseases, the impact of the host genome cannot be totally ruled out. The concordance of type 2 diabetes within the homozygote population is higher than between heterozygote twins [83, 84, 127] or in response to overfeeding [128, 129] suggesting the important role of the genetic background. The results reveal that the human gut microbiome is shared among family members but that each individual's gut microbial community varies in the specific bacterial lineages present, with a comparable degree of covariation between adult monozygotic and dizygotic twin pairs [126]. These results demonstrate that a diversity of organismal assemblages can however yield a core microbiome at a gene level and that deviations from this core are associated with different physiological states, for example, obese versus lean. However, the concordance of the metabolic phenotype is not absolute between twins – this could be linked to differences in gut microbiota since the adult monozygotic twins are no more similar to one another in terms of their gut bacterial community structure than are adult dizygotic twins [126, 130]. However, the impact of the host genome on the microbiota seems to depend on the microbiota location

considered. In the saliva twins resemble each other more closely than the whole population at all time points but become less similar to each other when they age and no longer cohabit [131]. The sequencing of gut microbiota from the general population and across countries and ethnic origin showed that shared features of the functional maturation of the gut microbiome are identified during the first 3 years of life [24], suggesting an imprinting from the mother which remains but is not absolute. Hence, room is available for the impact of environmental changes throughout life. Numerous studies now report that the influence of the genetic background is due to the impact of the immune system that shapes the microbial community [132–135]. Hence, the immunogenetic traits of an individual appear to be major regulators of gut microbiota. Mutations in the receptors to bacterial determinants such as TLRs which are pathogen-associated molecular pattern recognition receptors that recognize highly conserved microbial molecules (PAMPs) notably TLR2 [136], TLR4 [137], and TLR5 [132] and NLRs notably NOD2 [138–140] shape the microbiota although some controversies do exist [141].

11.3.1.3 Impact of Birth

Importantly, the host immune system to microbiota relationship continues to be educated throughout life since the immune system matures along with the microbiota at birth. The gastrointestinal tract of a normal fetus is sterile. During the birth process and rapidly thereafter, microbes from the mother and surrounding environment colonize the gastrointestinal tract of the infant until a dense, complex microbiota develops [9]. It is now clear that the composition and temporal patterns of the microbial communities vary widely from baby to baby [142]. The distinct features of each baby's microbial community are recognizable for intervals of weeks to months which show that each baby's microbiota, until the first year of life, follows an idiosyncratic law. However, it then converges toward a profile characteristic of the adult gastrointestinal tract. Therefore, during the first year of life, while the microbial ecology is not yet set up, the inheritance of gut microbiota could also be influenced during pregnancy and at delivery according to the influence of the mother's microbiota. Furthermore, the mode of delivery influences the infant's microbiota since it was shown that vaginally delivered infants acquire bacterial communities resembling their mother's vaginal microbiota, dominated by *Lactobacillus*, *Prevotella*, or *Sneathia* spp., whereas C-section infants harbored bacterial communities similar to those found on the skin surface, dominated by *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp. [11, 143]. However, the delivery mode did not influence the prevalence of obesity in children [144]. Conversely, antibiotic treatment during the first 6 months of life increased the risk of overweight in children with normal-weight mothers but reduced the risk of obesity in children with overweight mothers. Hence, at birth and during the first year of life, the early colonization of the gut by microbiota can influence the incidence of metabolic diseases [93]. Alterations in the microbiota composition of mothers may be transferred to infants and lead to an increased risk of weight gain. During pregnancy, gut

microbiota changes dramatically between the first and third trimesters, with a vast expansion of diversity among mothers, an overall increase in *Proteobacteria* and *Actinobacteria*, and reduced richness [145]. This change in gut microbiota during pregnancy was functional since colonization of germ-free mice with the microbiota from the third trimester induced greater adiposity and insulin resistance when compared to mice colonized with the microbiota from the first trimester [145]. This original observation could suggest that the change in gut microbiota during pregnancy programs infant body weight. It has also been suggested that the microbiota from obese mothers could influence the prevalence of obesity in children. This was further supported by the fact that the infants' fecal microbial composition was related to the weight and weight gain of their mothers during pregnancy [146]. The fecal *Bacteroides* and *Staphylococcus* concentrations were significantly higher in infants of overweight mothers, whereas the prevalences of *Akkermansia muciniphila*, *Staphylococcus*, and *Clostridium difficile* groups were lower in infants of normal-weight mothers and of mothers with normal weight gain during pregnancy. This was confirmed in that the concentration of the gram-negative family *Enterobacteriaceae* was significantly higher in obese/overweight children and the levels of *Desulfovibrio* and *Akkermansia muciniphila*-like bacteria were significantly lower when compared to lean controls [147]. Chapter 8 "Metabonomics in neonatal and pediatric research: Studying and modulating gut functional ecology for optimal growth and development" provides a comprehensive overview of the state of the art of metabonomics and gut microbiota studies in neonatal and pediatric research.

11.3.2 Molecular Crosstalk Between Gut Microbiota and the Host for the Control of Metabolic Diseases

11.3.2.1 The Lipopolysaccharides Hypothesis and Metabolic Inflammation

Whereas extensive analyses demonstrate the important impact of gut microbiota on host biology, the mechanisms of the crosstalk between the host and the microbiota remain to be delineated. As mentioned above, metabolic inflammation is a leading mechanism responsible for the impairment of glycemia and body weight regulation. One hypothesis involves bacterial factors from the gut, such as lipopolysaccharides (LPS). Lipopolysaccharides are components of the wall from gram-negative bacteria [1]. They are potent endotoxins, involved in the acute-phase response to bacterial infection, inducing a cytokine-mediated systemic inflammatory response that can cause shock and severe multiple organ failure [2, 3]. These bacterial antigens bind to their receptors TLR4 and CD14 on numerous cells types notably those of the immune system, i.e., macrophages and dendritic cells. Adipocytes also express TLR4 and can bind LPS and could be involved directly in the activation of intracellular inflammatory pathways [148, 149].

Mechanisms of High-Fat-Diet-Induced Blood Bacterial Molecular Patterns

LPS accumulates in blood and contributes to inflammation and insulin intolerance [6, 81]. A 1-month high-fat feeding in humans increased endotoxemia by 71 % [150], suggesting that therapeutic agents that reduce intestinal LPS permeability could control metabolic endotoxemia and hence systemic inflammation in patients with metabolic syndrome. Similarly, in type 1 diabetic patients, metabolic endotoxemia was associated with dyslipidemia, insulin resistance, obesity, and chronic inflammation [151]. The mechanism is associated with an increase of the gram-negative to gram-positive ratio within the intestinal microbiota [81]. LPS need to be transported across the intestinal epithelial barrier to reach the blood. A mechanism involving the synthesis and production of chylomicron is required [152]. The uptake of LPS is observed within hours of lipid absorption in human [153] and could hence be considered as a blood nutritional signal informing the body of a change in feeding behavior and in microbiota ecology. The accumulation of LPS in the blood is also linked to an increased intestinal permeability where tight junctions become leaky due to a reduction of the expression of specific proteins such as zonula occludens [81, 106] and the putative role of endocannabinoids [154] that could control intestinal permeability through a GLP-2-dependent mechanism [155]. This is reversed upon treatment with prebiotics [156, 157] or probiotics [107]. The increase of plasma LPS concentration is called metabolic endotoxemia. Lipoproteins bind LPS to attenuate the biological inflammatory response of this bacterial factor [158, 159] through all classes of lipoprotein (chylomicrons, VLDL, LDL, and HDL) [9]. Chylomicrons and VLDL have been shown to reduce LPS-induced toxicity in mice [10]. Similarly, the binding of LPS to LDL reduced endothelial cell activity [9, 11]. In one study performed in 10 individuals, HDL has been shown to be the main LPS carrier holding 60 % of the LPS, with LDL and VLDL carrying, respectively, 25 % and 12 % [14]. Among three ex vivo studies, two have indicated that LPS, in humans, was mainly located in VLDL and LDL [15, 16] and one has more precisely indicated that LDL was the major carrier for LPS [9]. Therefore, it is now evident that nutritional and lipid metabolism are tightly linked to the intestinal absorption of gut bacterial factors including LPS for the triggering of metabolic inflammation. The pharmacological control of intestinal LPS absorption could be of importance for the prevention or treatment of metabolic diseases. A change in nutrition involving an increase in fat content is a risk factor for metabolic endotoxemia as demonstrated in epidemiological studies in humans [151, 160, 161]. In the mouse the ablation of various pattern recognition receptors (PRRs) such as TLR4, CD14, and NLRP3 protects mice from diet-/obesity-induced inflammation and insulin resistance [6, 162–164]. In human myotubes, LPS increased JNK phosphorylation and MCP-1 and IL-6 gene expression [165] and could play a role in the pathogenesis of insulin resistance. Therefore, antagonists of CD14/TLR4 may improve insulin action in type 2 diabetic patients. In the mouse the chronic subcutaneous infusion of LPS at low rates favors liver, adipose tissue, and muscle cytokine production and macrophage accumulation showing that in vivo metabolic endotoxemia is an important risk factor of insulin resistance and hence metabolic diseases. Direct evidence

shows that the LPS to ligand crosstalk controls adipose tissue inflammation [6, 162, 164, 166], insulin sensitivity [148, 167], and obesity [6, 168] through molecular mechanisms most likely linked to the activation of NFkB and the transcription of genes coding for cytokines. The latter, such as TNFα, will then interfere with the insulin receptor leading to insulin resistance [169]. LPS are also involved in the reduction of adipogenesis [170], as well as hepatic steatosis [171, 172] again through mechanisms involving TNFα production or SREBP-1 activation [173]. Other bacterial components are involved in the inflammatory process characterizing metabolic inflammation. The peptidoglycan is a complex structure of the bacterial wall found mostly in gram-positive bacteria but also found, to a lower extent, in gram-negative bacteria. Its polysaccharide component is different between the two types of bacteria and binds to pattern recognition receptors such the nucleotide oligomerization domain (NOD)1 and NOD2. NOD1 and NOD2 are currently the only known sensors of bacterial cell wall peptidoglycan (PGN) that elicit inflammation by increasing cytokine production, defensin expression by Paneth cells [174–177], and stress kinase responses [178]. In addition to LPS, PGN could be positioned as a component of metabolic endotoxemia that contributes to inflammation and metabolic defects [27, 179]. This is important because gut-derived LPS alone appears to be insufficient for establishing glucose/insulin tolerance in poorly immune-responding germ-free mice [180]. Furthermore, PGN containing meso-DAP motifs (generally dominant in gram-negative bacteria) caused profound insulin resistance through actions on NOD1 directly in metabolic cells, including adipocytes [179]. The NOD2 activation with the minimal bioactive PGN motif, muramyl dipeptide (MDP), mostly present in gram-positive bacteria, elicited cell autonomous inflammation and impaired insulin action directly in muscle cells [181] and caused acute, peripheral insulin resistance in vivo [179]. Mice lacking NOD2 have improved insulin sensitivity during obesity illustrating the protective role of NOD2 as a sensor of bacterial motifs derived from the gut microbiota on the control of metabolic diseases [27, 179]. However, defects in NOD2 immunity have been associated with promoting other chronic proinflammatory pathologies, and human NOD2 variants have the highest risk associated with Crohn's disease [182–184]. NOD2 immunity is known to contribute to homeostasis of the gut microbiota demonstrating that the microbiota is in a tight relationship with the immune system that secondarily controls the metabolism [138].

Metabolic Endotoxemia and Gut Microbiota Induced Hepatic Steatosis

Possible mechanisms leading to hepatosteatosis in obese and type 2 diabetic patients that involve gut microbiota implicate intestinal permeability, low-grade inflammation and immune balance [124], the modulation of dietary choline and bile acid metabolism, and the production of endogenous ethanol [125]. A first hypothesis is proposed regarding the key role of the intestinal epithelium as a barrier between the environment, i.e., the luminal side of the intestine and the body. A leaky gut has been described in patients with NAFLD [185] and linked to a change in intestinal

microbiota [122, 186]. A second hypothesis is linked to the role of choline which is a major phospholipid component of the cell membrane involved in signal transduction and the control of lipoprotein metabolism, notably the very-low-lipoprotein assembly [187–189]. Therefore, a choline-deficient diet promotes liver steatosis. Such depletion could be induced by the gut microbiota that would convert dietary choline into toxic methylamines [190, 191]. It was shown that circulating levels of plasma phosphatidylcholine were low, whereas urinary excretion rates of methylamines dimethylamine, trimethylamine, and trimethylamine-N-oxide were high and were coprocessed by symbiotic gut microbiota and mammalian enzyme systems. An inflammatory feature is required to qualify hepatic steatosis (NASH) from a NAFLD phenotype. It could originate from the gut microbiota since it interacts with the overall immunity of the host via PAMPs TLRs and NLRs [175]. Similarly, the role of components from the inflammasome such as NLRP6 and NLRP3 has been demonstrated [124]. These inflammation sensors could modulate the gut microbiota and hence the crosstalk of host immunity and gut microbiota leading to the development of the pathology. Altogether, the triggering of innate immunity could lead to the production of cytokines which contribute to the inflammatory phenotype of NASH through a mechanism similar to that suggested for the development of type 2 diabetes and obesity.

11.3.2.2 The Energy Harvesting, Expenditure, and Short-Chain Fatty Acid Hypotheses

The change in intestinal microbiota (increased *Firmicutes* and decreased *Bacteroidetes*), observed during obesity, was associated with an increased efficiency of energy harvest in human and mouse [26, 105]. The fecal content in energy was lower in obese mice than in controls by about 150 kCal/day, suggesting that the energy was better absorbed by the gut. This observation was attributed to the excessive hydrolysis of polysaccharides into monosaccharides indispensable to the body as well as to the production of short-chain fatty acids (SCFA) [26]. However, it seems that the production of SCFA diminished over time during long-term high-fat feeding or aging in *ob*/*ob* mice [105]. The role of gut microbiota in the production of SCFA is however strongly supported since it was shown that germ-free mice are devoid of SCFAs [192] and could control AMP-activated protein kinase activity and macrophage infiltration in adipose tissue [193]. Human colonic butyrate producers are phylogenetically diverse, with the two most abundant groups related to *E. rectale*/*Roseburia* species and to *Faecalibacterium prausnitzii*. The precise SCFA responsible for the control of metabolism seems to be multiple with a significant role for acetate. In addition, SCFAs such as propionate can be used for de novo glucose or lipid synthesis and serve as an energy source for the host. It has also been demonstrated that butyrate lowers fatty acid content in liver and plasma, reduces food intake, exerts immunosuppressive actions, and probably improves tissue insulin sensitivity [194, 195]. The mechanism could involve notably the promotion of glucagon-like peptide 1 (GLP-1) secretion via the binding of SCFA to

G-protein-coupled receptors such as GPR41/43 at the surface of the enteroendocrine cells [195]. Cascades of events such as the control of glucagon and insulin secretion, gastric emptying, and satiety would secondary to GLP-1 secretion control glucose homeostasis. Butyrate is also involved in the improvement of the overall glucose metabolism as demonstrated by its supplementation to a high-fat diet [196]. Other experiments show that by high-pressure liquid chromatography analysis, authors identified significantly higher concentrations of butyrate and propionate in feces from obese versus normal-weight children [197]. Significantly lower concentrations of intermediate metabolites were detected in obese children suggesting exhaustive substrate utilization by obese gut microbiota [197]. Despite this evidence, the role of SCFA on metabolism still remains unclear. Recent data demonstrate that the activation of GPR43 by SCFA at the surface of adipocytes reduced insulin signaling [198]. This could certainly reduce insulin-induced lipid accumulation, and hence body weight gain, but could also be considered as a factor inducing adipose tissue insulin resistance which could prevent glucose to be taken up by adipocytes and hence accumulate in the blood or the liver to induce hyperglycemia and hepatic steatosis. Thus, data relating to the production of SCFA most likely cover other unidentified mechanisms that need to be identified to fully understand the benefit of polysaccharide fermentation.

The role of gut microbiota could also be on energy expenditure since germ-free mice are leaner despite a dramatically increased food intake [90]. The lean phenotype of these mice is associated with increased skeletal muscle and liver levels of phosphorylated AMP-activated protein kinase (AMPK) and its downstream targets involved in fatty acid oxidation such as acetylCoA carboxylase and carnitine palmitoyltransferase [199]. AMPk is a master switch considered to be a molecule recruited in case of stress and energy deprivation [200, 201]. Bacterial factors could activate this enzyme in muscles and the liver to increase energy expenditure. Conversely, the microbiota inhibits fasting-induced adipose factor (Fiaf) which downregulates circulating lipoprotein lipase. Hence, free fatty acids are stored in the liver of conventional mice which is not the case in germ-free animals [199].

Altogether, numerous eukaryotic targets are currently being identified using germ-free mice. An important matter will be to validate them in physiological conditions such as in conventional mice and in humans.

11.3.3 Gut Microbiota During Obesity Surgery

The last 40 years has seen the emergence of the treatment of obesity by surgery. Several types of surgery involving the stomach and the intestine are used. The Roux-en-Y gastric bypass (RYGB) surgery which is to date the major bariatric intervention to treat morbid obesity involves the direct connection of the jejunum to the stomach so that nutrients are no longer in contact with the duodenum. In addition, 90 % of the stomach is removed. The sleeve gastrectomy involves the removal

of a major part of the stomach so that food directly reaches the duodenum. Other types of surgery such as the ileal bypass transposition are being developed. All surgical procedures dramatically affect gut microbiota [202, 203]. A first set of experiments demonstrated that before surgery, *Firmicutes* were dominant in normal-weight and obese individuals but significantly decreased in post-gastric bypass [204]. Interestingly, the gut microbiota from patients after surgery was not similar to that of lean individuals since it was enriched in *Gammaproteobacteria* which are H(2)-producing *Prevotellaceae* [204]. These changes were independent of weight change and caloric restriction, were detectable throughout the length of the gastrointestinal tract, and were most evident in the distal gut, downstream of the surgical manipulation site [205]. A precise study identified 14 discriminant bacterial genera (7 were dominant and 7 were subdominant) and 202 genes changed in the white adipose tissue that correlated with RYGB as well as with both clinical phenotypes [206]. *Faecalibacterium prausnitzii* species was lower in patients with metabolic diseases but associated negatively with inflammatory markers even throughout the follow-up after surgery and independently with changes in food intake suggesting an imprinting of the microbiota that cannot be easily changed [207].

11.3.3.1 The Bile Acids to Microbiota Hypothesis

Intestinal microbiota also has a role in the metabolism of bile acids, which, with other sterols [208], are important regulators of metabolic diseases, as also described in Chaps. 13 and 14. Bile acid can control insulin secretion [209], GLP-1 secretion [210], energy expenditure [211], as well as atherosclerosis [212]. The role of bile acid is also suspected during bariatric surgery and could be the molecular link between the change in intestinal microbiota [213] and the improvement of metabolism [214] notably through the TGR5 receptor [215]. Bile acids are secreted into the duodenum and work to emulsify liposoluble dietary nutrients to facilitate their digestion and absorption. Studies have demonstrated that bile acid composition and secretion in response to fat intake modifies markedly gut microbiota thereby inducing a dysbiosis [216]. The dietary fat can alter the gut microbiota of mice indirectly by changing the animals' pool of bile acids and steroids that are produced by the liver and secreted into the intestine [216]. Bile acids are synthesized from cholesterol in the liver and further metabolized by the gut microbiota into secondary bile acids [217] which can change the metabolism by acting on the farnesoid X receptor to exert some negative feedback control. When compared to germ-free mice, the conventionally raised mice are characterized by a dramatic reduction in muricholic acid, but not cholic acid levels [218]. The microbiota controls fibroblast growth factor 15 in the ileum and cholesterol 7α-hydroxylase (CYP7A1) in the liver by FXR-dependent mechanisms thereby influencing the metabolism and secretion of bile acids.

Hence, a new ecology is expected from the host and the microbiota through bile acid metabolism.

11.3.3.2 The Immunomicrobiota Crosstalk and Metabolic Diseases

Metabolic diseases are now considered as low-grade immunomodulatory diseases [58–60]. The origin of the antigen is unknown but has been suspected to be from autoantigens, which notably form the adipose tissue [219–221]. The discovery of intestinal microbiota opens new avenues regarding the origin of the metabolic inflammation. This hypothesis stemmed from the observation that intestinal permeability was increased in high-fat-diet-fed mice, leading to accumulation in the blood of bacterial fragments such as LPS [6, 160]. Whereas, during diabetes and obesity, LPS and peptidoglycan were shown to be transported from the intestinal lumen to the blood through the intestinal epithelium, it was initially thought that whole commensal bacteria would be arrested by the mucosal layer and the immune system within Peyer's patches or the lamina propria [222, 223], thus preventing translocation across the intestinal epithelial layer [224, 225].

A deficiency in host immune defenses and increased permeability and damage to the intestinal mucosal barrier represent mechanisms through which bacteria of intestinal origin accumulate into the tissues. In the healthy situation macrophages, dendritic cells, and antibodies within the lumen restrain, along with the mucus layer, the bacteria within the luminal side of the gut to ensure a tight intestinal permeability. In the proximal intestinal segments where the microbiota is sparse and the mucus layer is thin or absent, commensal bacteria are in close contact with the epithelial cells, and host immunosuppression synergistically promotes bacterial translocation from the gastrointestinal tract resulting in accumulation of bacteria within the mesenteric lymph node [225]. Therefore, lymphocytes from mesenteric lymph nodes, Peyer's patches, intraepithelial cells, and the lamina propria are specifically educated to recognize commensal bacteria, which are therefore considered as self-antigens. The change of intestinal microbiota that occurs during a fat-enriched diet [81, 104] leads to the production of new antigens that are no longer recognized as self by the intestinal immune cells [134]. This change in gut microbiota can also be controlled by natural antibiotics such as defensins secreted by Paneth cells [226, 227].

11.3.3.3 Intestinal Bacterial Translocation and Metabolic Diseases

Bacterial translocation is defined as the passage of viable indigenous bacteria from the gastrointestinal tract to extra intestinal sites, such as the mesenteric-lymph-node complex, liver, spleen, and bloodstream [224]. This mechanism is largely observed during intestinal bacteria overgrowth leading to cirrhosis [228] or sepsis [229]. Although it is intuitively considered as deleterious for the organism, it could be suggested that the bacterial translocation helps the immune system to be prepared against infections. Another pathological situation of increased bacterial translocation is AIDS where, due to impaired intestinal immunosuppression, bacteria translocate toward tissues and could lead to inflammation and increased mortality [230].

The immune inhibitory receptor programmed death-1 (PD-1) regulates the function of CD8+ cells and the translocation of bacteria [230]. Importantly AIDS is associated with a dramatic increase in the incidence of diabetes and lipodystrophy [231, 232]. The CD4 T helper and Th17 cells appear to be critical for regulating gut mucosal immune responses to extracellular microbial pathogens and therefore could be involved in bacterial translocation [233]. Hence, an impaired intestinal immune system could lead to bacterial translocation and therefore to metabolic diseases. This hypothesis has been validated during high-fat diet-induced metabolic diseases [27]. First it was observed that a rapid augmentation of bacterial adherence to the intestinal epithelium layer occurred suggesting an impaired mucosal defense. Then CD11c positive phagocytes harvested the transepithelial bacteria and translocated with the live bacteria to adipose tissue. The bacteria were co-localized within the adipose depot with CD11c positive cells and most likely were intracellular. Importantly, the bacterial accumulation was considered as a predictive biomarker of type 2 diabetes [234]. The translocated bacteria were also detected in the blood in humans and conversely to the adipose tissue. The Proteobacteria phylum represented more than 80 % of the blood microbiota as assessed by 16S RNA DNA. Interestingly, other bacterial DNA fragments could predict the onset of cardiovascular events in a large cohort of type 2 diabetic patients [235]. The processes involved in bacterial translocation at the onset of type 2 diabetes could be related to molecular determinants involved in bacterial recognition. The NOD1 and LPS-CD14 bacterial receptors were involved in this translocation mechanism since their deletion dramatically reduced the amount of bacterial DNA present within the adipose tissue under high-fat diet [27]. Interestingly, both deletions were associated with improved insulin sensitivity and reduced glycemia suggesting that the tight control of bacterial translocation could be a master regulator of the onset of insulin resistance and diabetes. Conversely, the deletion of NOD2 induced over-accumulation of bacterial DNA within the tissues suggesting a protective effect. This was also observed in mice treated with NOD2 ligands [179]. The importance of the immune system in the translocation mechanism was also illustrated in mice carrying a deleted MyD88 gene and which were prone to diabetes [236]. These mice also displayed a dramatic accumulation of bacteria in the mesenteric lymph nodes and adipose tissue. Therefore, metabolic regulators of bacterial translocation could be linked to hormones controlling the immune system such as estrogens [237] or leptin [238]. This shift in the paradigm is supported by data which show that cardiovascular disease is associated with the role of microbiota in the control of lipid metabolism leading to the development of atherosclerosis [95, 239, 240]. Surprisingly, microbes associated with periodontitis were at the origin of the bacteria present in the plaques of atherosclerotic patients [240]. Studies in animals have revealed a mechanistic link between intestinal microbial metabolism of the choline moiety in dietary phosphatidylcholine (lecithin) and coronary artery disease through the production of a pro-atherosclerotic metabolite, trimethylamine-N-oxide (TMAO) [241, 242]. This has been confirmed in humans [243]. Importantly, antibiotic administration reduced the TMAO concentration suggesting that the microbiota was indeed the source of this

proatherogenic molecule. Furthermore, in addition to bacterial factors, whole bacteria have been identified within the atherosclerotic plaques. The taxa have been linked to periodontal diseases [240]. This finding bridges the gap between metabolic and cardiovascular diseases by means of opportunistic bacteria from the oral cavity. Its proportion increases in response to a change of diet [244]. In addition, genes closely involved in the regulation of cardiovascular diseases such as angiotensin I-converting enzyme (peptidyl-dipeptidase A) 2 (Ace2), which encodes a key regulatory enzyme of the renin-angiotensin system, also have an impact on gut microbiota leading to inflammation [245]. Furthermore, statins, classical anti-dyslipidemic agents prescribed to reduce the incidence of cardiovascular events, have been shown to be associated with microbial-derived agents that could be responsible for the cholesterol-lowering effect, explaining the efficacy of the treatment [246]. Cholesterol metabolism is indeed regulated by gut microbiota since there is a dramatic reduction of muricholic acid, a farnesoid X receptor (FXR) antagonist in the ileum, in conventional mice when compared with germ-free mice. Therefore, a given microbiota could be important for the efficacy of a drug treatment as well as for the susceptibility of developing cardiometabolic diseases, thus reconciling metabolic and cardiovascular diseases under the paradigm of the gut to tissue microbiota crosstalk.

11.3.4 Tissue Microbiota and Metabolic Diseases: The Paradigm Shift of Bacteria Translocation

Bacterial factors such as LPS, peptidoglycans, and bacterial DNA can be absorbed by the gut and found within the blood. These metafactors could serve as biomarkers and also as regulators since they can then activate cells from the immune system to generate inflammation. Pyrosequencing of the 16S RNA DNA from the stroma vascular fraction of human adipose tissue from lean, overweight, and obese patients showed that the diversity of the tissue microbiota was mainly related to *Firmicutes* and *Proteobacteria* (*in press*). Interestingly, although no changes were observed among the *Firmicutes* phylum, a major dysbiosis was detected within the *Proteobacteria* phylum and specifically the *Ralstonia* genus. *Ralstonia* was dramatically overrepresented within the family and, furthermore, its presence was proportional to increased BMI. This suggested that this bacterial genus might be causal. The bacterial translocation process also leads to the accumulation of bacterial DNA in the blood fraction [234], suggesting that either bacteria migrate through the blood to reach the tissues or are released by the tissues into the blood.

The role of this tissue microbiota is not known; similarly the proportion of live bacteria compared to dead bacteria or fragments is not perfectly identified and will most likely depend on diet, age, and other genetic and environmental factors. This paradigm shift is promising but will require much work to determine its physiological role.

11.3.4.1 Tissue Microbiota as Biomarkers of Metabolic and Cardiovascular Diseases

The blood tissue microbiota has been recently described in humans and mice [27, 234, 235]. The presence of bacterial DNA has been revealed by qPCR from human cohorts from the general population [234] and from diabetic patients [235]. It could be shown that the increased 16S RNA DNA concentration in blood predicts the onset of diabetes 6–9 years later [234], whereas the quantification of other bacterial factors could predict the onset of cardiovascular events in a population of type 2 diabetics [235]. In both cases it is suggested that the bacterial fragments which accumulate in the blood could be causal of the disease. Since the bacteria are inherited at birth, the accumulation of bacterial DNA initiates at a very early stage, linking the environment with the host genome. In risk situations, such as when feeding on a fat-enriched diet, the bacterial DNA increases reflecting the new nutritional situation and could be involved in the triggering of metabolic adaptation. Adipose tissue is targeted [6, 27, 137] and adipogenesis is induced [170]. Hence, these bacteria can be considered as nutrient sensors informing the tissues.

11.4 Therapeutic Perspectives

The therapeutic perspectives are huge but so far at their infancy, as presented and discussed in Chap. 19. One should separate the nutritional approaches aiming at maintaining health in the general population. This would first involve phenotyping of the human microbiome [21] and second performing studies to demonstrate the prevention of the risk of disease development. A second strategy would involve preventing the disease in patients at risk to develop metabolic diseases. In these patients subpopulations should be defined and studied in order to perfectly adapt the nutritional strategy. This should be a precisely directed strategy to treat patients for the prevention of developing a metabolic disease. This would involve overweight individuals, smokers, sedentary individuals, and hypertensive patients. With this aim pre- and probiotics selected for their capacity to influence all the above physiological mechanisms at the cross road of microbiota and the host should be delineated. Other nutritional approaches could be pursued aiming to target intestinal functions [115] by using antioxidants like polyphenols such as resveratrol. From a therapeutic point of view, pharmacological strategies can be envisaged that involve small molecules to target molecular mechanisms such as the immune system, mucosal defense, bile acid synthesis, incretin secretion, or the production of short-chain fatty acids. Again, they should address well-defined subgroups of patients characterized by their specific microbiota or blood biomarkers. Companion blood bacterial biomarkers could be used to monitor the impact of the intervention over a long-term treatment. This reasoned strategy should help to control metabolic diseases and associated cardiovascular events. The advent of next-generation sequencing strategies along with the development of bioinformatics and biostatistical skills are now available to set up these programs.

11.5 Conclusions

In the quest of a mechanism explaining the pandemic development of metabolic diseases, the consequent cardiovascular events, and further reconciling the key role of the adaptive and innate immune system, the role of the intestinal microbiota has emerged as a very promising candidate. The diversity and huge complexity of the microbiome precludes a rapid and clear identification of the molecular mechanisms at the crosstalk between the host and the microbiota. The molecular hypotheses for the explanation of the metabolic phenotype are numerous and probably related to the large number of molecular origins of the disease. This suggests that patients should be screened for their microbiota to host crosstalk. Thus, therapeutic strategies or preventive programs could be successful. We have entered a new era and one can no longer disregard the microbiome from the gut or other locations since it is now clearly involved at the onset and during the development of metabolic disease.

References

1. Haffner SM, Ruilope L, Dahlof B, Abadie E, Kupfer S, Zannad F. Metabolic syndrome, new onset diabetes, and new end points in cardiovascular trials. J Cardiovasc Pharmacol. 2006;47(3):469–75.
2. Juonala M, Magnussen CG, Berenson GS, Venn A, Burns TL, Sabin MA, et al. Childhood adiposity, adult adiposity, and cardiovascular risk factors. N Engl J Med. 2011;365(20):1876–85.
3. Franks PW, Hanson RL, Knowler WC, Sievers ML, Bennett PH, Looker HC. Childhood obesity, other cardiovascular risk factors, and premature death. N Engl J Med. 2010;362(6):485–93.
4. Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. Arterioscler Thromb Vasc Biol. 2000;20(5):1262–75.
5. Danaei G, Lawes CM, Vander Hoorn S, Murray CJ, Ezzati M. Global and regional mortality from ischaemic heart disease and stroke attributable to higher-than-optimum blood glucose concentration: comparative risk assessment. Lancet. 2006;368(9548):1651–9.
6. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes. 2007;56(7):1761–72.
7. Ford ES, Li C, Sattar N. Metabolic syndrome and incident diabetes: current state of the evidence. Diabetes Care. 2008;31(9):1898–904.
8. Dethlefsen L, Eckburg PB, Bik EM, Relman DA. Assembly of the human intestinal microbiota. Trends Ecol Evol. 2006;21(9):517–23.
9. Mackie RI, Sghir A, Gaskins HR. Developmental microbial ecology of the neonatal gastrointestinal tract. Am J Clin Nutr. 1999;69(5):1035S–45.
10. Penders J, Thijs C, Vink C, Stelma FF, Snijders B, Kummeling I, et al. Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics. 2006;118(2):511–21.
11. Azad MB, Konya T, Maughan H, Guttman DS, Field CJ, Chari RS, et al. Gut microbiota of healthy Canadian infants: profiles by mode of delivery and infant diet at 4 months. Can Med Assoc J. 2013.
12. Barrett E, Guinane CM, Ryan CA, Dempsey EM, Murphy BP, O'Toole PW, et al. Microbiota diversity and stability of the preterm neonatal ileum and colon of two infants. Microbiologyopen. 2013;2:215.

13. Gronlund MM, Lehtonen OP, Eerola E, Kero P. Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. J Pediatr Gastroenterol Nutr. 1999;28(1):19–25.
14. Orrhage K, Nord CE. Factors controlling the bacterial colonization of the intestine in breast-fed infants. Acta Paediatr Suppl. 1999;88(430):47–57.
15. Stratiki Z, Costalos C, Sevastiadou S, Kastanidou O, Skouroliakou M, Giakoumatou A, et al. The effect of a bifidobacter supplemented bovine milk on intestinal permeability of preterm infants. Early Hum Dev. 2007;83(9):575–9.
16. Claesson MJ, Cusack S, O'Sullivan O, Greene-Diniz R, de Weerd H, Flannery E, et al. Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc Natl Acad Sci U S A. 2011;108 Suppl 1:4586–91.
17. Maslowski KM, Mackay CR. Diet, gut microbiota and immune responses. Nat Immunol. 2011;12(1):5–9.
18. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464(7285):59–65.
19. Qin J, Li Y, Cai Z, Li S, Zhu J, Zhang F, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature. 2012;490(7418):55–60.
20. Dethlefsen L, McFall-Ngai M, Relman DA. An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature. 2007;449(7164):811–8.
21. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. Nature. 2007;449(7164):804–10.
22. Zoetendal EG, Akkermans AD, De Vos WM. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl Environ Microbiol. 1998;64(10):3854–9.
23. Biagi E, Candela M, Fairweather-Tait S, Franceschi C, Brigidi P. Aging of the human metaorganism: the microbial counterpart. Age (Dordr). 2012;34(1):247–67.
24. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. Nature. 2012;486(7402):222–7.
25. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell. 2006;124(4):837–48.
26. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 2006;444(7122):1027–31.
27. Amar J, Chabo C, Waget A, Klopp P, Vachoux C, Bermudez-Humaran LG, et al. Intestinal mucosal adherence and translocation of commensal bacteria at the early onset of type 2 diabetes: molecular mechanisms and probiotic treatment. EMBO Mol Med. 2011;3(9):559–72.
28. Chen X, D'Souza R, Hong ST. The role of gut microbiota in the gut-brain axis: current challenges and perspectives. Protein Cell. 2013;4:403.
29. Lebovitz HE. The relationship of obesity to the metabolic syndrome. Int J Clin Pract Suppl. 2003;134:18–27.
30. Chen G, Koyama K, Yuan X, Lee Y, Zhou YT, O'Doherty R, et al. Disappearance of body fat in normal rats induced by adenovirus-mediated leptin gene therapy. Proc Natl Acad Sci U S A. 1996;93(25):14795–9.
31. Shimabukuro M, Koyama K, Chen G, Wang MY, Trieu F, Lee Y, et al. Direct antidiabetic effect of leptin through triglyceride depletion of tissues [see comments]. Proc Natl Acad Sci U S A. 1997;94(9):4637–41.
32. Zhou YT, Shimabukuro M, Koyama K, Lee Y, Wang MY, Trieu F, et al. Induction by leptin of uncoupling protein-2 and enzymes of fatty acid oxidation. Proc Natl Acad Sci U S A. 1997;94(12):6386–90.
33. Shepherd PR, Gnudi L, Tozzo E, Yang H, Leach F, Kahn BB. Adipose cell hyperplasia and enhanced glucose disposal in transgenic mice overexpressing GLUT4 selectively in adipose tissue. J Biol Chem. 1993;268(30):22243–6.
34. Casteilla L, Planat-Benard V, Cousin B, Silvestre JS, Laharrague P, Charriere G, et al. Plasticity of adipose tissue: a promising therapeutic avenue in the treatment of cardiovascular and blood diseases? Arch Mal Coeur Vaiss. 2005;98(9):922–6.

35. Caspar-Bauguil S, Cousin B, Galinier A, Segafredo C, Nibbelink M, Andre M, et al. Adipose tissues as an ancestral immune organ: site-specific change in obesity. FEBS Lett. 2005;579(17):3487–92.
36. Galinier A, Carriere A, Fernandez Y, Carpene C, Andre M, Caspar-Bauguil S, et al. Adipose tissue proadipogenic redox changes in obesity. J Biol Chem. 2006;281(18):12682–7.
37. Sengenes C, Lolmede K, Zakaroff-Girard A, Busse R, Bouloumie A. Preadipocytes in the human subcutaneous adipose tissue display distinct features from the adult mesenchymal and hematopoietic stem cells. J Cell Physiol. 2005;205(1):114–22.
38. Maumus M, Peyrafitte JA, D'Angelo R, Fournier-Wirth C, Bouloumie A, Casteilla L, et al. Native human adipose stromal cells: localization, morphology and phenotype. Int J Obes (Lond). 2011;35:1141.
39. Planat-Benard V, Silvestre JS, Cousin B, Andre M, Nibbelink M, Tamarat R, et al. Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation. 2004;109(5):656–63.
40. Villaret A, Galitzky J, Decaunes P, Esteve D, Marques MA, Sengenes C, et al. Adipose tissue endothelial cells from obese human subjects: differences among depots in angiogenic, metabolic, and inflammatory gene expression and cellular senescence. Diabetes. 2010;59(11):2755–63.
41. Adachi A, Shimizu N, Oomura Y, Kobashi M. Convergence of hepatoportal glucose-sensitive afferent signals to glucose-sensitive units within the nucleus of the solitary tract. Neurosci Lett. 1984;46(2):215–8.
42. Burcelin R. The gut-brain axis: a major glucoregulatory player. Diabetes Metab. 2010;36 Suppl 3:S54–8.
43. Konturek SJ, Konturek JW, Pawlik T, Brzozowski T. Brain-gut axis and its role in the control of food intake. J Physiol Pharmacol. 2004;55(1 Pt 2):137–54.
44. Holst JJ. The physiology of glucagon-like peptide 1. Physiol Rev. 2007;87(4):1409–39.
45. Cabou C, Burcelin R. GLP-1, the gut-brain, and brain-periphery axes. Rev Diabet Stud. 2012;8:418–31.
46. Knauf C, Cani PD, Kim DH, Iglesias MA, Chabo C, Waget A, et al. Role of central nervous system glucagon-like peptide-1 receptors in enteric glucose sensing. Diabetes. 2008;57(10):2603–12.
47. Collins SM, Surette M, Bercik P. The interplay between the intestinal microbiota and the brain. Nat Rev Microbiol. 2012;10(11):735–42.
48. Forsythe P, Kunze WA, Bienenstock J. On communication between gut microbes and the brain. Curr Opin Gastroenterol. 2012;28(6):557–62.
49. Foster JA, McVey Neufeld KA. Gut-brain axis: how the microbiome influences anxiety and depression. Trends Neurosci. 2013;36:305.
50. DeFronzo RA, Ferrannini E, Simonson DC. Fasting hyperglycemia in non-insulin-dependent diabetes mellitus: contributions of excessive hepatic glucose production and impaired tissue glucose uptake. Metab: Clin Exp. 1989;38(4):387–95.
51. Baudon MA, Ferre P, Penicaud L, Maulard P, Ktorza A, Castano L, et al. Normal insulin sensitivity during the phase of glucose intolerance but insulin resistance at the onset of diabetes in the spontaneously diabetic BB rat. Diabetologia. 1989;32(12):839–44.
52. Burcelin R, Eddouks M, Maury J, Kande J, Assan R, Girard J. Excessive glucose production, rather than insulin resistance, accounts for hyperglycaemia in recent-onset streptozotocin-diabetic rats. Diabetologia. 1995;38(3):283–90.
53. Del Prato S, Bonadonna RC, Bonora E, Gulli G, Solini A, Shank M, et al. Characterization of cellular defects of insulin action in type 2 (non-insulin-dependent) diabetes mellitus. J Clin Invest. 1993;91(2):484–94.
54. Golay A, Felber JP, Jequier E, DeFronzo RA, Ferrannini E. Metabolic basis of obesity and noninsulin-dependent diabetes mellitus. Diabetes Metab Rev. 1988;4(8):727–47.
55. Hundal RS, Krssak M, Dufour S, Laurent D, Lebon V, Chandramouli V, et al. Mechanism by which metformin reduces glucose production in type 2 diabetes. Diabetes. 2000;49(12):2063–9.

56. Martin BC, Warram JH, Krolewski AS, Bergman RN, Soeldner JS, Kahn CR. Role of glucose and insulin resistance in development of type 2 diabetes mellitus: results of a 25-year follow-up study [see comments]. Lancet. 1992;340(8825):925–9.
57. Bergman RN, Ader M. Free fatty acids and pathogenesis of type 2 diabetes mellitus. Trends Endocrinol Metab. 2000;11(9):351–6.
58. Pickup JC, Crook MA. Is type II diabetes mellitus a disease of the innate immune system? Diabetologia. 1998;41(10):1241–8.
59. Shoelson SE, Lee J, Goldfine AB. Inflammation and insulin resistance. J Clin Invest. 2006;116(7):1793–801.
60. Hotamisligil GS. Inflammation and metabolic disorders. Nature. 2006;444(7121):860–7.
61. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. J Clin Invest. 2005;115(5):1111–9.
62. Manco M, Fernandez-Real JM, Equitani F, Vendrell J, Valera Mora ME, Nanni G, et al. Effect of massive weight loss on inflammatory adipocytokines and the innate immune system in morbidly obese women. J Clin Endocrinol Metab. 2007;92(2):483–90.
63. Dandona P, Aljada A, Bandyopadhyay A. Inflammation: the link between insulin resistance, obesity and diabetes. Trends Immunol. 2004;25(1):4–7.
64. Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, Ohsugi M, et al. CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. Nat Med. 2009;15(8):914–20.
65. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest. 2007;117(1):175–84.
66. Nguyen MT, Favelyukis S, Nguyen AK, Reichart D, Scott PA, Jenn A, et al. A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways. J Biol Chem. 2007;282(48):35279–92.
67. Ueki K, Kondo T, Kahn CR. Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms. Mol Cell Biol. 2004;24(12):5434–46.
68. Plomgaard P, Bouzakri K, Krogh-Madsen R, Mittendorfer B, Zierath JR, Pedersen BK. Tumor necrosis factor-alpha induces skeletal muscle insulin resistance in healthy human subjects via inhibition of Akt substrate 160 phosphorylation. Diabetes. 2005;54(10):2939–45.
69. Federici M, Hribal ML, Menghini R, Kanno H, Marchetti V, Porzio O, et al. Timp3 deficiency in insulin receptor-haploinsufficient mice promotes diabetes and vascular inflammation via increased TNF-alpha. J Clin Invest. 2005;115(12):3494–505.
70. Tanti J, Gual P, Gremeaux T, Gonzalez T, Barres R, Le Marchand-Brustel Y. Alteration in insulin action: role of IRS-1 serine phosphorylation in the retroregulation of insulin signalling. Ann Endocrinol. 2004;65:43–8.
71. Bouzakri K, Roques M, Gual P, Espinosa S, Guebre-Egziabher F, Riou JP, et al. Reduced activation of phosphatidylinositol-3 kinase and increased serine 636 phosphorylation of insulin receptor substrate-1 in primary culture of skeletal muscle cells from patients with type 2 diabetes. Diabetes. 2003;52(6):1319–25.
72. Bouloumie A, Curat CA, Sengenes C, Lolmede K, Miranville A, Busse R. Role of macrophage tissue infiltration in metabolic diseases. Curr Opin Clin Nutr Metab Care. 2005;8(4):347–54.
73. Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, et al. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. J Clin Invest. 2006;116(6):1494–505.
74. Suganami T, Tanimoto-Koyama K, Nishida J, Itoh M, Yuan X, Mizuarai S, et al. Role of the Toll-like receptor 4/NF-kappaB pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. Arterioscler Thromb Vasc Biol. 2007;27(1):84–91.
75. Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K, et al. CCR2 modulates inflammatory and metabolic effects of high-fat feeding. J Clin Invest. 2006;116(1):115–24.
76. Lumeng CN, Deyoung SM, Bodzin JL, Saltiel AR. Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity. Diabetes. 2007;56(1):16–23.

77. Charriere G, Cousin B, Arnaud E, Andre M, Bacou F, Penicaud L, et al. Preadipocyte conversion to macrophage. Evidence of plasticity. J Biol Chem. 2003;278(11):9850–5.
78. Charriere GM, Cousin B, Arnaud E, Saillan-Barreau C, Andre M, Massoudi A, et al. Macrophage characteristics of stem cells revealed by transcriptome profiling. Exp Cell Res. 2006;312(17):3205–14.
79. Winer S, Chan Y, Paltser G, Truong D, Tsui H, Bahrami J, et al. Normalization of obesity-associated insulin resistance through immunotherapy. Nat Med. 2009;15(8):921–9.
80. Feuerer M, Herrero L, Cipolletta D, Naaz A, Wong J, Nayer A, et al. Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. Nat Med. 2009;15(8):930–9.
81. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes. 2008;57(6):1470–81.
82. Drong AW, Lindgren CM, McCarthy MI. The genetic and epigenetic basis of type 2 diabetes and obesity. Clin Pharmacol Ther. 2012;92(6):707–15.
83. Medici F, Hawa M, Ianari A, Pyke DA, Leslie RD. Concordance rate for type II diabetes mellitus in monozygotic twins: actuarial analysis. Diabetologia. 1999;42(2):146–50.
84. Poulsen P, Grunnet LG, Pilgaard K, Storgaard H, Alibegovic A, Sonne MP, et al. Increased risk of type 2 diabetes in elderly twins. Diabetes. 2009;58(6):1350–5.
85. Vaarala O, Atkinson MA, Neu J. The "perfect storm" for type 1 diabetes: the complex interplay between intestinal microbiota, gut permeability, and mucosal immunity. Diabetes. 2008;57(10):2555–62.
86. Wen L, Ley RE, Volchkov PY, Stranges PB, Avanesyan L, Stonebraker AC, et al. Innate immunity and intestinal microbiota in the development of type 1 diabetes. Nature. 2008;455(7216):1109–13.
87. Markle JG, Frank DN, Mortin-Toth S, Robertson CE, Feazel LM, Rolle-Kampczyk U, et al. Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. Science. 2013;339:1084.
88. Nicholson JK, Holmes E, Wilson ID. Gut microorganisms, mammalian metabolism and personalized health care. Nat Rev Microbiol. 2005;3(5):431–8.
89. Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI. Molecular analysis of commensal host-microbial relationships in the intestine. Science. 2001;291(5505):881–4.
90. Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, et al. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A. 2004;101(44):15718–23.
91. Hooper LV, Gordon JI. Commensal host-bacterial relationships in the gut. Science. 2001;292(5519):1115–8.
92. Serino M, Chabo C, Burcelin R. Intestinal MicrobiOMICS to define health and disease in human and mice. Curr Pharm Biotechnol. 2012;13(5):746–58.
93. Backhed F. Programming of host metabolism by the gut microbiota. Ann Nutr Metab. 2011;58 Suppl 2:44–52.
94. Turnbaugh PJ, Gordon JI. The core gut microbiome, energy balance and obesity. J Physiol. 2009;587(Pt 17):4153–8.
95. Karlsson FH, Fak F, Nookaew I, Tremaroli V, Fagerberg B, Petranovic D, et al. Symptomatic atherosclerosis is associated with an altered gut metagenome. Nat Commun. 2012;3:1245.
96. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature. 2006;444(7122):1022–3.
97. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Human gut microbes associated with obesity. Nature. 2006;444:1022–3.
98. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proc Natl Acad Sci U S A. 2005;102(31):11070–5.
99. Karlsson FH, Tremaroli V, Nookaew I, Bergstrom G, Behre CJ, Fagerberg B, et al. Gut metagenome in European women with normal, impaired and diabetic glucose control. Nature. 2013;498:99.

100. Noble D, Mathur R, Dent T, Meads C, Greenhalgh T. Risk models and scores for type 2 diabetes: systematic review. BMJ. 2011;343:d7163.
101. van Dieren S, Beulens JW, Kengne AP, Peelen LM, Rutten GE, Woodward M, et al. Prediction models for the risk of cardiovascular disease in patients with type 2 diabetes: a systematic review. Heart. 2012;98(5):360–9.
102. Spor A, Koren O, Ley R. Unravelling the effects of the environment and host genotype on the gut microbiome. Nat Rev Microbiol. 2011;9(4):279–90.
103. Serino M, Luche E, Gres S, Baylac A, Berge M, Cenac C, et al. Metabolic adaptation to a high-fat diet is associated with a change in the gut microbiota. Gut. 2012;61(4):543–53.
104. Turnbaugh P, Ridaura V, Faith J, Rey F, Knight R, Gordon J. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. Sci Transl Med. 2009;1:6–14.
105. Murphy EF, Cotter PD, Healy S, Marques TM, O'Sullivan O, Fouhy F, et al. Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models. Gut. 2010;59(12):1635–42.
106. Moreira AP, Texeira TF, Ferreira AB, Peluzio Mdo C, Alfenas RC. Influence of a high-fat diet on gut microbiota, intestinal permeability and metabolic endotoxaemia. Br J Nutr. 2012;108(5):801–9.
107. Everard A, Belzer C, Geurts L, Ouwerkerk JP, Druart C, Bindels LB, et al. Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. Proc Natl Acad Sci U S A. 2013;498:99.
108. Pedersen R, Andersen AD, Molbak L, Stagsted J, Boye M. Changes in the gut microbiota of cloned and non-cloned control pigs during development of obesity: gut microbiota during development of obesity in cloned pigs. BMC Microbiol. 2013;13:30.
109. Yan H, Potu R, Lu H, Vezzoni de Almeida V, Stewart T, Ragland D, et al. Dietary fat content and fiber type modulate hind gut microbial community and metabolic markers in the pig. PLoS One. 2013;8(4):e59581.
110. Park DY, Ahn YT, Park SH, Huh CS, Yoo SR, Yu R, et al. Supplementation of lactobacillus curvatus HY7601 and lactobacillus plantarum KY1032 in diet-induced obese mice is associated with gut microbial changes and reduction in obesity. PLoS One. 2013;8(3):e59470.
111. Parks BW, Nam E, Org E, Kostem E, Norheim F, Hui ST, et al. Genetic control of obesity and gut microbiota composition in response to high-fat, high-sucrose diet in mice. Cell Metab. 2013;17(1):141–52.
112. Mujico JR, Baccan GC, Gheorghe A, Diaz LE, Marcos A. Changes in gut microbiota due to supplemented fatty acids in diet-induced obese mice. Br J Nutr. 2013;110:1–10.
113. de Wit N, Derrien M, Bosch-Vermeulen H, Oosterink E, Keshtkar S, Duval C, et al. Saturated fat stimulates obesity and hepatic steatosis and affects gut microbiota composition by an enhanced overflow of dietary fat to the distal intestine. Am J Physiol Gastrointest Liver Physiol. 2012;303(5):G589–99.
114. Liu T, Hougen H, Vollmer AC, Hiebert SM. Gut bacteria profiles of Mus musculus at the phylum and family levels are influenced by saturation of dietary fatty acids. Anaerobe. 2012;18(3):331–7.
115. Dao TM, Waget A, Klopp P, Serino M, Vachoux C, Pechere L, et al. Resveratrol increases glucose induced GLP-1 secretion in mice: a mechanism which contributes to the glycemic control. PLoS One. 2011;6(6):e20700.
116. Neyrinck AM, Van Hee VF, Bindels LB, De Backer F, Cani PD, Delzenne NM. Polyphenol-rich extract of pomegranate peel alleviates tissue inflammation and hypercholesterolaemia in high-fat diet-induced obese mice: potential implication of the gut microbiota. Br J Nutr. 2013;109(5):802–9.
117. Tachon S, Zhou J, Keenan M, Martin R, Marco ML. The intestinal microbiota in aged mice is modulated by dietary resistant starch and correlated with improvements in host responses. FEMS Microbiol Ecol. 2013;83(2):299–309.
118. Cani PD, Knauf C, Iglesias MA, Drucker DJ, Delzenne NM, Burcelin R. Improvement of glucose tolerance and hepatic insulin sensitivity by oligofructose requires a functional glucagon-like peptide 1 receptor. Diabetes. 2006;55(5):1484–90.

119. Serino M, Fernandez-Real JM, Fuentes EG, Queipo-Ortuno M, Moreno-Navarrete JM, Sanchez A, et al. The gut microbiota profile is associated with insulin action in humans. Acta Diabetol. 2012.
120. Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. Aliment Pharmacol Ther. 2011;34(3):274–85.
121. Wong VW, Tse CH, Lam TT, Wong GL, Chim AM, Chu WC, et al. Molecular characterization of the fecal microbiota in patients with nonalcoholic steatohepatitis - a longitudinal study. PLoS One. 2013;8(4):e62885.
122. Raman M, Ahmed I, Gillevet PM, Probert CS, Ratcliffe NM, Smith S, et al. Fecal microbiome and volatile organic compound metabolome in obese humans with nonalcoholic fatty liver disease. Clin Gastroenterol Hepatol: Off Clin Pract J Am Gastroenterol Assoc. 2013;11:868.
123. Le Roy T, Llopis M, Lepage P, Bruneau A, Rabot S, Bevilacqua C, et al. Intestinal microbiota determines development of non-alcoholic fatty liver disease in mice. Gut. 2012.
124. Henao-Mejia J, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, et al. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature. 2012;482(7384):179–85.
125. Aron-Wisnewsky J, Gaborit B, Dutour A, Clement K. Gut microbiota and non-alcoholic fatty liver disease: new insights. Clin Microbiol Infect. 2013;19(4):338–48.
126. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. Nature. 2009;457(7228):480–4.
127. Newman B, Selby J, King M, Slemenda C, Fabsitz R, Friedman G. Concordance for type 2 (non-insulin-dependent) diabetes mellitus in male twins. Diabetologia. 1987;30:763–8.
128. Bouchard C, Tremblay A, Despres J, Nadeau A, Lupien P, Theriault G, et al. The response to long-term overfeeding in identical twins. N Engl J Med. 1990;322(21):1477–82. 1477-82.
129. Bouchard C, Tremblay A, Despres J, Nadeau A, Lupien P, Moorjani S, et al. Overfeeding in identical twins: 5-year postoverfeeding results. Metab: Clin Exp. 1996;45:1042–50.
130. Turnbaugh PJ, Quince C, Faith JJ, McHardy AC, Yatsunenko T, Niazi F, et al. Organismal, genetic, and transcriptional variation in the deeply sequenced gut microbiomes of identical twins. Proc Natl Acad Sci U S A. 2010;107(16):7503–8.
131. Stahringer SS, Clemente JC, Corley RP, Hewitt J, Knights D, Walters WA, et al. Nurture trumps nature in a longitudinal survey of salivary bacterial communities in twins from early adolescence to early adulthood. Genome Res. 2012;22(11):2146–52.
132. Vijay-Kumar M, Aitken JD, Carvalho FA, Cullender TC, Mwangi S, Srinivasan S, et al. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. Science. 2010;328(5975):228–31.
133. Round JL, O'Connell RM, Mazmanian SK. Coordination of tolerogenic immune responses by the commensal microbiota. J Autoimmun. 2010;34(3):J220–5.
134. Burcelin R, Garidou L, Pomie C. Immuno-microbiota cross and talk: the new paradigm of metabolic diseases. Semin Immunol. 2012;24(1):67–74.
135. Natividad JM, Verdu EF. Modulation of intestinal barrier by intestinal microbiota: pathological and therapeutic implications. Pharmacol Res: Off J Ital Pharmacol Soc. 2013;69(1):42–51.
136. Caricilli AM, Picardi PK, de Abreu LL, Ueno M, Prada PO, Ropelle ER, et al. Gut microbiota is a key modulator of insulin resistance in TLR 2 knockout mice. PLoS Biol. 2011;9(12):e1001212.
137. Kim KA, Gu W, Lee IA, Joh EH, Kim DH. High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway. PLoS One. 2012;7(10):e47713.
138. Rehman A, Sina C, Gavrilova O, Hasler R, Ott S, Baines JF, et al. Nod2 is essential for temporal development of intestinal microbial communities. Gut. 2011;60(10):1354–62.
139. Mondot S, Barreau F, Al Nabhani Z, Dussaillant M, Le Roux K, Dore J, et al. Altered gut microbiota composition in immune-impaired Nod2(-/-) mice. Gut. 2012;61(4):634–5.
140. Rogler G. The importance of gut microbiota in mediating the effect of NOD2 defects in inflammatory bowel disease. Gut. 2010;59(2):153–4.

141. Robertson SJ, Zhou JY, Geddes K, Rubino SJ, Cho JH, Girardin SE, et al. Nod1 and Nod2 signaling does not alter the composition of intestinal bacterial communities at homeostasis. Gut Microbes. 2013;4(3):222–31.
142. Palmer C, Bik EM, Digiulio DB, Relman DA, Brown PO. Development of the human infant intestinal microbiota. PLoS Biol. 2007;5(7):e177.
143. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A. 2010;107(26):11971–5.
144. Ajslev TA, Andersen CS, Gamborg M, Sorensen TI, Jess T. Childhood overweight after establishment of the gut microbiota: the role of delivery mode, pre-pregnancy weight and early administration of antibiotics. Int J Obes (Lond). 2011;35(4):522–9.
145. Koren O, Goodrich JK, Cullender TC, Spor A, Laitinen K, Backhed HK, et al. Host remodeling of the gut microbiome and metabolic changes during pregnancy. Cell. 2012;150(3):470–80.
146. Collado MC, Isolauri E, Laitinen K, Salminen S. Effect of mother's weight on infant's microbiota acquisition, composition, and activity during early infancy: a prospective follow-up study initiated in early pregnancy. Am J Clin Nutr. 2010;92(5):1023–30.
147. Karlsson CL, Onnerfalt J, Xu J, Molin G, Ahrne S, Thorngren-Jerneck K. The microbiota of the gut in preschool children with normal and excessive body weight. Obesity (Silver Spring). 2012;20:2257.
148. Song MJ, Kim KH, Yoon JM, Kim JB. Activation of Toll-like receptor 4 is associated with insulin resistance in adipocytes. Biochem Biophys Res Commun. 2006;346(3):739–45.
149. Zu L, He J, Jiang H, Xu C, Pu S, Xu G. Bacterial endotoxin stimulates adipose lipolysis via toll-like receptor 4 and extracellular signal-regulated kinase pathway. J Biol Chem. 2009;284(9):5915–26.
150. Pendyala S, Walker J, Holt PR. A high-fat diet is associated with endotoxemia that originates from the gut. Gastroenterology. 2012;142:1100.
151. Lassenius MI, Pietilainen KH, Kaartinen K, Pussinen PJ, Syrjanen J, Forsblom C, et al. Bacterial endotoxin activity in human serum is associated with dyslipidemia, insulin resistance, obesity, and chronic inflammation. Diabetes Care. 2011;34(8):1809–15.
152. Ghoshal S, Witta J, Zhong J, de Villiers W, Eckhardt E. Chylomicrons promote intestinal absorption of lipopolysaccharides. J Lipid Res. 2009;50(1):90–7.
153. Laugerette F, Vors C, Geloen A, Chauvin MA, Soulage C, Lambert-Porcheron S, et al. Emulsified lipids increase endotoxemia: possible role in early postprandial low-grade inflammation. J Nutr Biochem. 2010;22:53.
154. Geurts L, Lazarevic V, Derrien M, Everard A, Van Roye M, Knauf C, et al. Altered gut microbiota and endocannabinoid system tone in obese and diabetic leptin-resistant mice: impact on apelin regulation in adipose tissue. Front Microbiol. 2011;2:149.
155. Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, et al. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut. 2009;58(8):1091–103.
156. Russo F, Linsalata M, Clemente C, Chiloiro M, Orlando A, Marconi E, et al. Inulin-enriched pasta improves intestinal permeability and modifies the circulating levels of zonulin and glucagon-like peptide 2 in healthy young volunteers. Nutr Res. 2012;32(12):940–6.
157. Stoidis CN, Misiakos EP, Patapis P, Fotiadis CI, Spyropoulos BG. Potential benefits of pro- and prebiotics on intestinal mucosal immunity and intestinal barrier in short bowel syndrome. Nutr Res Rev. 2010:1–9.
158. Kitchens RL, Thompson PA, Viriyakosol S, O'Keefe GE, Munford RS. Plasma CD14 decreases monocyte responses to LPS by transferring cell-bound LPS to plasma lipoproteins. J Clin Invest. 2001;108(3):485–93.
159. Vreugdenhil AC, Rousseau CH, Hartung T, Greve JW, van 't Veer C, Buurman WA. Lipopolysaccharide (LPS)-binding protein mediates LPS detoxification by chylomicrons. J Immunol. 2003;170(3):1399–405.

160. Amar J, Burcelin R, Ruidavets JB, Cani PD, Fauvel J, Alessi MC, et al. Energy intake is associated with endotoxemia in apparently healthy men. Am J Clin Nutr. 2008;87(5):1219–23.
161. Moreno-Navarrete JM, Manco M, Ibanez J, Garcia-Fuentes E, Ortega F, Gorostiaga E, et al. Metabolic endotoxemia and saturated fat contribute to circulating NGAL concentrations in subjects with insulin resistance. Int J Obes (Lond). 2010;34(2):240–9.
162. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. J Clin Invest. 2006;116(11):3015–25.
163. Vandanmagsar B, Youm YH, Ravussin A, Galgani JE, Stadler K, Mynatt RL, et al. The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. Nat Med. 2011;17(2):179–88.
164. Poggi M, Bastelica D, Gual P, Iglesias MA, Gremeaux T, Knauf C, et al. C3H/HeJ mice carrying a toll-like receptor 4 mutation are protected against the development of insulin resistance in white adipose tissue in response to a high-fat diet. Diabetologia. 2007;50(6):1267–76.
165. Liang H, Hussey SE, Sanchez-Avila A, Tantiwong P, Musi N. Effect of lipopolysaccharide on inflammation and insulin action in human muscle. PLoS One. 2013;8(5):e63983.
166. Suganami T, Mieda T, Itoh M, Shimoda Y, Kamei Y, Ogawa Y. Attenuation of obesity-induced adipose tissue inflammation in C3H/HeJ mice carrying a Toll-like receptor 4 mutation. Biochem Biophys Res Commun. 2007;354(1):45–9.
167. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest. 2003;112(12):1821–30.
168. Gerhardt CC, Romero IA, Cancello R, Camoin L, Strosberg AD. Chemokines control fat accumulation and leptin secretion by cultured human adipocytes. Mol Cell Endocrinol. 2001;175(1–2):81–92.
169. Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. Science. 1996;271(5249):665–8.
170. Wang L, Li L, Ran X, Long M, Zhang M, Tao Y, et al. Lipopolysaccharides reduce adipogenesis in 3T3-L1 adipocytes through activation of NF-kappaB pathway and downregulation of AMPK expression. Cardiovasc Toxicol. 2013;13:338.
171. Gabele E, Dostert K, Hofmann C, Wiest R, Scholmerich J, Hellerbrand C, et al. DSS induced colitis increases portal LPS levels and enhances hepatic inflammation and fibrogenesis in experimental NASH. J Hepatol. 2011;55(6):1391–9.
172. Tomofuji T, Ekuni D, Yamanaka R, Kusano H, Azuma T, Sanbe T, et al. Chronic administration of lipopolysaccharide and proteases induces periodontal inflammation and hepatic steatosis in rats. J Periodontol. 2007;78(10):1999–2006.
173. Endo M, Masaki T, Seike M, Yoshimatsu H. TNF-alpha induces hepatic steatosis in mice by enhancing gene expression of sterol regulatory element binding protein-1c (SREBP-1c). Exp Biol Med (Maywood). 2007;232(5):614–21.
174. Sansonetti P, Di Santo J. Debugging how bacteria manipulate the immune response. Immunity. 2007;26:149–61.
175. Kufer TA, Sansonetti PJ. Sensing of bacteria: NOD a lonely job. Curr Opin Microbiol. 2007;10(1):62–9.
176. Sansonetti P. Host-pathogen interactions: the seduction of molecular cross talk. Gut. 2002;50 Suppl 3:III2–8.
177. Stappenbeck TS, Hooper LV, Gordon JI. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. Proc Natl Acad Sci U S A. 2002;99(24):15451–5.
178. Carneiro LA, Magalhaes JG, Tattoli I, Philpott DJ, Travassos LH. Nod-like proteins in inflammation and disease. J Pathol. 2008;214(2):136–48.
179. Schertzer JD, Tamrakar AK, Magalhaes JG, Pereira S, Bilan PJ, Fullerton MD, et al. NOD1 activators link innate immunity to insulin resistance. Diabetes. 2011;60(9):2206–15.
180. Caesar R, Reigstad CS, Backhed HK, Reinhardt C, Ketonen M, Ostergren Lunden G, et al. Gut-derived lipopolysaccharide augments adipose macrophage accumulation but is not essential for impaired glucose or insulin tolerance in mice. Gut. 2012;61:1701.

181. Tamrakar AK, Schertzer JD, Chiu TT, Foley KP, Bilan PJ, Philpott DJ, et al. NOD2 activation induces muscle cell-autonomous innate immune responses and insulin resistance. Endocrinology. 2010;151(12):5624–37.
182. Ogura Y, Lala S, Xin W, Smith E, Dowds TA, Chen FF, et al. Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis. Gut. 2003;52(11):1591–7.
183. Ogura Y, Saab L, Chen FF, Benito A, Inohara N, Nunez G. Genetic variation and activity of mouse Nod2, a susceptibility gene for Crohn's disease. Genomics. 2003;81(4):369–77.
184. Inohara N, Ogura Y, Nunez G. Nods: a family of cytosolic proteins that regulate the host response to pathogens. Curr Opin Microbiol. 2002;5(1):76–80.
185. Miele L, Valenza V, La Torre G, Montalto M, Cammarota G, Ricci R, et al. Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. Hepatology. 2009;49(6):1877–87.
186. Lichtman SN, Keku J, Schwab JH, Sartor RB. Hepatic injury associated with small bowel bacterial overgrowth in rats is prevented by metronidazole and tetracycline. Gastroenterology. 1991;100(2):513–9.
187. Vance JE, Vance DE. The role of phosphatidylcholine biosynthesis in the secretion of lipoproteins from hepatocytes. Can J Biochem Cell Biol. 1985;63(8):870–81.
188. Ortu G, Rivabene R, Cantafora A, Bravo E. Age-related variations in hepatic biosynthesis of phosphatidylcholine: a study of choline metabolism with perfused rat liver. Proc Soc Exp Biol Med. 1997;216(1):44–51.
189. Vance DE. Role of phosphatidylcholine biosynthesis in the regulation of lipoprotein homeostasis. Curr Opin Lipidol. 2008;19(3):229–34.
190. Dumas ME, Barton RH, Toye A, Cloarec O, Blancher C, Rothwell A, et al. Metabolic profiling reveals a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice. Proc Natl Acad Sci U S A. 2006;103(33):12511–6.
191. Claus SP, Tsang TM, Wang Y, Cloarec O, Skordi E, Martin FP, et al. Systemic multicompartmental effects of the gut microbiome on mouse metabolic phenotypes. Mol Syst Biol. 2008;4:219.
192. Martin FP, Wang Y, Sprenger N, Yap IK, Lundstedt T, Lek P, et al. Probiotic modulation of symbiotic gut microbial-host metabolic interactions in a humanized microbiome mouse model. Mol Syst Biol. 2008;4:157.
193. Carvalho BM, Guadagnini D, Tsukumo DM, Schenka AA, Latuf-Filho P, Vassallo J, et al. Modulation of gut microbiota by antibiotics improves insulin signalling in high-fat fed mice. Diabetologia. 2012;55(10):2823–34.
194. Lin HV, Frassetto A, Kowalik Jr EJ, Nawrocki AR, Lu MM, Kosinski JR, et al. Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms. PLoS One. 2012;7(4):e35240.
195. Al-Lahham SH, Peppelenbosch MP, Roelofsen H, Vonk RJ, Venema K. Biological effects of propionic acid in humans; metabolism, potential applications and underlying mechanisms. Biochim Biophys Acta. 2010;1801(11):1175–83.
196. Gao Z, Yin J, Zhang J, Ward RE, Martin RJ, Lefevre M, et al. Butyrate improves insulin sensitivity and increases energy expenditure in mice. Diabetes. 2009;58(7):1509–17.
197. Payne AN, Chassard C, Zimmermann M, Muller P, Stinca S, Lacroix C. The metabolic activity of gut microbiota in obese children is increased compared with normal-weight children and exhibits more exhaustive substrate utilization. Nutr Diabetes. 2011;1:e12.
198. Kimura I, Ozawa K, Inoue D, Imamura T, Kimura K, Maeda T, et al. The gut microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor GPR43. Nat Commun. 2013;4:1829.
199. Backhed F, Manchester JK, Semenkovich CF, Gordon JI. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci U S A. 2007;104(3):979–84.
200. Hardie DG, Carling D, Carlson M. The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annu Rev Biochem. 1998;67:821–55.
201. Bergeron R, Russell 3rd RR, Young LH, Ren JM, Marcucci M, Lee A, et al. Effect of AMPK activation on muscle glucose metabolism in conscious rats. Am J Physiol. 1999;276(5 Pt 1):E938–44.

202. Aron-Wisnewsky J, Dore J, Clement K. The importance of the gut microbiota after bariatric surgery. Nat Rev Gastroenterol Hepatol. 2012;9(10):590–8.
203. Sweeney TE, Morton JM. The human gut microbiome: a review of the effect of obesity and surgically induced weight loss. JAMA Surg. 2013;148:1–7.
204. Zhang H, DiBaise JK, Zuccolo A, Kudrna D, Braidotti M, Yu Y, et al. Human gut microbiota in obesity and after gastric bypass. Proc Natl Acad Sci U S A. 2009;106(7):2365–70.
205. Liou AP, Paziuk M, Luevano Jr JM, Machineni S, Turnbaugh PJ, Kaplan LM. Conserved shifts in the gut microbiota due to gastric bypass reduce host weight and adiposity. Sci Transl Med. 2013;5(178):178ra41.
206. Kong LC, Tap J, Aron-Wisnewsky J, Pelloux V, Basdevant A, Bouillot JL, et al. Gut microbiota after gastric bypass in human obesity: increased richness and associations of bacterial genera with adipose tissue genes. Am J Clin Nutr. 2013.
207. Furet JP, Kong LC, Tap J, Poitou C, Basdevant A, Bouillot JL, et al. Differential adaptation of human gut microbiota to bariatric surgery-induced weight loss: links with metabolic and low-grade inflammation markers. Diabetes. 2010;59(12):3049–57.
208. Cummings JH, Macfarlane GT. Role of intestinal bacteria in nutrient metabolism. JPEN J Parenter Enter Nutr. 1997;21(6):357–65.
209. Seyer P, Vallois D, Poitry-Yamate C, Schutz F, Metref S, Tarussio D, et al. Hepatic glucose sensing is required to preserve beta cell glucose competence. J Clin Invest. 2013;123(4):1662–76.
210. Zarrinpar A, Loomba R. Review article: the emerging interplay among the gastrointestinal tract, bile acids and incretins in the pathogenesis of diabetes and non-alcoholic fatty liver disease. Aliment Pharmacol Ther. 2012;36(10):909–21.
211. Watanabe M, Morimoto K, Houten SM, Kaneko-Iwasaki N, Sugizaki T, Horai Y, et al. Bile acid binding resin improves metabolic control through the induction of energy expenditure. PLoS One. 2012;7(8):e38286.
212. Meissner M, Wolters H, de Boer RA, Havinga R, Boverhof R, Bloks VW, et al. Bile acid sequestration normalizes plasma cholesterol and reduces atherosclerosis in hypercholesterolemic mice. No additional effect of physical activity. Atherosclerosis. 2013;228(1):117–23.
213. Yokota A, Fukiya S, Islam KB, Ooka T, Ogura Y, Hayashi T, et al. Is bile acid a determinant of the gut microbiota on a high-fat diet? Gut Microbes. 2012;3(5):455–9.
214. Pournaras DJ, Glicksman C, Vincent RP, Kuganolipava S, Alaghband-Zadeh J, Mahon D, et al. The role of bile after Roux-en-Y gastric bypass in promoting weight loss and improving glycaemic control. Endocrinology. 2012;153(8):3613–9.
215. Keitel V, Haussinger D. Perspective: TGR5 (Gpbar-1) in liver physiology and disease. Clin Res Hepatol Gastroenterol. 2012;36(5):412–9.
216. Devkota S, Wang Y, Musch MW, Leone V, Fehlner-Peach H, Nadimpalli A, et al. Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10-/- mice. Nature. 2012;487(7405):104–8.
217. Zhao Y, Wu J, Li JV, Zhou NY, Tang H, Wang Y. Gut microbiota composition modifies fecal metabolic profiles in mice. J Proteome Res. 2013.
218. Sayin SI, Wahlstrom A, Felin J, Jantti S, Marschall HU, Bamberg K, et al. Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist. Cell Metab. 2013;17(2):225–35.
219. Hersoug LG, Linneberg A. The link between the epidemics of obesity and allergic diseases: does obesity induce decreased immune tolerance? Allergy. 2007;62(10):1205–13.
220. Lolmede K, Duffaut C, Zakaroff-Girard A, Bouloumie A. Immune cells in adipose tissue: key players in metabolic disorders. Diabetes Metab. 2011;37(4):283–90.
221. Decaunes P, Esteve D, Zakaroff-Girard A, Sengenes C, Galitzky J, Bouloumie A. Adipose-derived stromal cells: cytokine expression and immune cell contaminants. Methods Mol Biol. 2011;702:151–61.
222. Macpherson AJ, Geuking MB, McCoy KD. Immune responses that adapt the intestinal mucosa to commensal intestinal bacteria. Immunology. 2005;115(2):153–62.

223. Owen RL, Jones AL. Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles. Gastroenterology. 1974;66(2):189–203.
224. Berg RD. Bacterial translocation from the gastrointestinal tract. Trends Microbiol. 1995;3(4):149–54.
225. Berg RD, Wommack E, Deitch EA. Immunosuppression and intestinal bacterial overgrowth synergistically promote bacterial translocation. Arch Surg. 1988;123(11):1359–64.
226. Ouellette AJ, Bevins CL. Paneth cell defensins and innate immunity of the small bowel. Inflamm Bowel Dis. 2001;7(1):43–50.
227. Schenk M, Mueller C. The mucosal immune system at the gastrointestinal barrier. Best Pract Res Clin Gastroenterol. 2008;22(3):391–409.
228. Steed H, Macfarlane GT, Blackett KL, Macfarlane S, Miller MH, Bahrami B, et al. Bacterial translocation in cirrhosis is not caused by an abnormal small bowel gut microbiota. FEMS Immunol Med Microbiol. 2011;63(3):346–54.
229. Koh IH, Liberatore AM, Menchaca-Diaz JL, Ruiz-Silva M, Vilela-Oliveira L, Watanabe AY, et al. Bacterial translocation, microcirculation injury and sepsis. Endocr Metab Immune Disord Drug Targets. 2006;6(2):143–50.
230. Klatt NR, Funderburg NT, Brenchley JM. Microbial translocation, immune activation, and HIV disease. Trends Microbiol. 2013;21(1):6–13.
231. Jevtovic DJ, Dragovic G, Salemovic D, Ranin J, Djurkovic-Djakovic O. The metabolic syndrome, an epidemic among HIV-infected patients on HAART. BioMed Pharmacother. 2009;63(5):337–42.
232. Potthoff A, Brockmeyer NH, Gelbrich G, Neuhaus K, Esser S, Reinsch N, et al. Lipodystrophy – a sign for metabolic syndrome in patients of the HIV-HEART study. J Dtsch Dermatol Ges. 2010;8(2):92–8.
233. Dandekar S, George MD, Baumler AJ. Th17 cells, HIV and the gut mucosal barrier. Curr Opin HIV AIDS. 2010;5(2):173–8.
234. Amar J, Serino M, Lange C, Chabo C, Iacovoni J, Mondot S, et al. Involvement of tissue bacteria in the onset of diabetes in humans: evidence for a concept. Diabetologia. 2011;54(12):3055–61.
235. Amar J, Lange C, Payros G, Garret C, Chabo C, Lantieri O, et al. Blood microbiota dysbiosis is associated with the onset of cardiovascular events in a large general population: the D.E.S.I.R. study. PLoS One. 2013;8(1):e54461.
236. Hosoi T, Yokoyama S, Matsuo S, Akira S, Ozawa K. Myeloid differentiation factor 88 (MyD88)-deficiency increases risk of diabetes in mice. PLoS One. 2010;5:9.
237. Riant E, Waget A, Cogo H, Arnal JF, Burcelin R, Gourdy P. Estrogens protect against high-fat diet-induced insulin resistance and glucose intolerance in mice. Endocrinology. 2009;150(5):2109–17.
238. Otero M, Lago R, Gomez R, Lago F, Gomez-Reino JJ, Gualillo O. Leptin: a metabolic hormone that functions like a proinflammatory adipokine. Drug News Perspect. 2006;19(1):21–6.
239. Caesar R, Fak F, Backhed F. Effects of gut microbiota on obesity and atherosclerosis via modulation of inflammation and lipid metabolism. J Intern Med. 2010;268(4):320–8.
240. Koren O, Spor A, Felin J, Fak F, Stombaugh J, Tremaroli V, et al. Human oral, gut, and plaque microbiota in patients with atherosclerosis. Proc Natl Acad Sci U S A. 2011;108 Suppl 1:4592–8.
241. Griffin JL, Scott J, Nicholson JK. The influence of pharmacogenetics on fatty liver disease in the wistar and kyoto rats: a combined transcriptomic and metabonomic study. J Proteome Res. 2007;6(1):54–61.
242. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature. 2011;472(7341):57–63.
243. Tang WH, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, et al. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med. 2013;368(17):1575–84.

244. Blasco-Baque V, Serino M, Vergnes JN, Riant E, Loubieres P, Arnal JF, et al. High-fat diet induces periodontitis in mice through lipopolysaccharides (LPS) receptor signaling: protective action of estrogens. PLoS One. 2012;7(11):e48220.
245. Hashimoto T, Perlot T, Rehman A, Trichereau J, Ishiguro H, Paolino M, et al. ACE2 links amino acid malnutrition to microbial ecology and intestinal inflammation. Nature. 2012;487(7408):477–81.
246. Kaddurah-Daouk R, Baillie RA, Zhu H, Zeng ZB, Wiest MM, Nguyen UT, et al. Enteric microbiome metabolites correlate with response to simvastatin treatment. PLoS One. 2011;6(10):e25482.

Chapter 12
Role of Microbiota in Regulating Host Lipid Metabolism and Disease Risk

Isabel Bondia-Pons, Tuulia Hyötyläinen, and Matej Orešič

Abstract The gut microbiota is an environmental factor which affects host metabolism and correspondingly contributes to obesity and its cardiometabolic comorbidities. However, the mechanisms behind the regulation of host lipid metabolism by gut microbiota are poorly understood. The mechanistic in vivo studies over the past decade combining gnotobiotic animal models, metagenomics, and metabolomics have begun to decipher the role of the gut microbiota in the regulation of host physiology. However, since the animal studies cannot be easily extrapolated to humans, it still remains unclear if an altered microbiota associated with a specific disease in humans is a causative factor or merely a consequence of the disease state or both. Cheaper and more comprehensive sequencing tools for the analysis of gut microbiota, together with the recent advances in mass spectrometry-based analysis of molecular lipids, are expected to contribute to our understanding of the mechanisms linking gut microbiota, host lipid metabolism, and how they together contribute to metabolic comorbidities of obesity.

Keywords Analytics • Bile acids • Bioinformatics tools • Gnotobiotics • Lipid characterization • Lipid metabolism • Lipidomics • Mass spectrometry • Metabolomics • Metagenomics • Metataxonomics • Microbiota • Signaling molecules • Systems biology

12.1 The Link Between Lipids and Gut Microbiota

Advances in the study of microbiomes by culture-independent molecular methods based on the ribosomal 16S sequence have revolutionized the analysis of gut microbiota. Metagenomic sequencing has confirmed that the human gut microbiota is a very complex community of about 100 trillion archaeal and bacterial cells corresponding to over more than 1,000 species [1]. The community is dominated by bacteria belonging

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to three major groups (phyla), Firmicutes, Bacteroidetes, and Actinobacteria, that together represent >95 % of the total microbiota [2].

Diet, genetic background, and immune system status are among the factors affecting the composition of gut microbiota [3, 4], justifying the high interindividual variation observed in human microbiota. However, it is believed that at least a common core gut microbiota, and core microbiome, shared among individuals [3, 5] is needed for a correct functioning of the gut. Recent research shows that microbial community has indeed a beneficial role during normal homeostasis, modulating the host's immune system as well as influencing host development, physiology, and metabolism [6], but the underlying mechanisms remain largely unknown. By applying the "omics" approach, molecular snapshots of biological systems can be generated, allowing the study of comprehensive molecular profiles in time as dependent on genetic or environmental variation. Systems biology approach, as discussed in Chap. 1, is therefore essential in order to deal with the "omics data," thus shifting the research emphasis from single molecular components to how they together contribute as parts of a complex network to a specific phenotype or biological function [7]. In this context, changes in the concentration of specific groups of metabolites are sensitive and specific to pathogenically relevant factors including gut microbiota. As discussed from a nutritional perspective in Chap. 5, metabolomics is expected to help in understanding changes to gut microbiota populations and function. How do gut microbiota and other environmental factors affect the

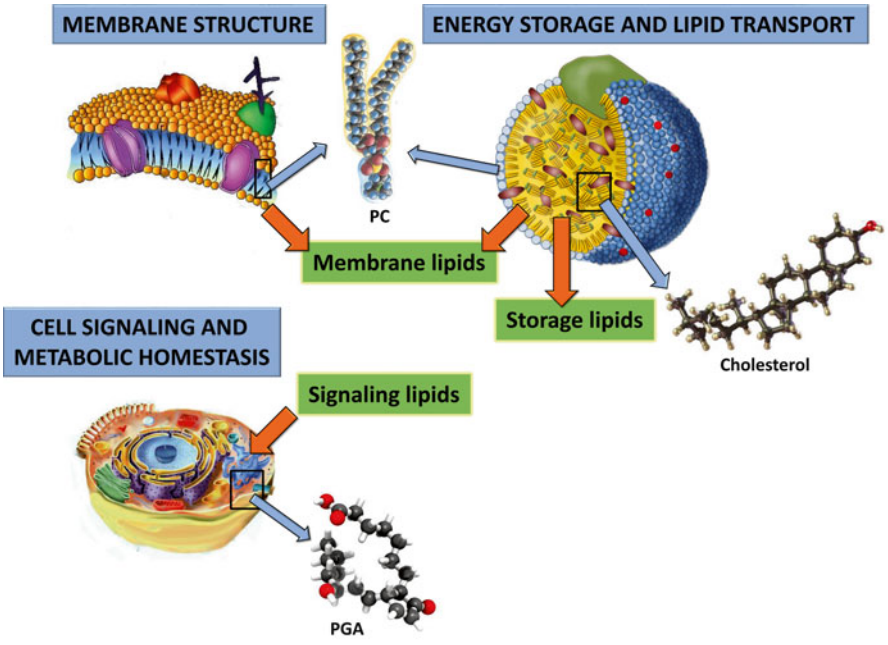

Fig. 12.1 Main functions of lipids in the human body. Lipids function as energy source, participate in essential signaling pathways, and constitute the cellular structural building blocks in cell and organelle membranes

lipidome and the underlying pathways is indeed one of the research questions to address in current and future studies in the nutrigenomics field.

Lipid homeostasis is fundamental to the maintenance of health. Lipids are a diverse group of compounds with many biological functions. The structural diversity of lipids is due to many combinatorial possibilities of how the lipid building blocks are put together, so that hundreds of thousands of distinct lipid species are theoretically possible [8, 9]. Lipids function as energy storage sources, participate in essential signaling pathways, and constitute the cellular structural building blocks in both cell and organelle membranes [10, 11] (Fig. 12.1). Lipids are thus directly involved in membrane trafficking, regulating membrane proteins, creating specific subcompartments in membranes that contribute to cellular function [12, 13], and in providing dynamic highly specialized molecular scaffolds for the construction of microscopic and macroscopic chemical assemblies needed for life processes [14]. Structurally, lipids are classified into eight main groups, namely, fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids and polyketides (derived from condensation of ketoacyl subunits), and sterol lipids and prenol lipids (derived from condensation of isoprene subunits) [15] (Fig. 12.2).

Category	Abbreviation	Sub-category
Fatty acyls	FA	Fatty acids and conjugates Octadecanoids Eicosanoids Docosanoids Fatty alcohols Fatty aldehydes Fatty esters
Glycerolipids	GL	Monoradylglycerols Diradylglycerols Triradylglycerols
Glycerophospholipids	GP	Phosphatidic acids Phosphatidylcholines Phosphatidylserines Phosphatidylglycerols Phosphatidylethanolamines Phosphatidylinositols Phosphatidylinositides Cardiolipins
Sphingolipids	SP	Sphingoid bases Ceramides Phosphosphingolipids Phosphonosphingolipids Neutral glycosphingolipids Acidic glycosphingolipids
Sterol lipids	ST	Sterols Steroids Secosteroids Bile acids and derivatives
Prenol lipids	PR	Isoprenoids Quinones and hydroquinones Polyphenols
Saccharolipids	SL	Acylaminosugars Acylaminosugar glycans Acyltrehaloses Acyltrehalose glycans
Polyketides	PK	Macrolide polyketides Aromatic polyketides Non-ribosomal peptide/polyketide hybrids

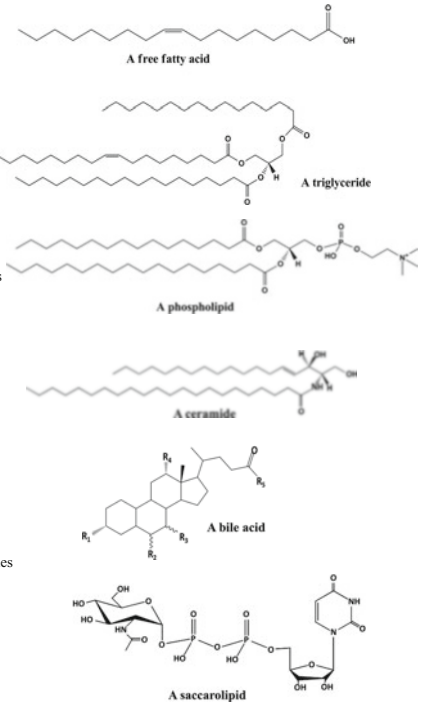

Fig. 12.2 Structural classification of lipids

12.2 Lipidomics

Lipidomics, a subdiscipline of metabonomics focused on the global study of molecular lipids, including pathways and networks of cellular lipids in biological systems [16], has been progressing rapidly over the past decade due to the advances in mass spectrometry (MS) [17, 18], computational methods [19, 20], and systems biology approaches [21]. These recent advances have highly influenced the evolution of lipidomics, together with the recognition of the major role that lipids play in health maintenance and in many metabolic diseases and their comorbidities. Lipidomic studies play indeed an essential role in defining the biochemical mechanisms of lipid-related diseases through identifying alterations in cellular lipid metabolism, trafficking, and homeostasis. Lipidomics is therefore envisaged to valuably contribute in a systems biology approach to understand the role that microbiota plays in the complex lipid host metabolism and, consequently, in human health and disease.

Increasing evidence has showed that lipid profiling might powerfully aid in clinical risk assessment. A simple example can be found in the study of triglycerides (TG). Owing to many possible combinations of acyl chains esterified to a glycerol backbone, many different molecular species of TG can be found in the human body. However, the current standard clinical measurement of TG relies on the measurement of total glycerol after acyl chain hydrolysis [22], obscuring this underlying diversity. Discriminating plasma lipids at a molecular level might shed insight on the intersection between dyslipidemia and metabolic risk in several metabolic diseases. For instance, the application of LC–MS-based lipidomic profiling has shown that specific TG containing FAs with lower carbon number and double bond content are associated with insulin resistance [23]. In agreement with the previous study, Rhee and colleagues found that the same TG signature was also predictive of type 2 diabetes in subjects who were followed up for over 12 years in the Framingham Heart Study [24].

The gut microbiota is one of the many environmental factors influencing the host lipid metabolism, but detailed lipid characterization is needed in future studies if we want to elucidate the complete mechanisms behind the regulation of the lipid host metabolism by the gut microbiota. Advances in this field can only be understood thanks to the recent (and future) advances in both analytical and computational methods and to its mandatory integration in a systems biology approach with other "omics" technologies such as metagenomics. The purpose of this section is to familiarize the reader with the core tools needed in lipidomics, by briefly overviewing the state-of-the-art and recent advances in the lipidomic field from an analytical perspective.

12.2.1 Analytical Lipidomics

Comprehensive analysis of molecular lipids requires specific analytical procedures [25] (Fig. 12.3). Generally, two types of analytical strategies are applied in lipidomic analysis: (a) the hypothesis-driven targeted selective analysis and,

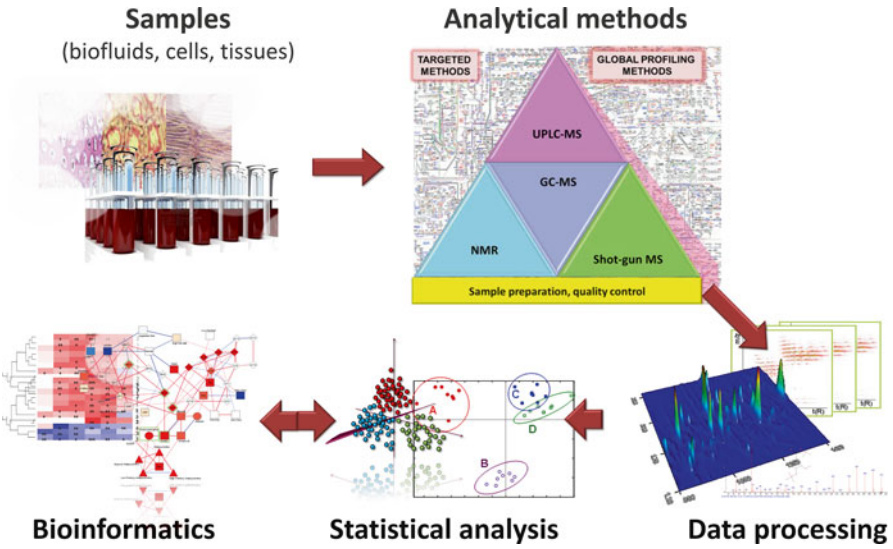

Fig. 12.3 Typical workflow used in lipidomics, starting from sampling, followed by sample preparation, analysis, data preprocessing, statistical analysis, and bioinformatics

more comprehensive, (b) the hypothesis-generating nontargeted profiling analysis. In the targeted analysis, only preselected lipids are analyzed. This approach allows very sensitive and robust determination of the selected metabolites, but it gives only limited information about the global lipidome. The nontargeted approaches aim to cover as many lipids as possible in a single analysis, across several lipid classes. As a drawback, the nontargeted approaches are typically only semiquantitative [26, 27].

Mass spectrometry has a central role in lipidomics [28]. Lipids are commonly analyzed either by direct infusion into the MS instrument (the so-called shotgun MS) or with MS in combination with a chromatographic separation method liquid chromatography (LC) or gas chromatography (GC). The LC–MS approaches are well suited for targeted as well as nontargeted analysis of lipids. While most of the complex lipids are too nonvolatile for the analysis by GC–MS, this approach is commonly applied for the analysis of FAs and sterols. Tandem or hybrid mass spectrometry (MS/MS) is used both for nontargeted analyses and for increasing the sensitivity and selectivity of quantitative analysis. In MS/MS experiments, the first analyzer is used to select a precursor ion which is fragmented in a collision cell. The product ions, i.e., the fragments of the precursor ion (collision-induced dissociation (CID)), are then detected in the second mass analyzer. For further identification, this MS/MS process can be iteratively repeated with sequential selection of resultant ions for fragmentation in MSn experiments. Suitable MS systems for CID include both quadruple-based tandem in-space instruments (e.g., triple quadrupole (QqQ) or quadrupole time-of-flight (QTOF)) and ion trap-based tandem in-time instruments (e.g., quadrupole-ion trap (QIT), linear trap quadrupole (LTQ)-Orbitrap,

or linear trap quadrupole Fourier-transform ion cyclotron resonance (LTQ-FTICR)). Among these, the QqQ is typically used for targeted quantitative analysis and the high-resolution systems for global lipid profiling as well as for the identification of lipids.

Novel hybrid high-resolution instruments, such as combination of ion mobility and TOFMS (IM–TOFMS), have recently been launched. They are promising tools to assist in the still great challenge of identification and quantification of all potential enantiomeric, stereoisomeric, and regioisomeric lipid species that can be found in biological systems. The main advantages and limitations of the recent analytical

Table 12.1 Main advantages and limitations of the recent analytical approaches used in lipidomics

Analytical approach	Main advantages	Main limitations	References
Shotgun lipidomics	Simple and fast approach	Ion suppression causes that compounds present in trace amounts are often not detected Its applicability to the search of novel, previously unknown lipids is relatively restricted	Gross and Han [29]; Isaac [30]
	Use of high-resolution mass spectrometers (QTOF-MS, FT-MS)		
	Novel hybrid high-resolution instruments, such as a combination of ion mobility (IM) and TOFMS, are suitable for the shotgun approach		
Lipidomics by ultrahigh performance liquid chromatography coupled to mass spectrometry (UHPLC–MS)	Versatile (widely used for both targeted and nontargeted analyses, using various types of mass spectrometers) Typically high sensitivity (especially when used in targeted approaches (picomoles))	Matrix effects (although less than in shotgun methods), as it is not possible to use labelled standards for all compounds	Nygren et al. [31]
		Memory effects due to, e.g., sample carryover	
	High-throughput analyses with high separation efficiency in a short analysis time		
	Identification of novel lipids is possible		
Lipidomics by structurally selective ion mobility coupled to mass spectrometry (IM-MS)	Ion mobility analysis has the ability to differentiate analytes which are isobaric in mass but differ in structure	Expensive instrumentation	Kliman et al. [32]
	Promising role for fundamental lipid characterization in future applications		

(continued)

Table 12.1 (continued)

Analytical approach	Main advantages	Main limitations	References
Comprehensive multidimensional approach	Combination of the advantages of both high-resolution instrumentation (i.e., Orbitrap MS, FT ion cyclotron resonance) and a two-dimensional chromatographic separation High resolving power Separation of isomers, conformers, and enantiomers	Ideally, comprehensive multidimensional approaches are expected to inherit the advantages of the existing methodologies and overcome the limitations of any individual, which further develop in the future	Guo and Lankmayr [33]; Han et al. [34]
		Complicated instrumentation not well suited for routine analysis	
Nuclear magnetic resonance (NMR) spectroscopy	Quantitative, nondestructive technique Useful in elucidation of molecular structures of purified lipids and structural analysis High-resolution magic-angle-spinning (HR-MAS) NMR spectroscopy potential use for metabolic profiling of intact tissues	Rather modest sensitivity The similarity of the spectra of lipids with respect to the limited structural carbon chain information is challenging NMR interpretation is complicated by the considerable number of spin-coupled multiplets that result in spectral crowding	Lindon and Nicholson [35]; Maher et al. [36]

approaches used in lipidomics are summarized in Table 12.1. The application of sophisticated histochemical, cytochemical, and physical imaging techniques [37] is also expected to contribute by adding valuable information in lipid localization in different cells and tissues.

As in other "omics" disciplines, data analysis plays a key role in lipidomics. Particularly in the global profiling approaches, the amount of data is large and it is challenging to interpret the data without proper bioinformatics. Advanced statistical analysis tools and strict quality assurance regimens are a must to provide reliable and meaningful lipidomic results. Before any statistical analysis can even be performed, data preprocessing is required, including signal processing, data normalization, and transformation, so that the raw data signals are transformed into the format which can be used for the statistical data analysis. Some techniques utilized in

lipidomics include artificial neural networks, self-organizing maps, and linear discriminant analysis, among many others [38–40].

12.3 Gut Microbiota and Host Lipid Metabolism

Research over the past decade has accumulated a large body of evidence linking alterations in the gut microbiota composition to several diseases [5, 6]. The gut microbiota is indeed currently accepted as an environmental factor that affects host metabolism and contributes to major disorders burdening the healthcare systems today, such as metabolic syndrome, cardiovascular diseases, and type 2 diabetes. Recent applications in relation to metabonomics and gut microbiota in diabetes are, for instance, discussed in detail in Chap. 12. From a mechanistic point of view, only a few recent studies have identified some key signaling pathways of the cross-species homeostatic regulation between the gut microbiota and its host [5]. In this section, the impact of the gut microbiota in lipid host metabolism is addressed from evidence accumulated in both animal and human studies.

12.3.1 In Vivo Studies

In the last decade, the combination of gnotobiotics (the study of animals living in a microbiologically defined environment, either germ-free or colonized with known bacteria) and metagenomics (the study of the metagenome, i.e., the collective genomic content of a microbiota) has elegantly begun to decipher the role of the gut microbiota in host metabolism and host physiology.

Bäckhed and colleagues [41] were the first to suggest that gut microbiota is an environmental factor affecting adiposity. The authors used normal and genetically engineered gnotobiotic mice (germ-free (GF), conventionally raised (CONV-R), and conventionalized (CONV-D) mice) to test the hypothesis that the microbiota acts through host signaling pathways to regulate energy storage in the host. GF mice are raised in the absence of any microbiota, while CONV-D mice are initially germ-free but then colonized with the microbiota from CONV-R donors. The conventionalization of adult germ-free (GF) mice with a normal microbiota harvested from the distal intestine of CONV-R mice resulted in 60 % increase in body fat content despite reduced food intake [41]. Conventionalization was accompanied by a significant increase in two liver mRNAs encoding key enzymes in the de novo fatty acid biosynthetic pathway, acetyl-CoA carboxylase (Acc1), and fatty acid synthase (Fas). Both enzymes are known targets of the two transcriptor factors, SREBP-1 (sterol response element binding protein 1) and ChREBP (carbohydrate response element binding protein), which mediate hepatocyte lipogenous responses to insulin and glucose [42]. Conventionalization clearly increased liver ChREBP mRNA and

to a lesser extent also SREBP-1 mRNA levels. Additionally, the presence of the microbiota promoted increased monosaccharide uptake from the gut, their increased delivery to the liver, and a resulting induction of de novo hepatic lipogenesis.

Lipoprotein lipase (LPL) is a key regulator of fatty acid release from triglyceride-rich lipoproteins in fat and muscle. Increased adipocyte LPL activity leads to increased cellular uptake of fatty acids and adipocyte TG accumulation. Fasting-induced adipocyte factor (Fiaf), a member of the angiopoietin-like family of proteins, is produced by brown and white fat, liver, and intestine [43]. Interestingly, Fiaf was selectively suppressed in the intestine epithelium of conventionalized mice. By using normal and Fiaf knockout mice, Fiaf was established as a circulating LPL inhibitor, highlighting that its suppression is essential for the microbiota-induced deposition of triglycerides in adipocytes [41]. The relevance of Fiaf expression, which is selectively suppressed in the gut epithelium by the microbiota, was established when Fiaf-deficient mice fed a Western diet gained significantly more weight and had significantly greater epididymal fat pads than their wild-type littermates [44].

The levels of leptin, which is an adipocyte-derived hormone known to reduce food intake and increase energy expenditure, were increased upon colonization and were proportional to the observed increase in body fat in [41]. The ob/ob mouse model, characterized by increased food consumption due to leptin deficiency, was then firstly used to provide evidence that the obesity-associated gut microbiome has an increased capacity for energy harvest from the diet [45]. The same group had shortly before revealed that the two most abundant bacterial divisions in mice were the phyla Firmicutes and Bacteroidetes and that their proportions were increased and reduced, respectively, in obese mice relative to their lean counterparts [46] in a study that can be considered the first approach of DNA sequencing focused on the 16S rRNA gene in the context of obesity. The next step was to apply shotgun pyrosequencing technology in the same mouse model [45]. In addition, in order to confirm the increased ratio of Firmicutes to Bacteroidetes in the obese mice, the predicted increased capacity for dietary energy harvest by the ob/ob microbiome was validated using biochemical assays and by transplantation of lean and obese cecal microbiotas into GF wild-type mouse recipients.

Interestingly, the persistently lean phenotype in GF animals, which are protected against the obesity that develops after consuming a Western-style, high-fat, sugar-rich diet, was associated with increased skeletal muscle and liver levels of phosphorylated AMP-activated protein kinase (AMPK) and its downstream targets involved in fatty acid oxidation, acetyl-CoA carboxylase, and carnitine palmitoyltransferase (Cpt1) [44]. AMPK is a heterotrimeric enzyme that functions as a "fuel gauge" monitoring cellular energy status, which is activated in response to metabolic stresses that result in an increased intracellular ratio of AMP to ATP, such as exercise, hypoxia, and glucose deprivation [47] and adipocyte-derived leptin levels [48]. All together, the findings reported by Gordon's group indicated that gut microbes can affect both sides of the energy balance equation, as a factor that influences the harvest of energy from components of the diet and as a factor that affects host genes that regulate how energy is expended and stored.

Wikoff and colleagues [49] were among the first demonstrating the large effect of the microbiome on mammalian plasma metabolic profile. The untargeted MS-based profiling of serum from GF and CONV-R mice showed that concentrations of many circulating metabolites are affected by the presence of the microbiome. Several pathways including the metabolic processing of indole-containing molecules were seen to particularly interact with the microbiome. Multiple organic acids containing phenyl groups were also greatly increased in the presence of gut microbes, and a broad, drug-like phase II metabolic response of the host to metabolites generated by the microbiome suggested that the gut microbiota has also a direct impact on the drug metabolism capacity of the host [49]. In another study, global analysis of polar metabolites and molecular lipids in serum, white adipose tissue, and liver of GF and CONV-R mice was applied to delineate how the gut microbiota affects host energy and lipid metabolism [50]. Analysis of the serum metabolome showed that energy metabolites were increased in CONV-R mice, which was consistent with higher-energy metabolism in the presence of gut microbiota [51]. Notably, lipidomic analysis highlighted systemic effects of gut microbiota on host lipid metabolism, especially in a large number of individual TG species and in several phosphatidylcholine species. TG levels were lower in serum but higher in adipose tissue and liver of CONV-R mice, consistently with increased lipid clearance. Thus, the study demonstrated that gut microbiota affects both energy-storing and signaling lipids.

The investigation of the microbial communities from both wild-type and resistin-like molecule (RELM) β knockout mice fed a standard chow diet and a high-fat diet [52] demonstrated the importance of diet as a determinant of gut microbiome composition and suggests the need to control for dietary variation when evaluating the composition of the human gut microbiome. The expression of the RELMβ gene has been shown to be dependent on the gut microbiome and can be induced by a high-fat diet [53]. In Hilderbrant et al. study [52], the RELMβ knockout mice consuming the HFD remained lean, whereas the corresponding wild-type mice became obese. Higher levels of RELMβ expression were observed in HFD mice when compared with mice fed a standard chow diet. Further analysis also revealed that the expression of a collection of genes encoding ABC transporters was increased in wild-type mice fed the HFD when compared with expression of the same genes in wild-type mice on a standard chow diet. The corresponding proteins are responsible for the transport of lipids, sugars, and peptides. Altogether and because the general changes in the composition of the gut microbiota were similar in both types of mice, the authors concluded that the HFD, and not the obese state, accounted for the alteration in the gut microbial communities, highlighting therefore the dominant effect of diet.

Research in Nicholson's group added further knowledge to understand the bidirectional interaction between the host metabolism and its symbionts. Firstly, the metabolic phenotypes of GF and conventional C3H mice were characterized by applying an NMR-based metabolic profiling, providing evidence that the microbiota status affects the systemic metabolism of host modulating the metabolic fingerprint of topographically remote organs such as the liver and the kidney [54].

In a second study, the adaptive mechanisms of gut colonization by microbiota using a similar systems biology approach in the same mouse strain were explored by combining NMR-based profiling to gut microbial monitoring by 16SrRNA gene pyrosequencing [55]. This kind of approach allows the simultaneous study of the composition of the microbial ecosystem with the modifications of the host metabolism induced by the colonization process. In particular, the study focused on the evolution of liver metabolism, but similar research in other tissue-specific metabolism would highly benefit the global understanding of the complex lipid host metabolism.

Claus et al. study [55] findings can be considered as the first evidence of an in vivo association between a family of bacteria and hepatic lipid metabolism. Acquisition of the gut microbiota was associated with rapid increase in body weight over the first days of colonization with parallel changes in multiple pathways in all compartments analyzed (liver, kidney, colon, urine, and plasma). The colonization process stimulated glycogenesis in the liver prior to triggering TG increases in hepatic TG synthesis. These changes were associated with modifications of the expression of hepatic Cyp8b1 (sterol 12α-hydroxylase), and the subsequent alteration of bile acid metabolites, which are essential regulators of lipid absorption, and will be further discussed later in this chapter. Statistical regression OPLS-based models between hepatic metabolic profiles and microbial composition revealed strong associations of the Coriobacteriaceae family with hepatic TG, glucose, and glycogen levels. Two bacterial phyla (Actinobacteria and Tenericutes) were significantly predicted by the liver metabolic profiles and were both associated with high hepatic levels of TG and low hepatic levels of glycogen and glucose. A complementary study performed on hamsters [56] previously reported a strong correlation between unidentified bacteria of the same Coriobacteriaceae family and non-HDL plasma cholesterol when the metabolism was challenged using grain sorghum lipid extract to improve the HDL/non-HDL ratio.

Brown adipose tissue (BAT) is a central organ involved in the regulation of energy expenditure in mice. BAT is increasingly considered as a new target in the battle against obesity after recent discovery of BAT in humans [57, 58]. BAT and its specific uncoupling protein UCP1 have been linked to the development of obesity in C57B1/6J mice ablated for the UCP1 [59]; and interestingly, the regulator role that BAT plays for TG-rich lipoprotein clearance and for the control of blood lipid abundance has been recently demonstrated [60], highlighting the potential role of BAT, with its main depots localized in the supraclavicular and neck regions, for reducing the risk of metabolic syndrome [61]. In this context, an NMR spectroscopy-based metabolic profiling approach was applied in order to investigate the influence of GF state and gender on energy metabolism in urine, plasma, liver, and BAT of C3H mouse [62]. Interestingly, conventional male mice had a significantly higher total body fat content compared to conventional female mice, whereas this sexual dimorphism disappeared in GF animals. Among the metabolite changes, choline, phosphocholine, and glycerophosphocholine were observed in higher levels in GF males' BAT metabolic profiles when compared to their conventional counterparts. As it is well known, these metabolites are involved in the

formation and maintenance of cellular membranes through structural lipids, and according to [62], their observed increased levels may indicate a variation of brown adipocyte sizes between GF and conventional male animals. It is then likely that conventional BAT contains larger mature adipocytes and GF display a higher number of small undifferentiated preadipocytes.

Higher levels of (D)-3-hydroxybutyrate and lower levels of lactate were also observed in GF mice compared to their conventional counterparts. On one hand, a strong elevation of (D)-3-hydroxybutyrate was also observed in plasma and liver of the GF mice. 3-hydroxybutyrate is the major ketone body produced in the mitochondria of adipocytes and hepatocytes, initiated by the condensation of two molecules of acetyl-CoA derived from the beta-oxidation of lipids. This ketone body plays an important role in the host energy metabolism, acting on noradrenaline receptors to inhibit BAT thermogenesis and to regulate appetite in mice [63]. On the other hand, adipose tissue is a major site of glucose conversion to lactate, and lactate overproduction could be associated with metabolic abnormalities related to obesity development. In addition, decreased circulating VLDL levels in GF mice suggested hepatic inhibition of lipogenesis. Altogether, the findings in [62] indicate that GF BAT overactivated the catabolism of lipids compared to conventional animals.

Recently, fatty liver showed to accompany an increase in *Lactobacillus* species in the hindgut of C57BL/6 mice fed an HFD [64]. The C57BL/6 mouse model of obesity uses a diet high in total fat, and particularly n-6 FA, to produce outcomes similar to those observed in obese humans, namely, increased adiposity, production of proinflammatory cytokines, and fatty infiltration of the liver. Emerging evidence suggests that hindgut microbiota may contribute to liver pathology [65, 66]. It is well known that HF feeding increases the secretion of bile acids, which are important regulators of hepatic lipid metabolism that are believed to be a determinant of the gut microbiota in response to HFD [67]. The obesity-related inflammatory fatty liver caused by the HFD in the previously mentioned model was interestingly accompanied by a large increase in hindgut L. gasseri and/or L. taiwanensis, both of which are part of L. acidophilus species group of bacteria. Both bacteria have been recently suggested to play a role in body weight control [68]. Further studies with GF mice are therefore needed to determine the mechanistic role of these bacteria in the development of inflammatory liver fat due to HF feeding.

These last 10 years of gnotobiotics research have highly contributed in the understanding of the interactions of microbiota and host lipid metabolism (Fig. 12.4). Nevertheless, it is important to point out that the results obtained in gnotobiotics-based studies cannot be automatically extrapolated to humans, and it still remains unclear if an altered microbiota associated with a disease in humans is causing, contributing, or merely a consequence of the disease state. Cheaper sequencing and improved bioinformatics tools for the analysis of the gut microbiota, together with the recent advances in detailed lipid characterization, are expected to contribute to the future studies that are still needed to elucidate the complete mechanisms connecting microbiota and human metabolism.

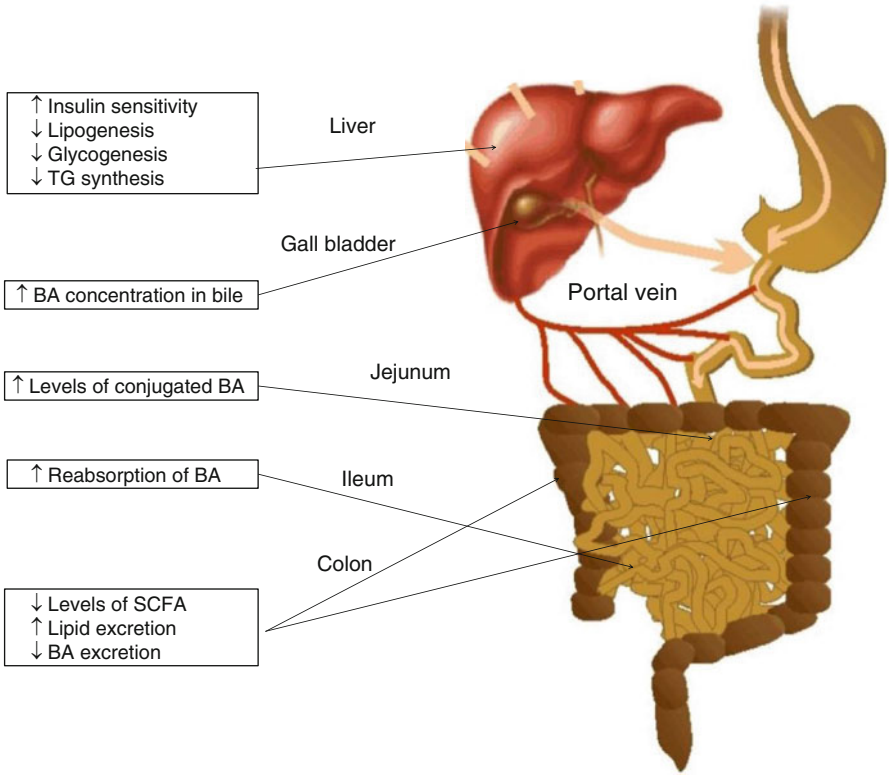

Fig. 12.4 Effect of microbiota in lipid metabolism of germ-free mice (*GF*) vs. conventionally raised (*CONV-R*) mice according to gnotobiotics studies. Arrows mean increased or decreased levels in GF vs. CONV-R mice

12.3.2 Human Studies

Accumulating evidence indicates that the gut microbiota may be a future target for treating metabolic diseases [69]. Supplementing the diet with nondigestible foods, or prebiotics, that stimulate the expansion of specific microbes to improve metabolic regulation can be a nutritional therapy for overweight and obesity management [70]. However, to determine the effects of these therapies, appropriate human intervention studies are still required. Human studies are helping to show what effect the gut microbiota has on host metabolism by improving energy yield from food and modulating dietary or the host-derived metabolites that alter host metabolic pathways. Due to the heterogeneous etiology of lipid metabolism-related diseases, such as obesity and diabetes, the role of gut microbiota in the development of these disorders is still unclear. Many studies so far are underpowered, include participants with diverse ethnic origin and dietary habits, and have used different methods with specific biases to profile the microbiota. In addition, studies in humans tend to be associative, so the mechanistic role of the microbiota in obesity and its comorbidities in humans remains

to be proven. Relevant human studies in the field are briefly discussed in this section. Results from bariatric surgery studies are not included as they are discussed in detail in another chapter of this book.

Gut microbiota composition is altered in people who are obese, and it can respond to changes in body weight. Ley and colleagues [71] revealed that the Bacteroidetes and Firmicutes divisions dominate the human microbiota, with obese subjects having lower levels of Bacteroidetes and higher levels of Firmicutes than lean subjects. Later on, a study with monozygotic and dizygotic twins and their mothers showed that the composition of the gut microbiota is more similar between family members than unrelated individuals [3]. Each individual's gut microbiota was thought distinct, and a similar degree of covariation existed between adult monozygotic and dizygotic twin pairs. The previously reported lower levels of Bacteroidetes in obese than lean subjects were confirmed, but no significant differences in Firmicutes levels were detected. Remarkably, the microbial population was in general less diverse in obese individuals.

Some other studies have shown discrepancies in the Firmicutes and Bacteroidetes proportions with respect to obesity in humans [72, 73]. The different clinical criteria, such as the level of obesity, degree of weight loss, and duration of caloric restriction, together with different geographical locations, ages, population sizes, and microbiota-profiling methodologies, can be responsible of the differences observed between studies. A low-cost clinical method for monitoring the variations of bacterial phyla of the gut using real-time PCR assay was able to confirm a reduction in the Bacteroidetes community in obese subjects and found an increase in *Lactobacillus* species in obese subjects and an increase in methanogens (M. smithii) in anorexic patients [74]. This preliminary data that links *Lactobacillus* levels with obesity needs further study with other advanced methodologies.

Consumption of lactic acid bacteria marketed as probiotics is indeed a common approach to maintain health [75]. *Lactobacillus rhamnosus* GG is one of the most widely used probiotic bacteria that is assumed to interact with the host via binding to human mucus via its extracellular pili [76]. However, further molecular details of probiotics signaling are not yet understood. In a recent study, high-throughput screening of the intestinal microbiota was performed using a phylogenetic HITChip microarray and qPCR methodology and integrated with serum lipidomic profiling data to study the impact of probiotic intervention on the intestinal ecosystem and to explore the associations between the intestinal bacteria and serum lipids [77]. Healthy subjects received either L. rhamnosus GG or placebo for a 3-week period following a randomized, double-bind intervention design. While a specific increase in the L. rhamnosus-related bacteria was observed during the intervention, no other changes in the composition or stability of the microbiota were detected. The most prevailing association between the gut microbiota and lipid profiles was a strong positive correlation between uncultured phylotypes of *Ruminococcus gnavus* group and polyunsaturated serum TG species of dietary origin. Actinomycetaceae correlated negatively with TG of highly unsaturated FA while a set of Proteobacteria showed negative correlation with ether PCs. Altogether, these results suggest that several members of the Firmicutes, Actinobacteria, and Proteobacteria may be

involved in the metabolism of dietary and endogenous lipids. This data supports the concept that the overall lipid content in human serum is a composite of host and microbial metabolic activity, and the intestinal commensals are implicated in the metabolism of various lipid species that the human body uses for membranes, energy storage, and signaling. Considering that a single gene in an intestinal bacterium could alter host FA composition [78], the potential metabolic capacity and the functional consequences from the million genes in the intestinal microbiome are overwhelming. Future studies combining metagenomics and lipidomics, involving controlled diet and dyslipidemic subjects, are indeed needed to provide further insights on the role of intestinal microbiota on human lipid metabolism.

The human intestinal microbiota is immensely complex and includes thousands of species that have a collective genome of close to five million genes. High throughput of this metagenome is increasingly replacing the characterization of individual microbes [79]. Notably, the MetaHIT Consortium was the first in addressing the feasibility of comparative metagenomics of the human gut across cohorts and protocols and in obtaining first insights into commonalities and differences between gut microbiomes across different populations [80]. They sequenced 22 European metagenomes from Danish, French, Italian, and Spanish subjects and combined them to existing Japanese [81] and American [3, 82] datasets. Three enterotypes were identified on the basis of variations in the relative levels of Bacteroidetes, *Prevotella*, and *Ruminococcus*, which were not nation or content specific. Interestingly, the Enterotype 1 is enriched in *Bacteroides* and the co-occurring *Parabacteroides*, which derived energy mainly from carbohydrates and proteins by fermentation [83]. Enterotypes appear complex, but there are functional markers such as genes or modules that correlate remarkably well with individual features. This might potentially be used for diagnostic and prognostic tools for numerous human disorders, including those related to lipid metabolism.

The combination of metagenomic analysis with clinical data is the base of the recently emerging metagenome-wide association studies (MGWAS), which are important contributions to reveal the associations of gut microbiota with health and disease. To date, the metagenomes of relatively few individuals have been sequenced.

Recently, Karlsson and colleagues [84] applied shotgun sequencing to characterize the fecal metagenome of 145 European 70-year-old women with normal, impaired, or diabetic glucose control. Interestingly, the study reported compositional and functional alterations in the metagenomes of women with type 2 diabetes, such as increases in the abundance of four *Lactobacillus* species and decreases in the abundance of five Clostridium species. In the total cohort, *Lactobacillus* species correlated positively with fasting glucose and glycosylated hemoglobin (HbA1c), which is a long-term measure of blood glucose control. By contrast, *Clostridium* species correlated negatively with fasting glucose, HbA1c, insulin, C-peptide, and plasma TG and positively with adiponectin and HDL. These correlations are relevant for T2D because high TG and low HDL levels are components of the dyslipidemia typically found in T2D, and reduced levels of adiponectin have been reported in people at risk of T2D [85]. Interestingly, the authors developed a mathematical model based on metagenomic clusters (MGC) to test whether the microbiota

composition can identify diabetes status. MGC identified T2D more accurately than species, indicating that several important gut species still need to be characterized. L. gasseri had the highest score for the identification of T2D women in the model. Notably, the model identified *Roseburia* and *Faecalibacterium prausnitzii* as highly discriminant for T2D. These bacteria are known human gut colonizers and butyrate producers [86] and have been linked to improved insulin sensitivity and diabetes amelioration in studies of the human fecal microbiota [87, 88]. Gut microbiota transplantations from lean donors to recipients with metabolic syndrome have indeed been shown to increase *Roseburia* and butyrate levels together with improved insulin sensitivity [87].

The pathways that showed the highest scores for enrichment in T2D metagenomes included KEGG orthologues for starch and glucose metabolism, fructose and mannose metabolism, and ABC transporters for amino acids, ions, and simple sugars. These findings agree with previous studies showing an increase in microbial functions for energy metabolism and harvest in the obese microbiome [45]. Other metabolic pathways containing KEGG orthologues enriched in women with T2D included glycerolipid metabolism and fatty acid biosynthesis [84]. When applying the model to a recently described Chinese cohort [89], the authors observed that the most discriminatory MCG differed between the European and Chinese T2D cohorts. This observation underscores the need to sample human populations and perform parallel studies in different continents.

Given that the cost for sequencing is rapidly declining, the major challenge in metagenome studies will be data analysis rather than data generation [90]. Detailed studies on the metagenome early in life as well as regional metagenomes will be needed in the future to determine whether or not it can be programmed. In addition, future studies might benefit from the use of tracers to look at lipid metabolic functions in order to have a readout to investigate host-bacterial co-metabolism and signaling.

12.4 Microbiota and Host Lipid Metabolism: Potential Role of Signaling Molecules

Animal and in vitro studies have shown that the intestinal microbiota can regulate host lipid metabolism via numerous microbial activities [91]. So far, short-chain fatty acids (SCFA) have shown to be key microbial products with multiple effects on host metabolism, while the best characterized mechanism of microbiota and host lipid metabolism is through the biotransformation of bile acids [92]. Combination of metagenomics and metataxonomics with comprehensive metabolomics of biofluids and stool samples has a potential to identify novel metabolites associated with specific microbes. These could be functionally studied in vitro and in vivo for their potential role in the regulation of host metabolism.

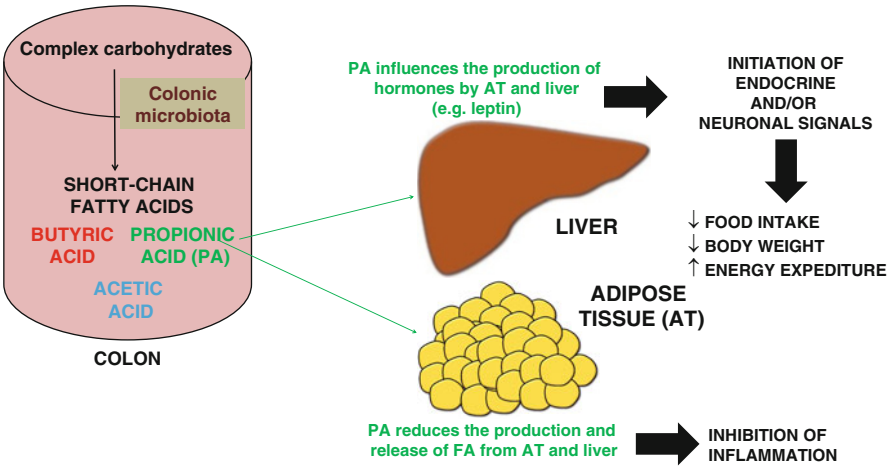

Fig. 12.5 Main metabolic effects of the short-chain fatty acid propionic acid (*PA*) produced by colonic fermentation

12.4.1 Microbiota and Short-Chain Fatty Acids

Short-chain fatty acids are clearly one of the most important microbial products and have shown to affect a range of host processes, including energy utilization, host-microbiome signaling, and control of colonic pH [93]. Complex carbohydrates can be digested and subsequently fermented in the colon by gut microbes into SCFA, such as acetate, propionate, and butyrate. Their profile in the gut reflects the metabolic cooperation between different microbial types because no genus of bacteria can hydrolyze all substrates and none produce all four SCFA upon carbohydrate fermentation [94]. SCFA have different metabolic features. Among them, butyrate is used as an energy substrate for colonocytes, whereas acetate is potentially used as a cholesterol or fatty acid precursor [95]. Propionate is a gluconeogenetic substrate in the liver, but it may also counteract de novo lipogenesis from acetate or glucose in the same tissue [96] (Fig. 12.5).

More recently, SCFA have been identified as the physiological ligands of two receptors for fatty acids, the G protein-coupled receptors GPR41 and GPR43, which are expressed in several cell types (immune cells, endocrine cells, and adipocytes) and in a wide variety of host tissues [97]. Both receptors show differences in SCFA specificity, intracellular signaling, and tissue localization [98]. GPR41 knockout mice colonized with a specific fermentative microbial community have shown to resist fat mass gain compared to their wild-type littermates [99]. By using GPR45 knockout animals, it was shown that the activation of GPR43 in adipocytes leads to inhibition of lipolysis and results in the reduction of plasma FFA levels in vivo [100]. These findings therefore suggest a potential role for GPR43 in regulation of plasma lipid profiles, but its use as a potential target for the treatment of dyslipidemia

requires further research. By using GPR43-deficient mice fed an HFD, the activation of GPR43 by the acetate and propionate has also shown to contribute to adipocyte differentiation [101], thereby promoting the expansion of adipose tissue, which is in agreement with a gnotobiotics studies performed by Bäckhed et al. [41].

Interestingly, studies adding fermentable carbohydrates with prebiotic properties, such as insulin-type fructans, into the diet did not increase but even lessened fat mass development in obese mice and humans [102, 103]. Notably, the supplementation with those prebiotics blunted the overexpression of GPR43 occurring in HFD-fed animals, a phenomenon that contributes to lower adiposity [104]. It is well known that specific changes in the gut microbiota composition by using prebiotics strongly promote SCFA production [105]. Studies related to the potential of metabonomics and metagenomics to promote health via prebiotics are discussed in Chap. 9. Altogether the previous studies support the idea that SCFA coming from the gut act not only as energy substrates but also as important metabolic regulators.

12.4.2 Microbiota and Bile Acid Metabolism

The interaction between bile acids and gut microbiota is complex. However, recent studies have added novel insights into the regulation of BA metabolism by gut microbiota. Bile acids (BA) play an important role in lipid metabolism. They function as detergents by forming micelles that facilitate solubilization, digestion, and absorption of dietary lipids and lipid-soluble vitamins and represent the major route of cholesterol excretion, which is critical in whole body sterol metabolism [106]. Remarkably, recent research has highlighted the role of BA as signaling molecules. The discovery that specific BA differentially activates three nuclear receptors, namely, farnesoid X receptor (FXR), pregnane X receptor (PXR), and vitamin D receptor (VDR), and one G protein-coupled receptor (TGR5) identified BA as hormones that alter multiple metabolic pathways [107]. The activation of these receptors alters gene expression in multiple tissues, leading to changes not only in BA metabolism but also in glucose homeostasis, lipid and lipoprotein metabolism, energy expenditure, inflammation, and liver regeneration processes.

Bile acids are produced in hepatocytes, stored in the gallbladder, and released into the duodenum upon ingestion of food. After having traveled through the small intestine, >95 % of all liver-secreted BA are reabsorbed in the ileum to be taken up by the liver in a process known as enterohepatic circulation [108]. Only a small part of the BA pool escapes the enterohepatic cycle and travels toward the large intestine to be excreted in the feces. This excretion is accompanied by microbial deconjugation of glycine(predominant in humans)- and taurine(predominant in mice)-conjugated bile acids [54]. Intestinal microbiota readily deconjugate and 7-α-dehydroxylate the primary BA (cholic acid (CA) and chenodeoxycholic acid (CDCA) in humans, and CA and β-muricholic acid (βMCA) in mice [109], converting them into secondary BA (mainly DCA, UDCA, and LCA in humans) [109] (Fig. 12.6).

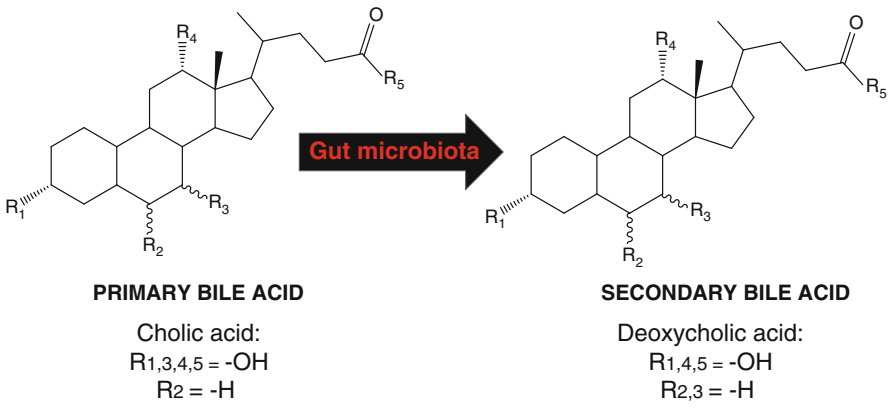

Fig. 12.6 Conversion of primary bile acids to secondary bile acids by the action of gut microbiota. The basic C24-bile acid structure is illustrated in the figure, showing the conversion of cholic acid into deoxycholic acid

Recent animal studies have shown that the microbiome affects not only the composition of the BA pool but also the expression of genes controlled by the BA-activated nuclear receptor FXR [110]. Bile acid synthesis is indeed under negative feedback control through activation of FXR in the ileum and liver [111]. At least 14 liver enzymes have been reported to be required in the BA synthesis from cholesterol. Briefly, the rate-limiting enzyme cholesterol 7α-hydroxylase (CYP7A1) initiates the classic pathway for BA synthesis, while CYP27A1 initiates the alternative pathway [112]. Sterol 12α-hydroxylase (CYP8B1) is needed for CA synthesis, and the hepatic expression of both, CYP7A1 and CYP8B1, is regulated by FXR [113].

Already 40 years ago, Wostmann et al. showed that the BA concentration in bile was three times increased and cholesterol absorption was 25 % greater in the absence of gut microbiota in GF rats when compared to their conventionally raised counterparts [114]. The cholesterol accumulation was thought to be due to an increase in intestinal BA reabsorption. Follow-up studies supported Weismann's hypothesis by showing that GF animals have elevated levels of conjugated BA throughout the intestine with no deconjugation and strongly decreased fecal excretion [115]; and later it was also confirmed in studies with mice treated with antibiotics [116]. Those previous studies showed that the gut microbiota reduces the bile acid pool size with its greatest effect on βMCA rather than CA levels. However, the molecular mechanisms for how the gut microbiota suppresses BA synthesis are currently unknown. A comprehensive profile of the BA composition of CONV-R and GF mice through the enterohepatic system and in serum identified a profound role of the gut microbiota not only on secondary BA but also as regulator of hepatic BA synthesis [108]. In addition to confirm a dramatic reduction in MCA, but not CA, levels in CONV-R mice, rederivatization of FXR-deficient mice as GF demonstrated that the gut microbiota regulated expression of fibroblast growth factor 15 (Fgf15)

in the ileum and CYP7A1 in the liver, by FXR-dependent mechanisms. Remarkably, tauro-conjugated beta- and alpha-MCA were identified as potent FXR antagonist, proposing that the higher levels of MCA in GF mice at least partially account for the lower expression of FXR-dependent genes in the ileum of GF mice [117].

In addition to FXR-based studies, TGR5 is the only G protein-coupled receptor that has been reported to respond to BAs by the production of cAMP and the subsequent activation of PKA signaling pathways [118]. By treating brown adipocytes and human skeletal myocytes with BA, Watanabe et al. [119] showed an increase in type 2 iodothyronine deiodinase (D2) activity and oxygen consumption, highlighting that bile acids can induce energy expenditure by promoting intracellular thyroid hormone activation. Since then, the BA–TGR5–cAMP–D2 signaling pathway has been considered as a key mechanism for fine-tuning energy homeostasis that can be targeted to improve metabolic control.

Interestingly, a recent study in which treatment of primary rodent hepatocytes with conjugated BA led to activation of extracellular regulated kinase (ERK) 1/2 and protein kinase B (AKT), in a sphingosine-1-phosphate receptor-dependent pathway [120], suggests that other BA receptors remain to be identified. The generation of new knockout mice lacking potential BA receptors is therefore needed. Nevertheless, accumulating evidence has proved that the modulation of FXR and TGR5 activity either directly by BA or pharmacological compounds or indirectly by intestinal BA sequestration has helped to unravel the function of these BA receptors in metabolic control. Consequently, both receptors might be promising targets for the treatment of metabolic disorders associated with the metabolic syndrome as recently reviewed by Porez et al. [121]. Although some clinical trials in phases I–III have been already performed by using synthetic FXR agonists, large-scale clinical trials will be needed to objectively assess their therapeutic possibilities in the treatment of type 2 diabetes, metabolic syndrome, or nonalcoholic steatohepatitis.

The understanding of the role of BA plays in human health and disease will also likely benefit from recent advances in technology that enables genome-wide association studies. Some inflammatory genes has been, for instance, identified in the GWAS for primary biliary cirrhosis [122, 123], and some genes involved in BA metabolism have been associated with other traits such as the association of CYP7A1 with total and LDL cholesterol [124]. Further studies will probably open up new insights for discovery of novel genes involved in BA metabolism. Similar scientific strategies may also be applied to study the role of other bioactive lipids in the regulation of host lipid metabolism. Comprehensive metabolomics of biofluids and stool samples, associated with genomic and metagenomic strategies, may help identify the microbes as well as genes associated with the specific lipids. These lipids may then be studied in the context of their role in health and disease, and mechanistic studies similarly as described above may contribute to the elucidation of the mechanisms behind their regulation of host metabolism.

12.5 Future Directions

In order to account for the enormous functional and structural diversity of lipids and their complex regulation at multiple spatial and temporal scales, a systems biology approach is needed for the study of lipids. Lipid signaling pathways are complex and the therapeutic potential of modulation of intracellular and systemic lipid metabolism is well recognized [10, 125]. Understanding the integrated lipidomic networks and decoding the coordinately regulated pathways will therefore constitute major goals for the following years in applied lipidomic research.

A better understanding of the lipidome at the physiological level lipids does not only have to include lipid modelling at the level of biological pathways, but also at the level of the biophysical systems the lipids are part of, such as cellular membranes and lipoproteins particles [20]. Rapidly accumulating information about the importance of gut microbiota in many lipid-related disorders together with the advances in lipid analytical technologies and modelling approaches are likely to contribute to better understanding of the role gut microbiota plays in the regulation of cellular and systemic lipid metabolism, and vice versa, and how the dysregulation of these physiological systems may contribute to many devastating diseases.

Future studies should rely on a systems medicine approach, where instead of focusing on each disease individually, the aim is to account for the complex gene–environment, socioeconomic interactions and comorbidities that lead to individual-specific complex phenotypes. An in-depth understanding of the metabolic phenotypes in health and disease is crucial if one is to implement personalized medicine and nutrition. How different metabolic phenotypes can be implemented as diagnostic tools in clinic needs therefore to be addressed at a clinical translational research level, as pointed out in the last chapter of this book.

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References

1. Qin J, Li R, Raes J, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464:59–65.
2. Greiner T, Bäckhed F. Effects of the gut microbiota on obesity and glucose homeostasis. Trends Endocrinol Metab. 2011;22:117–23.
3. Turnbaugh PJ, Hamady M, Yatsunenko T, et al. A core gut microbiome in obese and lean twins. Nature. 2009;457:480–4.
4. Benson AK, Kelly SA, Legge R, et al. Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. Proc Natl Acad Sci U S A. 2010;107:18933–8.
5. Tremaroli V, Backhed F. Functional interactions between the gut microbiota and host metabolism. Nature. 2012;489:242–9.
6. Sommer F, Backhed F. The gut microbiota – masters of host development and physiology. Nat Rev Microbiol. 2013;11:227–38.

7. Ideker T, Galitski T, Hood L. A new approach to decoding life: systems biology. Annu Rev Genomics Hum Genet. 2001;2:343–72.
8. Yetukuri L, Ekroos K, Vidal-Puig A, Oresic M. Informatics and computational strategies for the study of lipids. Mol BioSyst. 2008;4:121–7.
9. Buckingham J, editor. Dictionary of natural products on CD-ROM, version 19.1. London: Chapman and Hall; 2010.
10. Orešič M, Hänninen VA, Vidal-Puig A. Lipidomics: a new window to biomedical frontiers. Trends Biotechnol. 2008;26:647–52.
11. Fahy E, Cotter D, Sud M, Subramaniam S. Lipid classification, structures and tools. Biochim Biophys Acta. 1811;2011:637–47.
12. German JB, Gillies LA, Smilowitz JT, Zivkovic AM, Watkins SM. Lipidomics and lipid profiling in metabolomics. Curr Opin Lipidol. 2007;18:66–71.
13. Shevchenko A, Simons K. Lipidomics: coming to grips with lipid diversity. Nat Rev Mol Cell Biol. 2010;11:593–8.
14. Klose C, Ejsing CS, García-Sáez AJ, et al. Yeast lipids can phase-separate into micrometer-scale membrane domains. J Biol Chem. 2010;285:30224–32.
15. Fahy E, Subramaniam S, Brown HA, et al. A comprehensive classification system for lipids. J Lipid Res. 2005;46:839–62.
16. Wenk MR. Lipidomics: new tools and applications. Cell. 2010;143:888–95.
17. Jung HR, Sylvänne T, Koistinen KM, Tarasov K, Kauhanen D, Ekroos K. High throughput quantitative molecular lipidomics. Biochim Biophys Acta. 1811;2011:925–34.
18. Murphy RC, Axelsen PH. Mass spectrometric analysis of long-chain lipids. Mass Spectrom Rev. 2011;30:579–99.
19. Niemelä PS, Castillo S, Sysi-Aho M, Orešič M. Bioinformatics and computational methods for lipidomics. J Chromatogr B. 2009;877:2855–62.
20. Orešič M. Informatics and computational strategies for the study of lipids. Biochim Biophys Acta Mol Cell Biol Lipids. 1811;2011:991–9.
21. Gross R, Han X. Lipidomics at the interface of structure and function in systems biology. Chem Biol. 2011;18:284–91.
22. Stein EA, Myers GL. National cholesterol education program recommendations for triglyceride measurement: executive summary. The National Cholesterol Education Program Working Group on lipoprotein measurement. Clin Chem. 1995;41:1421–6.
23. Kotronen A, Johansson LE, Johansson LM, Roos C, et al. A common variant in PNPLA3, which encodes adiponutrin, is associated with liver fat content in humans. Diabetologia. 2009;52:1056–60.
24. Rhee EP, Cheng S, Larson MG, Walford GA, Lewis G, McCabe E, Yang E, Farrell L. Lipid profiling identifies a triacylglycerol signature of insulin resistance and improves diabetes prediction in humans. J Clin Invest. 2011;121:1402–11.
25. Wang JH, Byun J, Pennathur S. Analytical approaches to metabolomics and applications to systems biology. Semin Nephrol. 2010;30:500–11.
26. Navas-Iglesias N, Carrasco-Pancorbo A, Cuadros-Rodríguez L, From lipid analysis towards lipidomics, a new challenge for the analytical chemistry of the 21st century. TrAC. 2009;28:393–403.
27. Hyötyläinen T, Bondia-Pons I, Orešič M. Lipidomics in nutrition and food research. Mol Nutr Food Res. 2013;57:1306–18.
28. Harkewicz R, Dennis EA. Applications of mass spectrometry to lipids and membranes. Annu Rev Biochem. 2011;80:301–25.
29. Gross RW, Han X. Shotgun lipidomics of neutral lipids as an enabling technology for elucidation of lipid-related diseases. Am J Physiol Endocrinol Metab. 2009;297:E297–303.
30. Isaac G. Electrospray ionization tandem mass spectrometry (ESI-MS/MS)-based shotgun lipidomics. Methods Mol Biol. 2011;708:259–75.
31. Nygren H, Seppänen-Laakso T, Castillo S, Hyötyläinen T, Orešič M. Liquid chromatography-mass spectrometry (LC-MS)-based lipidomics for studies of body fluids and tissues. Methods Mol Biol. 2011;708:247–57.

32. Kliman M, May JC, McLean JA. Lipid analysis and lipidomics by structurally selective ion mobility-mass spectrometry. Biochim Biophys Acta. 1811;2011:935–45.
33. Guo X, Lankmayr E. Multidimensional approaches in LC and MS for phospholipid bioanalysis. Bioanalysis. 2010;2:1109–23.
34. Han X, Yang K, Gross RW. Multi-dimensional mass spectrometry-based shotgun lipidomics and novel strategies for lipidomic analyses. Mass Spectrom Rev. 2012;31:134–78.
35. Lindon JC, Nicholson JK. Spectroscopic and statistical techniques for information recovery in metabonomics and metabolomics. Annu Rev Anal Chem (Palo Alto Calif). 2008;1:45–69.
36. Maher AD, Lindon JC, Nicholson JK. ^{1}H NMR-based metabonomics for investigating diabetes. Future Med Chem. 2009;1:737–47.
37. Bou Khalil M, Hou W, Zhou H, et al. Lipidomics era: accomplishments and challenges. Mass Spectrom Rev. 2010;29:877–929.
38. Katajamaa M, Orešič M. Data processing for mass spectrometry-based metabolomics. J Chromatogr A. 2007;1158:318–28.
39. Sumner LW, Urbanczyk-Wochniak E, Broeckling CD. Metabolomics data analysis, visualization, and integration. Methods Mol Biol. 2007;406:409–36.
40. Wheelock CE, Goto S, Yetukuri L, D'Alexandri FL, Klukas C, Schreiber F, Orešič M. Bioinformatics strategies for the analysis of lipids. Methods Mol Biol. 2009;580:339–68.
41. Bäckhed F, Ding H, Wang T, et al. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A. 2004;101:15718–23.
42. Dentin R, Pegorier JP, Benhamed F, Foufelle F, Ferre P, et al. Hepatic glucokinase is required for the synergistic action of ChREBP and SREBP-1c on glycolytic and lipogenic gene expression. J Biol Chem. 2004;279:20314–26.
43. Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI. Molecular analysis of commensal host-microbial relationships in the intestine. Science. 2001;291:881–4.
44. Bäckhed F, Manchester JK, Semenkovich CF, Gordon JI. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci U S A. 2007;104:979–84.
45. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 2006;444:1027–131.
46. Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proc Natl Acad Sci U S A. 2005;102:11070–5.
47. Kahn BB, Alquier T, Carling D, Hardie DG. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metab. 2005;1:15–25.
48. Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Müller C, Carling D, Kahn BB. Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. Nature. 2002;415:339–43.
49. Wikoff WR, Anfora AT, Liu J, et al. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proc Natl Acad Sci U S A. 2009;106:3698–703.
50. Velagapudi VR, Hezaveh R, Reigstad CS, et al. The gut microbiota modulates host energy and lipid metabolism in mice. J Lipid Res. 2010;51:1101–12.
51. Wostmann B, Larkin C, Moriarty A, Bruckner-Kardoss E. Dietary intake, energy metabolism, and excretory losses of adult male germfree wistar rats. Lab Anim Sci. 1983;33:46–50.
52. Hildebrandt MA, Hoffmann C, Sherrill-Mix SA, Keilbaugh SA, Hamady M, Chen YY, Knight R, Ahima RS, Bushman F, Wu GD. High-fat diet determines the composition of the murine gut microbiome independently of obesity. Gastroenterology. 2009;137:1716–24.
53. Shojima N, Ogihara T, Inukai K, et al. Serum concentrations of resistin-like molecules are elevated in high-fat-fed and obese db/db mice, with increased production in the intestinal tract and bone marrow. Diabetologia. 2005;48:984–92.
54. Claus SP, Tsang TM, Wang Y, et al. Systemic multicompartmental effects of the gut microbiome on mouse metabolic phenotypes. Mol Syst Biol. 2008;4:291.
55. Claus SP, Ellero SL, Berger B, et al. Colonization-induced host-gut microbial metabolic interaction. MBio. 2011. doi:10.1128/mBio.00271-10.
56. Martinez AN, Lahiri R, Pittman TL, Scollard D, Truman R, Moraes MO, Williams DL. Molecular determination of Mycobacterium leprae viability by use of real-time PCR. J Clin Microbiol. 2009;47:2124–30.

57. Virtanen KA, Lidell ME, Orava J, et al. Functional brown adipose tissue in healthy adults. N Engl J Med. 2009;360:1518–25.
58. Lidell ME, Enerback S. Brown adipose tissue – a new role in humans? Nat Rev Endocrinol. 2010;6:319–25.
59. Feldmann HM, Golozoubova V, Cannon B, Nedergaard J. UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. Cell Metab. 2009;9:203–9.
60. Bartelt A, Bruns OT, Reimer R, et al. Brown adipose tissue activity controls triglyceride clearance. Nat Med. 2011;17:200–5.
61. Nedergaard J, Bengtsson T, Cannon B. New powers of brown fat: fighting the metabolic syndrome. Cell Metab. 2011;13:238–40.
62. Mestdagh R, Dumas M, Rezzi S, et al. Gut microbiota modulate the metabolism of brown adipose tissue in mice. J Proteome Res. 2012;11:620–30.
63. Canas X, Fernandez-Lopez J, Remesar X, Alemany M. 3-hydroxybutyrate inhibits noradrenaline-induced thermogenesis in lean but not in obese zucker rats. Int J Obes Relat Metab Disord. 1998;22:734–40.
64. Zeng H, Liu J, Jackson MI, Zhao F, Yan L, Combs GF. Fatty liver accompanies an increase in lactobacillus species in the hind gut of C57BL/6 mice fed a high-fat diet. J Nutr. 2013;143:627–31.
65. Compare D, Coccoli P, Rocco A, et al. Gut–liver axis: the impact of gut microbiota on non alcoholic fatty liver disease. Nutr Metab Cardiovasc Dis. 2012;22:471–6.
66. Quigley EMM, Stanton C, Murphy EF. The gut microbiota and the liver. Pathophysiological and clinical implications. J Hepatol. 2013;58:1020–7.
67. Yokota A, Fukiya S, Islam KBMS, et al. Is bile acid a determinant of the gut microbiota on a high-fat diet? Gut Microbes. 2012;3(5):455–9.
68. Million M, Angelakis E, Paul M, Armougom F, Leibovici L, Raoult D. Comparative meta-analysis of the effect of lactobacillus species on weight gain in humans and animals. Microb Pathog. 2012;53:100–8.
69. Murphy EF, Cotter PD, Hogan A, et al. Divergent metabolic outcomes arising from targeted manipulation of the gut microbiota in diet-induced obesity. Gut. 2013;62:220–6.
70. Delzenne NM, Neyrinck AM, Cani PD. Gut microbiota and metabolic disorders: how prebiotic can work? Br J Nutr. 2013;109 Suppl 2:81–5.
71. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature. 2006;444:1022–3.
72. Duncan SH, Lobley GE, Holtrop G, et al. Human colonic microbiota associated with diet, obesity and weight loss. Int J Obes. 2008;32:1720–4.
73. Schwiertz A, Taras D, Schäfer K, et al. Microbiota and SCFA in lean and overweight healthy subjects. Obesity. 2010;18:190–5.
74. Armougom F, Henry M, Vialettes B, Raccah D, Raoult D. Monitoring bacterial community of human gut microbiota reveals an increase in Lactobacillus in obese patients and Methanogens in anorexic patients. PLoS One. 2009. doi:10.1371/journal.pone.0007125.
75. Saxelin M, Tynkkynen S, Mattila-Sandholm T, de Vos WM. Probiotic and other functional microbes: from markets to mechanisms. Curr Opin Biotechnol. 2005;16:204–11.
76. Kankainen M, Paulin L, Tynkkynen S, et al. Comparative genomic analysis of lactobacillus rhamnosus GG reveals pili containing a human- mucus binding protein. Proc Natl Acad Sci U S A. 2009;106:17193–8.
77. Lahti L, Salonen A, Kekkonen RA, Salojärvi J, Jalanka-Tuovinen J, Palva A, Orešič M, de Vos WM. Associations between the human microbiota, Lactobacillus rhamnosus GG and serum lipids indicated by integrated analysis of high-throughput profiling data. Peer J. 2013. doi:10.7717/peerj.32.
78. Rosberg-Cody E, Liavonchanka A, Gobel C, et al. Myosin-cross-reactive antigen (MCRA) protein from bifidobacterium breve is a FAD-dependent fatty acid hydratase which has a function in stress protection. BMC Biochem. 2011;12:9.
79. De Voos WM, Nieuwdorp M. A gut prediction. Nature. 2013;498:48–9.

80. Arumugam M, Raes J, Pelletier E, et al. Enterotypes of the human gut microbiome. Nature. 2011;473:174–80.
81. Kurokawa K, Itoh T, Kuwahara T, et al. Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes. DNA Res. 2007;14:169–81.
82. Gill SR, Pop M, DeBoy RT, et al. Metagenomic analysis of the human distal gut microbiome. Science. 2006;312:1355–9.
83. Martens EC, Koropatkin NM, Smith TJ, Gordon JI. Complex glycan catabolism by the human gut microbiota: the bacteroidetes sus-like paradigm. J Biol Chem. 2009;284:24673–7.
84. Karlsson FH, Tremaroli V, Nookaew I, et al. Gut metagenome in European women with normal, impaired and diabetic glucose control. Nature. 2013;498:99–103.
85. Marques-Vidal P, Schmid R, Bochud M, et al. Adipocytokines, hepatic and inflammatory biomarkers and incidence of type 2 diabetes. The CoLaus study. PLoS One. 2012. doi:10.1371/journal.pone.0051768.
86. Louis P, Young P, Holtrop G, Flint HJ. Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene. Environ Microbiol. 2010;12:304–14.
87. Vrieze A, Van Nood E, Holleman F, et al. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. Gastroenterology. 2012;143:913–6.
88. Furet J, Kong L, Tap J, et al. Differential adaptation of human gut microbiota to bariatric surgery–induced weight loss: links with metabolic and low-grade inflammation markers. Diabetes. 2010;59:3049–57.
89. Qin J, Li Y, Cai Z, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature. 2012;490:55–60.
90. Bäckhed F. Programming of host metabolism by the gut microbiota. Ann Nutr Metab. 2011;58 Suppl 2:44–52.
91. Fava F, Lovegrove JA, Gitau R, Jackson K, Tuohy K. The gut microbiota and lipid metabolism: implications for human health and coronary heart disease. Curr Med Chem. 2006;13:3005–21.
92. Ridlon JM, Kang D, Hylemon PB. Bile salt biotransformations by human intestinal bacteria. J Lipid Res. 2006;47:241–59.
93. Musso G, Gambino R, Cassader M. Interactions between gut microbiota and host metabolism predisposing to obesity and diabetes. Annu Rev Med. 2011;62:361–80.
94. Louis P, Flint HJ. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS Microbiol Lett. 2009;294:1–8.
95. Delzenne NM, Cani PD. Interaction between obesity and the gut microbiota: relevance in nutrition. Annu Rev Nutr. 2011;31:15–31.
96. Al-Lahham SH, Peppelenbosch MP, Roelofsen H, Vonk RJ, Venema K. Biological effects of propionic acid in humans; metabolism, potential applications and underlying mechanisms. Biochim Biophys Acta. 2010;1801:1175–83.
97. Stoddart LA, Smith NJ, Jenkins L, Brown AJ, Milligan G. Conserved polar residues in transmembrane domains V, VI, and VII of free fatty acid receptor 2 and free fatty acid receptor 3 are required for the binding and function of short chain fatty acids. J Biol Chem. 2008;283:32913–24.
98. Brown AJ, Goldsworthy SM, Barnes AA, et al. The orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J Biol Chem. 2003;278:11312–9.
98. Samuel BS, Shaito A, Motoike T, et al. Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. Proc Natl Acad Sci U S A. 2008;105:16767–72.
100. Ge H, Li X, Weiszmann J, et al. Activation of G protein-coupled receptor 43 in adipocytes leads to inhibition of lipolysis and suppression of plasma free fatty acids. Endocrinology. 2008;149:4519–26.
101. Bjursell M, Admyre T, Göransson M, et al. Improved glucose control and reduced body fat mass in free fatty acid receptor 2-deficient mice fed a high-fat diet. Am J Physiol Endocrinol Metab. 2011;300:E211–20.

102. Cani PD, Delzenne NM. Interplay between obesity and associated metabolic disorders: new insights into the gut microbiota. Curr Opin Pharmacol. 2009;9:737–43.
103. Cani PD, Delzenne NM. The role of gut microbiota in energy metabolism and metabolic disease. Curr Pharm Des. 2009;15:1546–58.
104. Dewulf EM, Cani PD, Neyrinck AM, et al. Inulin-type fructans with prebiotic properties counteract GPR43 overexpression and PPARγ-related adipogenesis in the white adipose tissue of high-fat diet-fed mice. J Nutr Biochem. 2011;22:712–22.
105. Roberfroid M, Gibson GR, Hoyles L, et al. Prebiotic effects: metabolic and health benefits. Br J Nutr. 2010;104 Suppl 2:1–63.
106. Lefebvre P, Cariou B, Lien F, Kuipers F, Staels B. Role of bile acids and bile acid receptors in metabolic regulation. Physiol Rev. 2009;89:147–91.
107. de Aguiar VT, Tarling E, Edwards P. Pleiotropic roles of bile acids in metabolism. Cell Metab. 2013;17:657–69.
108. Kootte RS, Vrieze A, Holleman F, et al. The therapeutic potential of manipulating gut microbiota in obesity and type 2 diabetes mellitus. Diabetes Obes Metab. 2012;14:112–20.
109. Russell DW. The enzymes, regulation, and genetics of bile acid synthesis. Annu Rev Biochem. 2003;72:137–74.
110. Swann JR, Want EJ, Geier FM, Spagou K, Wilson ID, Sidaway JE, Nicholson JK, Holmes E. Systemic gut microbial modulation of bile acid metabolism in host tissue compartments. Proc Natl Acad Sci U S A. 2011;108 Suppl 1:4523–30.
111. Russell DW. Fifty years of advances in bile acid synthesis and metabolism. J Lipid Res. 2009;50 Suppl 1:S120–5.
112. Chiang JYL. Bile acids: regulation of synthesis. J Lipid Res. 2009;50:1955–66.
113. Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G, Gonzalez FJ. Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. Cell. 2000;102:731–44.
114. Wostmann BS. Intestinal bile acids and cholesterol absorption in the germfree rat. J Nutr. 1973;103:982–90.
115. Jones BV, Begley M, Hill C, Gahan CGM, Marchesi JR. Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. Proc Natl Acad Sci U S A. 2008;105:13580–5.
116. Miyata M, Sakaida Y, Matsuzawa H, Yoshinari K, Yamazoe Y. Fibroblast growth factor 19 treatment ameliorates disruption of hepatic lipid metabolism in farnesoid X receptor (fxr)-null mice. Biol Pharm Bull. 2011;34:1885–9.
117. Sayin S, Wahlström A, Felin J, et al. Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist. Cell Metab. 2013;17(2):225–35.
118. Kawamata Y, Fujii R, Hosoya M, et al. A G protein-coupled receptor responsive to bile acids. J Biol Chem. 2003;278:9435–40.
119. Watanabe M, Houten SM, Mataki C, et al. Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. Nature. 2006;439:484–9.
120. Studer E, Zhou X, Zhao R, et al. Conjugated bile acids activate the sphingosine-1-phosphate receptor 2 in primary rodent hepatocytes. Hepatology. 2012;55:267–76.
121. Porez G, Prawitt J, Gross B, Staels B. Bile acid receptors as targets for the treatment of dyslipidemia and cardiovascular disease: thematic review series: new lipid and lipoprotein targets for the treatment of cardiometabolic diseases. J Lipid Res. 2012;53:1723–37.
122. Liu X, Invernizzi P, Lu Y, et al. Genome-wide meta-analyses identify three loci associated with primary biliary cirrhosis. Nat Genet. 2010;42:658–60.
123. Mells GF, Floyd JAB, Morley KI, et al. Genome-wide association study identifies 12 new susceptibility loci for primary biliary cirrhosis. Nat Genet. 2011;43:329–32.
124. Teslovich TM, Musunuru K, Smith AV, et al. Biological, clinical and population relevance of 95 loci for blood lipids. Nature. 2010;466:707–13.
125. Quehenberger O, Dennis EA. The human plasma lipidome. N Engl J Med. 2011;365:1812–23.

Chapter 13
Role of the Gut Microbiota in Maintaining GI Health: Highlights on Inflammatory Bowel Disease

Lisa Gruber and Dirk Haller

Abstract The human gut harbors trillions of microbes, with an area of at least 300 m^2 intestinal tissue in constant contact with this microbial ecological system (Artis, Nat Rev Immunol 8(6):411–420, 2008, Ley et al. Cell 124(4):837–848, 2006). The intestinal epithelium with a number of different cell types shapes the frontier between microbes and the host. Intestinal epithelial cells (IECs) as well as cells of the immune system guard the local interface of microbes and host and actively tolerate selected commensal microbiota while mounting an adequate inflammatory response toward pathogens in the context of infection or disease. Although there is no clear definition of a "normal" intestinal microbiota as such, it is apparent that perturbations of a certain homeostatic system may lead to a dysregulated interaction between microbes and the host intestinal mucosal immune system, resulting in aberrant or disproportionate inflammatory conditions.

The following chapter gives a general introduction to the role of the microbiota in gut health, focuses on aberrations in microbe-host mutualism that are implicated in the etiopathology of inflammatory bowel diseases, and then briefly addresses the possibilities of dietary modulation of intestinal microbiota in the context of inflammatory bowel diseases.

Keywords Adaptive immunity • Antibiotics • Antigen-presenting cells • Crohn's disease • Barrier • Bile acids • Dendritic cells • Diet • Elemental diet • Enteral nutrition • Fecal microbial transplantation • Fat • Genome-wide association studies • Gnotobiology • IBD • Inflammatory bowel disease • Innate immunity

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• Intestinal health • Iron • Metabonomics • Metagenomics • Metaproteomics • Metatranscriptomics • Microbiota • Mouse models • Nutrigenetics • Nutrition • Pathobionts • Permeability • Phylogeny • Prebiotics • Probiotics • Short-chain fatty acids • Ulcerative colitis

13.1 Host-Microbe Mutualism in Gut Health

Microbial organisms and the host mucosal immune system have established fine-balanced interactions during coevolution. In the last decades, the use of gnotobiology has brought forward the studies of host-microbe interactions to a substantial extent. These experiments in rodents manipulate the microbiota by introducing selected bacteria. Comparative studies allow the investigation of factors directing the establishment and maintenance of bacterial communities in the intestine, as well as investigating the impact of microbial factors on gastrointestinal functions. It is now known that the presence and composition of microbiota affect gut morphology; metabolic, absorptive, nerval, and endocrine functions; as well as mucosal and systemic immune functions.

However, the specific microbial and host factors that regulate the aspects of mutualism in a complex and changing environment are poorly understood. The most important and best studied host-microbe metabolic interactions will be presented in the following chapters.

13.1.1 Colonization: A Matter of Interaction

Besides major time-dependent variation of the intestinal microbiome and its spatial distribution along the intestinal tract, there are also major differences between mucosa-associated resident bacteria and fecal bacteria [3], deriving from different abilities of the microbiota to persist in and attach to a given host environment. While there exist at least 55 divisions of bacteria, the human gut microbial community is dominated by members of only four phyla, namely, Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria. It is suggested that a few early colonizers of the human intestine have established an exclusive population and then diversified into a high variety of species and strains [2, 4].

From the viewpoint of bacteria, colonization is driven mainly by the availability of nutrients and the ability to attach to surfaces for building up residency. Many species possess large numbers of genes encoding for carbohydrate metabolism enzymes and can switch between different energy sources depending on their availability, also including protein sources [5]. Some strains are capable of turning to host-derived mucus when dietary saccharides are rare, as can *Akkermansia muciniphila* [6, 7], *Bacteroides acidifaciens* [7], or *Bacteroides thetaiotaomicron* [8, 9]. Although microbial organisms benefit differentially from components of

host-secreted compounds, the activity of host foragers was shown to depend on other community members [7]. The mucus layer also serves as a major source of attachment sites [10–12]. Given the high variability among mucins and glycoprotein structures on the host cell surface and the spatial distribution of the different forms, this suggests a mechanism of host-microbe interaction for the regulation of microbial composition.

At a second stage, the ability of a bacterial strain to persist in the gut ecosystem also depends on its power to compete with other strains. Microbes can compete with bacteria or parasites by the competition for nutrients ("exploitation competition"); by direct inhibition, for example, via toxic substances ("interference competition"); or by the induction of immune responses that target competitors ("apparent competition") [13]. Apparently, a driving force for successful colonization is to keep the own vulnerability toward host-derived defense mechanisms low (also see Sect. 13.1.3) while promoting immune responses toward competitors.

13.1.2 Immune Maturation Processes upon Microbial Stimulation

Microbial organisms derive clear advantage from ensuring tolerance of the mucosal immune system toward them and therefore have evolved to influence the host immune system development profoundly. Studies in germfree animals show disrupted organization of mesenteric lymph nodes and Peyer's patches and reduced numbers of dendritic cells (DCs), T cells, and B cells in the lamina propria [14–20]. These findings implicate that stimulation from microbes and/or microbial structures or metabolites is needed for functional development of the immune system.

Antigen-presenting cells such as DCs constantly sample and process luminal compounds and can be regarded as major directors of subsequent modulation of immune response. Reduced numbers of intestinal DCs are observed in germfree animals, whereas microbial stimulation by mono-colonization with *Escherichia coli* was sufficient to recruit DCs to the intestine [20]. Different subsets of DCs are thought to exert specialized functions during antigen sampling and information dissemination, as visualized for the murine system in Fig. 13.1a. Murine intestinal CX3CR1+ antigen-presenting cells extend protrusions into the lumen for uptake of dietary and microbial antigens [21, 22]. Signals from the intestinal epithelium shape the properties of DCs [23]. Conditioned antigen-presenting cells then pass on information to T cells which, depending on co-stimulatory factors, differentiate into one of the major phenotypes upon this stimulation: T helper cell type (Th) 1 and Th17, both implicated in inflammation; Th2, implicated in allergic reactions; as well as Treg, regulating inflammatory responses. Other types of Th cells such as Th9 or Th22 are less well described today. Specialized CD103+ DCs develop a tolerogenic phenotype after antigen sampling and upon stimulation by IEC-derived factors such as retinoic acid or transforming growth factor-β (TGF-β). In contrast

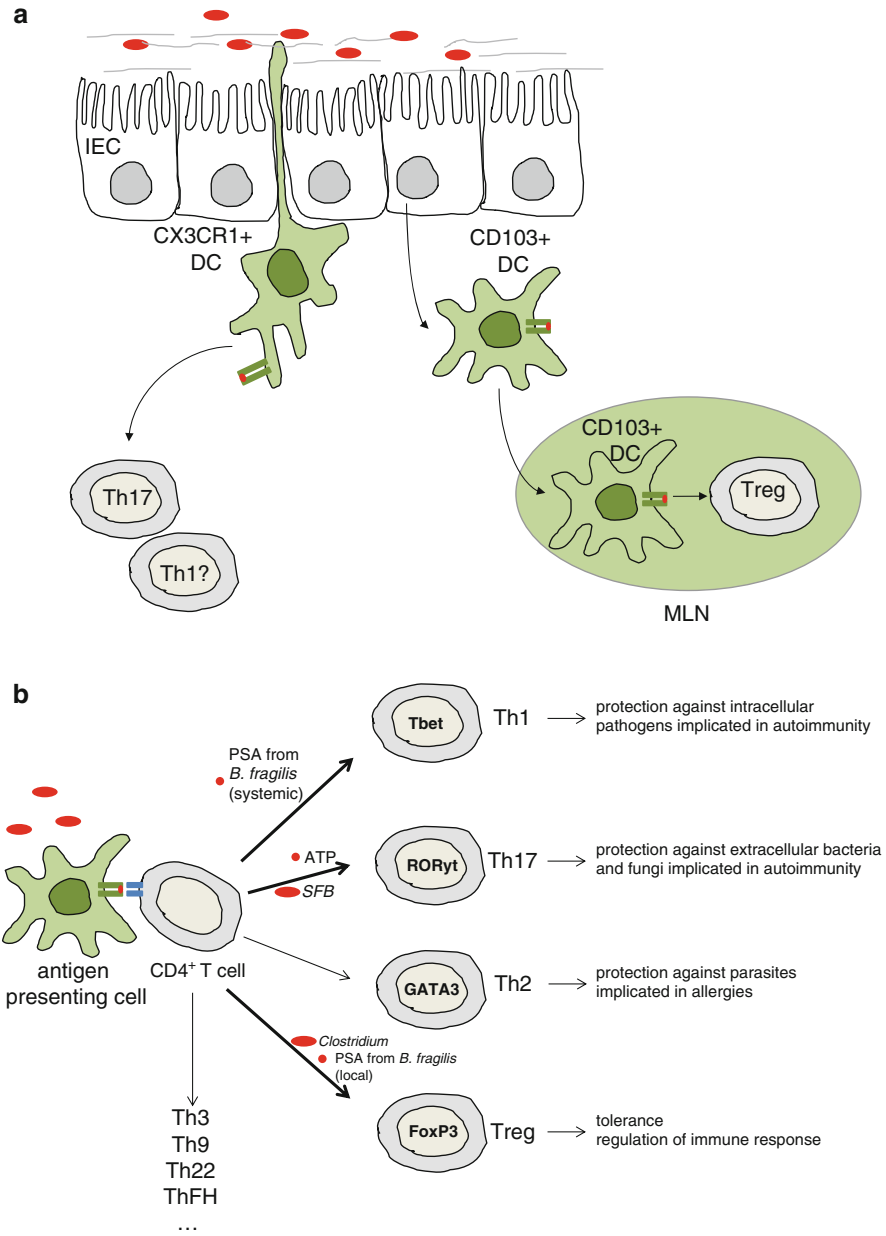

Fig. 13.1 (**a**) Antigen sampling and information dissemination by murine lamina propria antigen-presenting cell subsets. CD103−CX3CR1+ cells sample bacteria and induce Th17 and probably also Th1 cell differentiation, without migration to lymph nodes. In the presence of RA, TGF-β, and TSLP released by IEC, CD103+ DCs develop a tolerogenic phenotype and induce Treg cell differentiation after their migration to the mesenteric lymph nodes. (**b**) Microbes and microbial compounds can induce T-cell differentiation into one of the major T helper cell types: Th1, Th17, Th2, and Treg. Differentiation requires the induction of transcription factors – the most important are outlined in the figure. The bacteria or bacteria-derived factors that have been shown to promote the induction of a particular Th subtype are indicated in the figure. Each Th subset then has a specific function as indicated. *IEC* intestinal epithelial cell, *Th* T helper cell, *Treg* regulatory T cell, *MLN* mesenteric lymph node, *ATP* adenosine 5′-triphosphate, *SFB* segmented filamentous bacteria, *PSA* polysaccharide A

to CX3CR1+ cells, these CD103+ DCs then migrate to the mesenteric lymph nodes and generate Tregs, inducing tolerance to constantly sampled, harmless antigens [24, 25]. Far less is known about subsets of human specialized antigen-presenting cells. While CD103+SIRPα+ DCs correspond to murine CD103+CD11b+ DCs and account for the majority of small intestinal dendritic cells [26, 27], CD103-SIRPα+ DCs, likely derived from blood monocytes, are more abundant in the large intestine and accumulate during inflammation [27]. CD141+CD103+ SIRPα- with a repertoire of expressed proteins involved in the induction of cytotoxic T-cell response have been described in the human small intestine and likely correspond to murine CD103+CD11b- DCs [27].

In general, germfree mice display reduced total numbers of T cells, bias toward Th2 response [28], and especially low numbers of Th17 cells [29, 30]. Specific microbes and microbial compounds have been shown to profoundly shape the balances of T-cell subsets, presumably via conditioning of both IECs and antigen-presenting cells (Fig. 13.1b). Segmented filamentous bacteria (SFB) promote Th17 response [30, 31]. Colonization with SFB confers resistance to *Citrobacter rodentium* infection, indicating that Th17-cell induction by SFB is responsible for this protective immune response. Bacteria-derived flagellin triggers the differentiation of Th1 and Th17 cells after stimulation of DCs [32]. Also commensal bacteria-derived adenosine 5′-triphosphate has been found to activate a unique subset of lamina propria DCs, resulting in differentiation of Th17 cells [29]. *Clostridia*, particularly of clusters IV and XIVa, are capable of inducing Tregs [33]. Polysaccharide A, derived from the commensal *Bacteroides fragilis*, also mediates Treg cell responses and suppresses Th17, resulting in protection from inflammation [28, 34]. However, a Th1-/Th17-driven pro-inflammatory response is induced upon the systemic presence of *Bacteroides fragilis* [35, 36]. This example highlights the plasticity of immune responses toward microbial antigens and the fine regulation of tolerogenic and inflammatory mechanisms.

The induction of B-cell maturation and recruitment is also mediated by the microbiota. The organized structures in lymphoid organs where differentiation and maturation of B cells occur are disrupted in germfree animals [17, 37], and they exhibit reduced plasma cell numbers and decreased levels of secretory immunoglobulin A (sIgA) [38]. In contrast, allergy-associated IgE is increased in germfree rats [39].

As another aspect of microbial influence on the host immune system, microbial metabolites, especially short-chain fatty acids (SCFA), exert anti-inflammatory actions on the intestinal immune system. SCFA are produced by microbes upon fermentation of polysaccharides. Butyrate, propionate, and acetate are the most abundant and described SCFA, with almost all the produced amount absorbed in the colon. Butyrate is regarded as the primary energy source for colonic IEC but also displays anti-inflammatory effects such as decreasing cytokine production [40, 41]. Indeed, treatment with acetate or butyrate resulted in the amelioration of inflammation in rodents [40, 42] and humans [43].

Interestingly, the immune maturation processes depend on the presence of coevolved host-specific microbiota, as cross-species colonization cannot induce full expansion of lymphocytes in mice and also fails to protect against *Salmonella* infection compared to self-species colonization [44].

13.1.3 Shaping and Confining the Gut Microbiota

Despite the beneficial mutual effects, the host has to keep the microbiota at bay and therefore has developed a broad range of innate protection mechanisms. Besides the selection of colonizing strains, the host also actively shapes the resident community by strategies related to innate immune response – probably in part as a result of bacteria modulating the host.

Antimicrobial peptides (AMPs) form a barrier with maximal activity in the intestinal crypts as well as in the mucus layer, preventing contact or penetration of the host epithelium [45, 46]. AMPs are a diverse group of compounds, including defensins, cathelicidins, lectins, and angiogenins, exerting their effect on bacteria by destructing bacterial cell wall integrity. AMPs are secreted by Paneth cells upon stimulation by live bacteria, bacterial components, or metabolites [46–50]. Especially Ang4 and REGIIIγ expression is induced by mono-colonization of germfree mice [48] or during weaning [51, 52]. Both of them exclusively target Gram-positive bacteria while sparing Gram negatives, thus influencing microbial composition and presumably contributing to the shift observed during weaning. Mice expressing a human α-defensin gene show a loss of SFB and fewer IL17-producing T cells [53], providing a clear example for the bidirectional effects of host immune system and microbial community.

Bacteria also induce the production and secretion of sIgA by B cells [17, 38]. SIgA translocates into the lumen, binding bacteria or antigens of other origin, and therefore prevents attachment on IEC and regulates colonization [54]. IgA can also bind to bacteria that have gained access to the serosal side and translocate them back to the lumen. Bound to bacteria, sIgA induces the clearance of the bacteria by DCs, phagocytes, or neutrophils [55]. DCs then can retain bacteria and selectively induce the secretion of IgA. These activated DCs are restricted to the mucosal immune compartment by the mesenteric lymph nodes, ensuring a local immune response [56]. Taking into consideration that IgA deficiency results in intestinal dysbiosis [57] and that bacterial species are differentially capable of inducing IgA [58], IgA secretion is a means of both confining bacteria and shaping the intestinal ecology.

Cells of the innate immune system constantly recognize conserved microbe-associated molecular patterns, mainly via Toll-like receptors (TLRs) or nucleotide-binding oligomerization domain (NOD)-like receptors. In a healthy host, commensal bacteria thereby direct a tolerogenic immune response. On the other hand, the binding of bacteria-derived flagellin or lipopolysaccharides induces TRL5 and TLR4 signaling in DCs, respectively, alerting the immune system [59]. NODs initiate innate immune responses upon intracellular sensing of bacterial cell wall components, which results in elimination of intruded bacteria. Although the targeted structure muramyl dipeptide is highly conserved throughout bacterial phyla, NOD2 has been found to shape the intestinal microbiota in mice [60–62] and IBD patients (also see Fig. 13.7) [63, 64]. In this context, it has been reported that a colitogenic microbial ecology shaped by a certain host physiology is even transferring disease from this host into another: the dysbiosis reported for NOD2$^{-/-}$ mice results in

enhanced disease risk in wild-type mice [65]. In other examples for host genotype shaping microbial composition, multidrug resistance gene-deficient mice exhibit altered microbiota independent of their intestinal inflammation (also see Fig. 13.7) [66]. Furthermore, a deficiency of T-bet, implicated in directing host innate immunity, resulted in colitis that was antibiotic sensitive and communicable to wild-type mice [67].

It thus becomes evident that the microbiota plays a critical role in inducing host mechanisms for the defense against pathogens and that at the same time host responses toward microbiota shape the intestinal ecology. The host genetic background associated with the function of Paneth cells which secrete antimicrobial compounds into the lumen is considered especially critical in influencing the intestinal microbial composition [68]. In conclusion, the physiology and genetic background of the host determine the ecology of the intestinal tract (though with scope for modulation by environmental factors), and the microbiota is under control in a healthy individual [69].

13.1.4 Inflammatory Bowel Disease: Loss of Homeostasis

A loss of homeostasis concerning microbiota and host mucosal immune system is the basis for the pathogenesis of inflammatory conditions of the gastrointestinal tract, including the complex etiology of inflammatory bowel disease (IBD). IBD, with its two main idiopathic pathologies, ulcerative colitis (UC) and Crohn's disease (CD), is regarded as a multifactorial disease in which a certain host susceptibility regarding perturbations of barrier or microbe-host interactions combined with microbial aggressiveness leads to inappropriate host immune response toward the microbiota. The microbiota is affected in composition and functionality by both environmental triggers and host genetic background at the same time. Environmental triggers such as diet, drugs, or infectious agents can also affect the host condition and thereby impact on disease development. Figure 13.2 gives an overview of all these aspects in the current paradigm of IBD development. Alternating phases of active disease (relapse) and freeness of symptoms (remission) are characteristics of IBD.

Although described by the collective term IBD, UC and CD are two diseases with different symptoms and distinct pathogenesis. CD potentially extends to the submucosa and may occur anywhere along the gastrointestinal tract – with a predominantly ileal phenotype referred to as ICD and a colonic phenotype as CCD. In UC, inflammation involves mucosa only and is restricted to the large intestine. Interestingly, the inflammatory profiles of UC and CD are very distinct. UC displays predominantly Th2 phenotype, associated with IL5 and TGF-β as predominant cytokines, whereas CD is associated with type Th1 and Th17 immune responses dominated by IL12, IL23, IFNγ, and TNF.

The prevalence of CD and UC has risen considerably over the last decades. In the 1990s and 2000s, reported incidence rates for CD in Western countries were 4 [70]

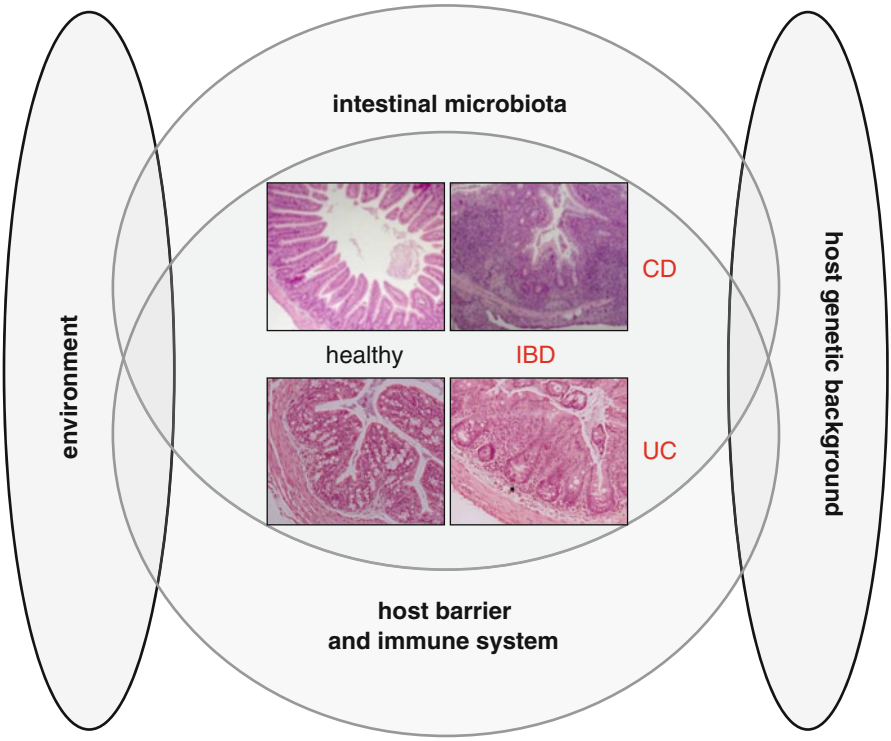

Fig. 13.2 IBD is a multifactorial disease, resulting from aberrant host responses to intestinal microbiota. The host genetic background establishes susceptibility in regard to barrier function and immune response. This can result in an aberrant response toward intestinal microbiota. At the same time, the host condition and genetic background shape the intestinal microbiota. Environmental triggers can affect both barrier and immune functions of the host and composition as well as functionality of intestinal microbiota. *IBD*, inflammatory bowel disease, *CD* Crohn's disease, *UC* ulcerative colitis. The histological pictures show distal ileum tissues of TNFΔARE/WT mice displaying CD-like ileitis and the corresponding wild-type mouse, as well as proximal colon tissues of IL10$^{-/-}$ mice developing UC-like colitis and the corresponding wild-type mouse

to 8 cases [71] per 100,000 person-years with a tendency of higher rates in women and an incidence peak among 15- to 30-year-olds [70, 72–75]. UC is more prevalent than CD, with North America and northern Europe showing the highest incidence rates varying from 9 [71] to 20 cases [76] per 100,000 person-years, showing a similar age distribution to CD without sex differences.

Despite the lack of data for many areas (especially Africa), there seems to be a north-south axis of IBD prevalence within continents or even countries [77–79]. In Asia, incidence rates of IBD have been low in the past but are now rising as well [80, 81]. The fact that genetic factors are critical in IBD pathogenesis is stressed by the discrepancies in data sets of Caucasian versus Asian populations (see Sect. 13.2) [82, 83]. Most importantly, concordance rates for CD in monozygotic twins are 20–50 % in northern Europe, meaning that the relative risk is at least 40-fold higher

for individuals with an affected twin compared to the general population [84–88]. According to these studies, concordance rates are lower for UC. Indeed, the disease variance that can be explained by genetic variations is higher in CD than UC [89], implicating that genetic factors might contribute less significantly than in CD or that environmental and lifestyle triggers overwrite genetics.

Irrespective of the differences in pathophysiology and genetic contribution, there is remarkable evidence for a role of the intestinal microbiota and loss of host tolerance toward it during the pathogenesis of both IBD etiologies [90, 91]. Surgical bypass of the ileum prevents inflammation in patients with active CD, and inflammation reoccurs rapidly after the reinfusion of the bypassed segments [92]. IBD patients develop serological responses toward their microbiota [93, 94], and much higher rates of colonic bacteria are coated with immunoglobulin in patients with active CD compared to controls or patients in remission [95]. In addition, antibiotic treatment may result in the amelioration or even abrogation of inflammation in CD patients [96–98]. Finally, as listed in Table 13.1, treatment with broad-spectrum antibiotics as well as housing under germfree conditions ameliorates or abrogates inflammation in genetically engineered rodent models of intestinal inflammation. These studies provide substantial evidence for the role of microbiota and the development of chronic intestinal inflammatory diseases and in addition suggest a crucial role for the host genetic background in modeling susceptibility.

13.2 IBD Susceptibility Genes: Mucosal Immunology and Microbial Defense

According to the current paradigm, IBD results from a disturbed host-microbe interaction and loss of tolerance to nonpathogenic microbiota, leading to a chronic inflammatory response. The concept of homeostasis in this context not necessarily means the absence of inflammatory responses, but the ability of the host to mount an appropriate response toward any changes of the microbial community. The necessary fine-balanced interaction of microbe and host physiology can be perturbed by primary alterations on both sides. In the following chapter, possible disturbances of this homeostasis on host side will be highlighted, with a focus on genes that have been assigned as susceptibility factors for the development of IBD.

More than 160 genetic loci have been revealed implicated in IBD by genome-wide association studies (GWAS) up to date [89]. In CD 13.6 % of the disease variance can be explained by the means of variations in these genes and 7.5 % in UC [89]. It is noteworthy that the ethnicity of the study population has to be kept in mind when looking at the data sets: along with differences in IBD prevalence, the identification of relevant susceptibility genes varies in Asians versus Caucasians [82, 83, 99] and in Jewish versus non-Jewish populations [100–102].

GWAS highlight the impact of microbiota and microbial components in IBD pathogenesis. A substantial number of genes are implicated in both CD and UC, many of which involved in barrier function, primary defense mechanisms such as

Table 13.1 Rodent models of inflammatory intestinal diseases and the effect of manipulation of their microbiota

Animal model	Phenotype	Manipulation of microbiota	Effect
IL10$^{-/-}$	Colitis	GF	No disease [132]
		GF + *E. faecalis*	Recurrence of disease [230, 313]
		AB† (before onset of disease)	No disease [314–316]
IL2$^{-/-}$	Colitis	GF	Decreased severity [317]
HLA-B27 (rat)	Colitis	GF	No disease [177, 318]
Tcrα$^{-/-}$	Colitis (Th2 phenotype)	GF	No disease [319]
	Colitis (Th2 phenotype)	GF + *L. plantarum*, *S. faecalis*, *S. faecium*, *E. coli*	No disease [319]
SAMP1/Yit	CD-like ileitis	GF	Decreased severity [320]
		AB*	Decreased severity [321]
TRUC	Colitis	AB‡	No disease [67]
K8$^{-/-}$	Colitis	AB‖	No disease [322]
Mdr1a$^{-/-}$	Colitis	AB$^{\#}$	No disease [323]
dnTGFβRII x IL10R2$^{-/-}$	Colitis	AB*	No disease [324]
		AB* + *B. thetaiotaomicron*	Recovery of disease [168]
		AB* + *E. coli*	No disease [168]
NOD2$^{-/-}$ + AOM	Colitis and inflammation-associated colorectal cancer	AB$^{\circ}$	Reduced severity [65]
STAT3-IKO	Colitis and inflammation-associated colorectal cancer	AB‡	Decreased severity, no tumor formation [325]
SCID + CD44RB^{high}	Lymphoid cell accumulation and hyperplasia	AB	Decreased severity [326]

GF germfree conditions, *AB* antibiotic treatment; antibiotics used are indicated with the following symbols, if described

*Ciprofloxacin and metronidazole

$^{\circ}$Streptomycin, gentamicin, ciprofloxacin, and bacitracin

‖Vancomycin and imipenem

$^{\#}$Streptomycin, neomycin, bacitracin, and amphotericin

‡Vancomycin, neomycin, metronidazole, and ampicillin

†The following combinations: ciprofloxacin, neomycin and metronidazole, metronidazole alone, vancomycin and imipenem, and roxithromycin

Note that this list may not be complete and not every reference applicable for a certain example may be indicated

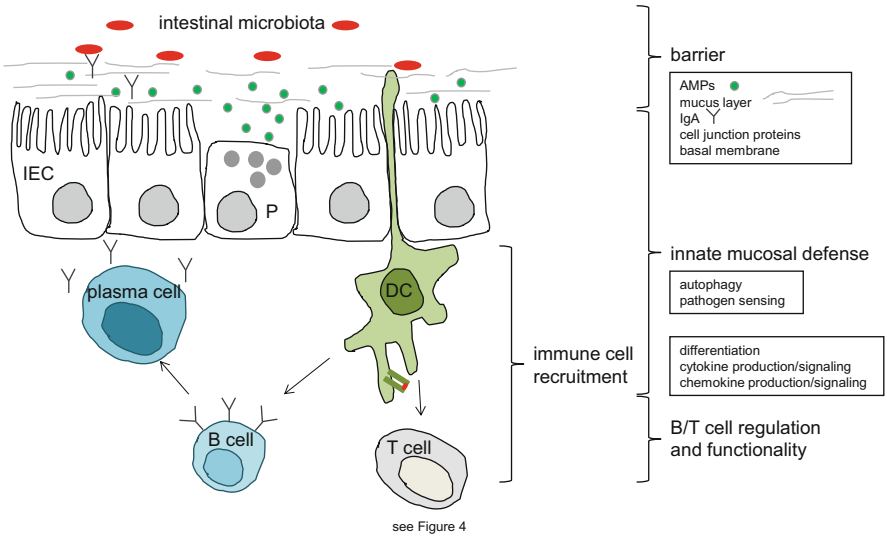

Fig. 13.3 GWAS reveal a critical role of microbe-host interactions on several levels. A compromised barrier function of the host, whether resulting from impaired AMP production (e.g., due to Paneth cell dysfunction), mucus layer aberrations, inadequate IgA production, loss of cell junction proteins, or disorganized basal membrane structure, may lead to intestinal inflammation as observed in IBD. Inadequate innate mucosal defense mechanisms involving autophagy and pathogen sensing can lead to inappropriate responses of the host immune system. Immune cell recruitment and lymphocyte regulation and functionality are regulated by a complex network of cytokines, chemokines, and differentiation processes, and a perturbation of these interactions can result in IBD. *AMPs* antimicrobial peptides, *IgA* immunoglobulin A, *DC* dendritic cell, *IEC* intestinal epithelial cell, *P* Paneth cell

sensing and processing of bacteria, cytokine signaling, and adaptive immunity [90, 103]. The stratification of these processes and tasks in the intestinal cell network is depicted in Fig. 13.3. A selection of susceptibility genes is introduced in the following sections, focusing on the just mentioned functions.

13.2.1 Decreased Barrier Function

The mucosal barrier is built up by different components: commensal microbiota blocking niches for pathogens, IgA and AMPs secreted to the lumen, mucins above the epithelial layer, and barrier proteins sealing the IEC layer. Several IBD-associated loci suggest a critical role for barrier integrity in IBD susceptibility.

GWAS report significant association of mutation of CDH1, resulting in truncated forms of E-cadherin, with CD [104]. Intestinal biopsies of patients carrying mutated alleles show defective E-cadherin localization [104]. MUC19, encoding for a secreted protein forming a chemical barrier together with other mucins and

embedded AMPs, is also implicated in IBD [105–107]. Further, Paneth cell function is critical in maintaining barrier integrity, as they are the producers of AMPs. Paneth cell dysfunction is a common consequence of mutations of IBD susceptibility genes, such as NOD2, ATG16L1, or XBP1 [108] (also see Sect. 13.2.2). Extracellular matrix proteins ECM1 and LAMB1 are also implicated in IBD, predominantly in UC [89, 103]. As laminins are the major non-collagenous constituent of basement membranes, defective variants could allow the penetration of microbes [109]. UC patients exhibited abrogation of laminin in the epithelial basement membranes surrounding the crypts in affected tissues [110].

13.2.2 Impaired Pathogen Sensing and Processing/Cellular Innate Immunity

Bacteria that have penetrated the epithelial barrier have to be sensed and processed for the induction of defense mechanisms. Upon intruding into the cell, bacteria undergo lysis in autophagolysosomes, and their structures are sensed by NOD-like receptors. NODs contain caspase recruitment domains (CARD) which mediate downstream signaling pathways such as nuclear factor κB (NFκB) and by this activate an appropriate immune response, resulting in the clearance of the infection [111].

GWAS identified various polymorphisms of NOD2/CARD15 as more prevalent in Caucasian CD patients compared to healthy controls [112–114]. NOD2 malfunctioning leads to inefficient clearance of intruded bacteria, likely in part mediated by reduced expression of Paneth cell-derived defensins. Paneth cell dysfunction and reduced AMP production have also been reported for CD patients with NOD2 mutations [115, 116]. Although NOD2-deficient mice do not spontaneously develop IBD, studies reveal increased susceptibility toward *Listeria monocytogenes* and *Helicobacter hepaticus* infection [62, 117].

Other CARD structures are implicated as susceptibility genes: variants of CARD11 are significantly associated with UC and variants of CARD9 with both IBD etiologies [89, 106]. Besides NOD2, ATG16L is regarded as one of the strongest genetic contributors to CD [107]. ATG16L1 is critical for the formation of autophagosomes and thus for the degradation and processing of microbial proteins [118]. Patients with either NOD2 or ATG16L1 mutations show ineffective induction of autophagy and bacterial processing [119, 120]. Indeed, bacterial killing is abrogated in IEC with disease-associated ATG16L mutation after stimulation with muramyl dipeptide [121], affirming a functional link of NOD2 and ATG16L1. In addition, other factors mediating autophagy, such as T-cell protein tyrosine phosphatase (PTPN2) [122–124] and interferon-inducible protein 1 (IRGM) [89, 114, 124, 125], are implicated in IBD. IRGM-deficient mice exhibit decreased bacterial killing in macrophages and are more susceptible to infections [126], whereas $PTPN2^{-/-}$ mice exhibit compromised T-cell functions [127, 128], systemic inflammation [127], and increased susceptibility to chemically induced colitis [129].

This convergence of several strong genetic risk factors highlights the importance of pattern recognition and autophagic processes in the clearance of bacteria and thus maintenance of intestinal health.

13.2.3 Aberrant T Helper Cell Immune Response

Upon sensing and processing of bacteria by innate mechanisms, a complex network of signals leads to the induction of tolerance or immune response toward the respective organisms. Of note, it was found that human IECs promote the differentiation of tolerogenic DCs driving the development of adaptive Foxp3+ Treg cells, as mentioned above, and that this mechanism is lost in patients with CD, with concomitantly reduced expression of tolerogenic factors by IECs [23].

Chemokines recruit immune cells and cytokines regulate inflammatory activity of adaptive immune cells. In general, the regulation of cytokine production seems to be the most overrepresented functionality in IBD GWAS (Fig. 13.4), especially for

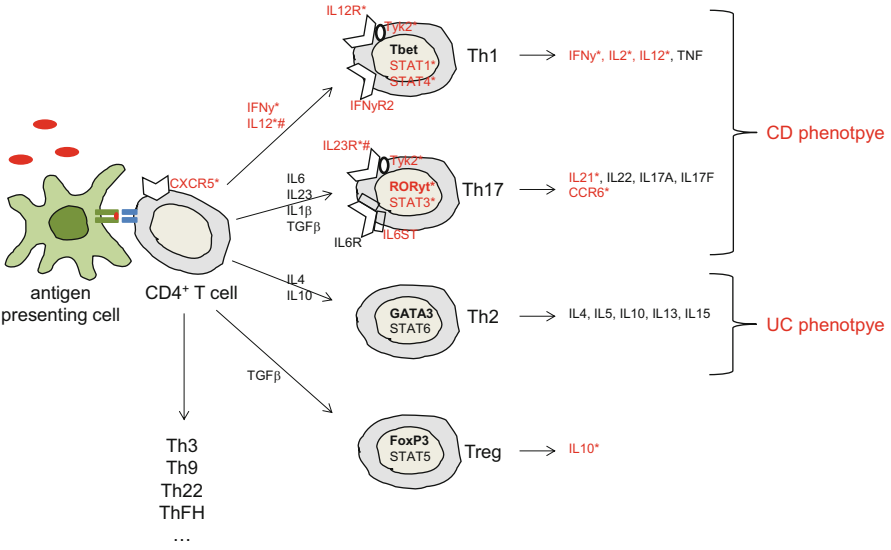

Fig. 13.4 GWAS reveal a critical role of the induction of T-cell differentiation and responses in IBD development. Antigen-presenting cells can induce T-cell differentiation into one of the major T helper cell types: Th1, Th17, Th2, and Treg. Differentiation depends on co-stimulatory factors of the antigen-presenting cell surface or by the network of cytokines present that are sensed by receptors on the T-cell surface. Lineage commitment then requires the induction of transcription factors – the most important of them are outlined in *bold* in the figure. The different T-cell subsets are characterized by cytokine profiles. Reported susceptibility genes for the development of CD or UC are printed in *red*. Association of a gene with both etiologies is indicated by *. If there are functional studies available for a gene, this is indicated by #. *IEC* intestinal epithelial cell, *Th* T helper cell, *Treg* regulatory T cell, *UC* ulcerative colitis, *CD* Crohn's disease

IFNγ, IL12, TNF, and IL10 signaling [89]. IFNγ, IL12, and TNF are the predominant cytokines in Th1 immune response, whereas IL10 is a major anti-inflammatory cytokine. IL10 signaling is required for the generation of regulatory T-cell responses via FoxP3 [130]. The impact of IL10 functionality on intestinal health is further emphasized by the fact that IL10-deficient mice develop colitis when colonized and have become a widely used model of IBD [131, 132].

Genes annotated to the ontology term "regulation of interleukin-17 production" are strongly enriched in GWAS data sets (Fig. 13.4). Among these are genes driving lineage commitment toward Th17 (RORγt, STAT3, IL23R, IL12B, IL6ST) and genes related to Th17 signaling (IL21) and chemotaxis (CCR6) [89, 103, 105, 114, 133]. A subunit of IL12, a cytokine driving Th1 differentiation, also serves as a subunit of IL23 and therefore can be regarded as interface between Th1- and Th17-directed cell commitment [134], as can TYK2, processing both signaling from IFNγ/IL12 and IL23. Further, Th1-associated genes identified as susceptibility loci include genes encoding for factors driving Th1 differentiation (STAT1) and mediating Th1 signaling such as IL2, IFNG with respective receptor forms, and a broad range of receptor and signaling proteins associated with TNF [89, 103].

Most of these genes are implicated in both etiologies of IBD, although only CD displays Th17 and Th1 immune response, whereas UC is Th2 dominated. These genetic observations support the view that the chronic inflammation in IBD is a consequence of innate immune dysfunction toward microbial stimuli, with environmental risk factors shaping the relationship between microbiota and the immune system. A substantial number of the susceptibility genes for IBD mentioned here overlap with other diseases, such as Coeliac disease, atopic dermatitis and psoriasis [89, 103], all of which result from faulty response of the immune system toward nonpathogenic microbiota or antigens.

Together with subsequent functional analyses, the results of GWAS have contributed to a great extent to the understanding of IBD pathogenesis, although they have not revealed potential for diagnostics as the predictive power is low, even at combination of all genetic susceptibility loci known today.

13.3 Microbial Diversity, Composition, and Function in Health and IBD

The comparison between health and diseased state helps to better understand the aspects of the host-microbe mutualism that sustain health, and the studies on microbial ecology performed in IBD patients offer a broad range of indications for mechanisms involved in health maintenance versus pathogenesis.

Despite the obvious contribution of microbiota and microbe-host interactions to the development of IBD, there is no specific microorganism described which can be consistently isolated in each IBD case and is absent in healthy hosts. Hypotheses that a particular bacterial agent causes IBD can therefore not be

supported. Several bacterial species are reported to correlate with the disease, although no evidence exists for any of them to be the causative factor of human IBD. Adherent-invasive *E. coli* (AIEC), for example, are found in abnormal numbers in ileal mucosa of CD patients, presumably due to enhanced expression of adherence factors [135, 136]. Besides this example of one defined species, IBD may in general be caused by overall changes in the composition and functionality of the intestinal microbiota, termed dysbiosis. A change of phenotypic features of the microbiota has been observed in IBD patients. Also the functional activity of these organisms has major impact on host-microbe signaling and is studied using global approaches such as metagenomics, metatranscriptomics, metaproteomics, and metabonomics. The phenomena observed so far on different levels are summarized in the following sections.

13.3.1 Gut Microbial Phenotyping: Alterations in Microbial Composition in IBD

There are many studies addressing the mucosa-associated microbial diversity in IBD patients but with major discrepancies concerning the study specimen and the way of data analysis. First, while some studies do not see differences between CD and UC samples and therefore regard them as one sample set, others demonstrate shifts in certain phyla specific for one of the etiologies. Second, the localization of the biopsy sample taken is critical and differs depending on the etiology studied. The definition of the control group is critical and may vary from unaffected people to unaffected relatives to samples from an unaffected site of an IBD patient. In conclusion, only the findings that have so far been reproduced in different experiments can be addressed here.

As a high-throughput method, 16SrDNA-based sequencing allows the rapid and parallel processing of many samples under the same conditions and is therefore commonly used in studies focusing on the microbial environment associated with IBD. Regarding the total numbers of bacterial population, no differences have been observed for fecal samples. Recently, studies have focused on mucosa-associated microbiota as the population with proximal contact to the host. This is important as luminal and mucosal communities have been shown to be distinct [3]. There are discrepancies in the data for the total numbers of mucosa-associated bacteria [137, 138], but dysbiosis and reduced diversity are a common feature of this compartment in the context of IBD.

Several studies have demonstrated reduced diversity of the intestinal ecology in CD [138–141] and UC [140] compared to unaffected control patients. This reduction in diversity is connected to reduced abundance of the dominant members of the human gut microbiota in IBD patients. In general, a decrease of Firmicutes [138, 140, 142, 143], and often Bacteroidetes [140, 142, 144], with concomitant increase of Proteobacteria [141–143, 145], and Actinobacteria [141, 142], was reported for mucosal biopsy samples or feces of IBD patients.

13.3.1.1 Firmicutes and Bacteroidetes

For the Firmicutes phylum, there is in particular substantial evidence for decreased abundance of populations belonging to *Clostridia IXa* and *IV* [139, 142, 146–148]. The depletion of *Faecalibacteria*, especially *F. prausnitzii*, seems to be a common feature in IBD patients, especially in CD [142, 145, 149–152]. With *F. prausnitzii* being a member of the Firmicutes<*Clostridia*, this could therefore contribute to the relative loss of this class observed in IBD. Supplementation of live *F. prausnitzii* or its supernatant ameliorated chemically induced colitis in animal studies and tended to correct the connected dysbiosis [151].

Regarding Bacteroidetes abundance, findings are not as consistent as for Firmicutes. While some comparisons find Bacteroidetes depleted in IBD [140, 142, 144], others do not observe significant alterations [64] or even report increased abundance [138].

13.3.1.2 Actinobacteria and Proteobacteria

Despite an overall tendency to relative increase in members of the Actinobacteria phylum, a reduction in populations of *Bifidobacteria* has been reported for UC patients [153]. It has been observed that the abundance of *Bifidobacteria* inversely correlates with abdominal pain in healthy subjects [154]. Probiotic approaches therefore consider *Bifidobacteria* as potential candidate for therapeutic use in IBD (see Sect. 13.13.2).

The growth of sulfate-reducing bacteria (SRB) seems increased in IBD, especially UC, along with the rate of sulfidogenesis [155–157]. With about 23 genera, the largest group of SRB is found among the Deltaproteobacteria, for example, within the orders Desulfovibrionales and Desulfobacterales [158]. An increase in relative amounts of SRB could thus contribute to the often observed increases in Proteobacteria abundance. SRB can metabolize sulfate via dissimilatory reduction and use it as the terminal electron acceptor in the electron transport chain. Sulfate or elemental sulfur is also converted into hydrogen sulfide, which can act on IECs by inhibiting butyrate utilization [159], as well as proliferation [160]. Desulfovibrionales member *Bilophila wadsworthia* utilizes taurine for energy generation [161] and is involved in high-fat diet-associated colitis in IL10$^{-/-}$ mice [162]. There is another important aspect of the possible involvement of SRB in colitis: dextran sodium sulfate (DSS) is used to chemically induce colitis in rodent models. Indeed, colonotoxic effects of sulfur compounds were initially reported with the observation that DSS treatment induces colitis and colorectal tumors [163, 164]. The microbial reduction of sulfate in the DSS molecule to the inflammatory, barrier-breaking hydrogen sulfide may be an initial trigger for the development of colitis [165]. DSS alters the microbial community already before the onset of intestinal pathology [166], possibly by the selection of bacteria that can degrade DSS or metabolize sulfur, such as *Proteus mirabilis* [167] or *Akkermansia muciniphila* (Verrucomicrobiae) – the latter is only detectable

in mice treated with DSS [166]. These findings emphasize the role of SRB in the induction of inflammation.

Concerning the increase in relative abundance of Proteobacteria in IBD, there is also particular evidence for the overgrowth of facultative anaerobe Enterobacteriaceae [138, 141, 143, 144, 147]. However, this family does not seem to be associated with disease in a causative way, as Enterobacteriaceae (*E. coli*) isolates did not induce colitis in antibiotic-pretreated susceptible mice despite robust colonization [168]. This example emphasizes that IBD-associated alterations in microbial composition do not necessarily reflect a causative relation.

13.3.1.3 Conclusions on Phylogenic Approaches

Many of the studies investigating dysbiosis in IBD revealed ICD as very distinct from healthy and UC state, whereas CCD and UC were often not distinguishable from each other or from healthy state. It is noteworthy that the donor patients of specimen used to study the microbiota often have different backgrounds, as ICD is more likely to be treated with immunosuppressant therapy and less likely to be treated with mesalamine or antibiotics than colonic phenotypes [169, 170]. These confounders might strongly affect the correlations seen between microbial composition and disease state. Basically, only findings for increased abundance in Enterobacteriaceae for CD, *Faecalibacterium* for ICD specifically, and *Clostridia* in both etiologies could be upheld after correction for all available covariates [169].

So far, all reported findings are correlations only and it therefore cannot be ruled out that dysbiosis may simply be a consequence of inflammation, with microbiota adapting to a changing environment. Related to the abovementioned example, the growth of Enterobacteriaceae can be enhanced by an inflammatory state per se or by genetic predisposition of the host in animal models [66, 171]. The overgrowth of an introduced species of *Salmonella enterica* (*S. typhimurium*) was also observed in an adoptive transfer model of colitis, in which cytotoxic T cells destroy the epithelium [172]. This highlights the fact that there is a clear impact of host genotypes and phenotypic conditions on microbial composition. Strikingly, genotypic effects on bacterial ecology have also been reported for humans since unaffected twins from UC patients also showed lower bacterial diversity than healthy, unrelated individuals and the same tendencies concerning relative increase of Actinobacteria [144]. This again implicates that a certain host genetic background may select pathogenic microbiota, comparable to the observations associated with NOD2 polymorphisms mentioned above.

Another drawback of using phylogenic characterization of the microbiota as a tool to study host-microbe interactions in health versus disease are the substantial intra- and interindividual variances in microbial composition, often outranging the differences between the study groups [138]. Defining alterations in relative abundance of certain bacteria and their possible relation to disease is therefore difficult. Still, the characterization of the intestinal microbial composition may be a promising tool to aid in diagnostics of IBD. A noninvasive method applying

16S-based sequencing and mapping to a database of healthy and IBD samples was successful in identifying pediatric patients with IBD (in contrast to patients with other gastrointestinal symptoms) and could even distinguish between ICD and UC both with reasonable sensitivity and specificity [173].

13.3.2 Metagenomics, Transcriptomics, and Proteomics: Microbial Gene Repertoire and Activity in IBD

Compositional analyses reflect the abundance of different microbial communities, but cannot give information on their activity or functionality. When aiming to elucidate the possible impact of the microbiota on gut health, the logical consequence is to study the functionality of the present populations. A higher transcriptional activity suggests higher cell replication rate or increased protein expression. Using this approach, it was observed that the prevalence of Enterobacteriaceae and in particular *E. coli* also translated to a high transcriptional activity [143, 174].

Metagenomic approaches study the whole repertoire of genes present in the microbiome. In the next steps, metatranscriptomics can give a picture of what genes are expressed, and finally, metaboproteomics reveal the resultant protein expression. However, in comparison to compositional analysis, metagenomics as well as metatranscriptomics and metaproteomics have rarely been applied in the study of microbial contribution to IBD until now.

The human intestinal microbiome contains an estimated number of 3.3 million genes, referred to as the metagenome [175]. Considering that there are about 150-fold more microbial genes present than human genes, it seems plausible to study the prospective functional activity assigned to the intestinal microbiota when aiming to elucidate host-microbe interactions. Indeed, microbial function might be more consistently perturbed than composition in IBD [169]. Generally speaking, metagenomic approaches have shown topological shifts of the microbiome associated with IBD [176]. The enzymes correlating with the host state were related to metabolic processes likely to use or produce metabolites that build an interface between bacterial and host metabolism, therefore probably affecting microbe-host interaction in the gut environment. Interestingly, similar changes were observed for IBD and obesity.

Chip-based metatranscriptomics have been applied in studying the transcriptional profiles of bacteria when present in different hosts, thereby substantiating the concept that microbes are affected by host genotype and the created intestinal environment. For example, the gene expression profile of *Bacteroides thetaiotaomicron* varied depending on whether it was mono-associated into transgenic, inflamed HLA-B27, or non-inflamed wild-type rats, with a downregulation of pathways involved in bacterial growth and metabolism in transgenic rats with colitis [177]. In contrast, bacterial genes in the ontology of the molecular function "receptor activity," mostly encoding nutrient binding proteins, were significantly upregulated in this colitis model. In another approach, nonpathogenic *E. coli* exhibited upregulated

expression of stress-response-associated genes upon mono-association into inflamed IL10$^{-/-}$ compared to healthy wild-type mice [178].

First attempts in applying untargeted shotgun proteomics to the microbiome could show a common core metaproteome, enriched in proteins related to translation, energy production, and carbohydrate metabolism in comparison to what could be predicted from metagenomics [179, 180]. Furthermore, the metaproteome of an individual proved to be reasonably stable throughout time [180]. Metagenomic and metaproteomic analyses of stool samples of a cohort of twin pairs, for which data on bacterial diversity were available, have reported a consistency in the findings of these three approaches regarding microbial alterations in IBD [141, 181]. The metagenomic reads of *F. prausnitzii* were significantly reduced in ICD, and in tendency also the reads of *Roseburia*, in accord with reduced abundance of these species confirmed by phylogenic sequencing. In addition to the metagenomic data, also proteome analysis indicated the depletion of proteins derived from these species. Especially bacterial proteins assigned to processes of replication, recombination, and repair were enriched in CD, whereas those related to energy production and nutrient transport and metabolism were depleted. In particular, in metagenomic as well as proteomic approach, SCFA production pathways were underrepresented in ICD. In total, there were significantly less microbial genes expressed and translated to proteins in CD compared to healthy state (2 % vs. 8 %). This is consistent with the finding that the richness of the metagenome is reduced in CD.

13.3.3 Metabonomics: Microbial Metabolic Products in IBD

In contrast to limited data from metatranscriptomics and metaproteomics, metabonomics have been more frequently used to study the role of microbiota in maintaining gut health and also in addressing differences between healthy state and inflammatory situation. Despite sophisticated approaches [182], no early IBD biomarkers could be established by the means of metabonomics up to now, but the applications of metabonomics certainly helped to describe molecular patterns. Fecal water, intestinal tissue, urine, or plasma is often used as specimen. As not all metabolites originating from bacteria will translocate or are absorbed and can be found in the plasma or urine, the type of specimen influences the outcome of analysis. In the following chapters, metabolites implicated in IBD are therefore discussed as grouped according to the respective compartment in which they have been described as regulated, with emphasis on the potential contribution of the microbiota to the metabonome profiles. An overview of the findings discussed in the following is given in Fig. 13.5. Sample specimens obtained by invasive methods (plasma and intestinal tissue) have been used less frequently in IBD studies, probably explaining a certain lack of described regulations for these compartments. Regardless of the sample type, all the differences described can either stem from metabolic profiles assigned to the host system or from bacteria if their metabolites are absorbed unless the respective metabolite is uniquely produced by only one of the involved parties.

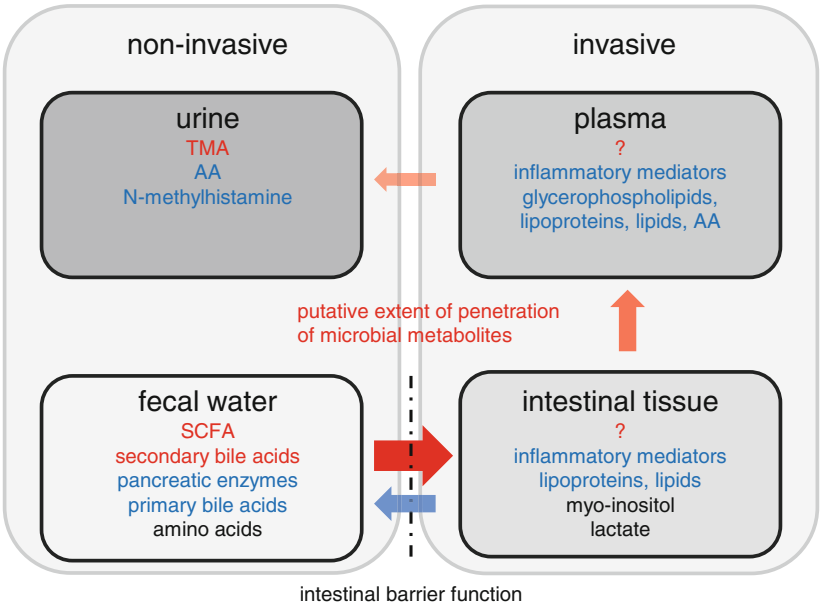

Fig. 13.5 Samples of different origins are used for metabonomic approaches studying IBD-related changes, describing the regulation of both host-derived (*blue*) and microbiota-derived (*red*) metabolites. Bacteria-derived metabolites may penetrate the intestinal barrier, depending on their size, chemical properties, and the intestinal permeability. Their abundance and the extent to which they are found regulated decreases in the subsequent compartments of the intestinal tissue, plasma, and urine compared to fecal water. Host metabolites implicated in IBD in the respective sample types are written in *blue*, bacteria-derived metabolites in *red*, and metabolites of unidentified origin in *black*. *TMA* trimethylamine, *AA* amino acids, *SCFA* short-chain fatty acids

If conclusions shall be deduced for IBD etiopathology, it is therefore necessary to validate any correlations by targeted approaches and in regard to the origin of the metabolite.

13.3.3.1 Fecal Water

The analysis of metabolites in fecal water seems a direct approach to link the composition of bacteria to their activity reflected by their metabolites and to draw conclusions on how this might affect host intestinal health. Studies on the fecal microbiota in twin cohorts have revealed that the respective metabonomes of fecal water cluster in the same way as data for phylogenic composition and metaproteomics for the same samples do [182]. All these approaches highlight the fact that CD samples are distinct from healthy samples but also that CCD samples differ

from ICD. Differences between CCD and healthy samples were not reported by other -omics approaches in the same samples, indicating that the groups were more distinct in metabolite profiles than other microbe-associated profiles. This suggests that the fecal water metabonome might be a good means of studying potential mechanisms in IBD etiopathology.

Amino Acids

The abundance of amino acids in fecal water is increased in IBD patients [183]. This might be due to reduced absorption of dietary amino acids or due to catabolic actions of the host epithelium, but also an alteration of microbial metabolism could result in changes in amino acids. Besides this ambivalent finding, also clearly bacteria-derived metabolites are reportedly regulated in fecal water of IBD patients.

Bile Acids

Metabolites related to bile acid (BA) synthesis and metabolism are often described as differentially regulated between healthy and IBD samples and even between ICD and CCD. Especially in ICD but also in irritable bowel syndrome or mouse models of colitis, luminal contents of total BA are significantly elevated [182, 184, 185]. This increase in the total luminal BA concentration in ICD might reflect a loss of transport activity as most of the BA should be reabsorbed in the ileum and undergo enterohepatic circulation. Associations of specific microbial populations with the respective metabolic profile of the feces were successfully established, with bacteria of higher abundance in ICD correlating to BA and fatty acid metabolites. As bacteria are differentially prone to antimicrobial effects exerted by BA and are differentially able to metabolize BA species, alterations in BA concentration or composition presumably result in concomitant shifts in microbial composition. Indeed, feeding of BA-enriched diets altered microbial composition in rodents profoundly [162, 186]. Targeted quantifications have revealed particular implication of tauroconjugated (-SO_3H) BA species in the lumen of UC patients [187]. Studies on animal models of colitis have reported that higher abundance of taurocholic acid [162] promotes the growth of SRB pathobionts that aggravate disease. Interestingly, certain luminal BA also decrease barrier function [188–190], probably via epidermal growth factor receptor and/or modulation of the nuclear farnesoid X receptor in IEC [191]. One study reported that the anti-inflammatory effect potentially exerted by secondary BA is abolished upon sulfation of the respective species [187]. As impaired barrier integrity and inept inflammatory response are the main features of IBD, BA may trigger disease progression double-tracked – via direct action on IEC and indirectly via modulation of the microbiota.

SCFA

The amount of SCFA, in particular butyrate, was markedly reduced in the fecal water of CD patients, and to a lesser extent UC patients, compared to healthy controls [183, 192]. The importance of butyrate for IEC functionality is described in Sect. 13.1.2. The depletion of these bacteria-derived metabolites may reflect alterations in microbial composition. In general, butyrate-producing bacteria have been reported as diminished in IBD [142]. Especially *Faecalibacterium prausnitzii* is regarded as an important butyrate producer and as outlined above is depleted in IBD-affected mucosa. Also metagenomic and metaproteomic approaches have revealed a depletion of butyrate and other SCFA production pathways in IBD [181] and concomitant reduction of *Faecalibacterium prausnitzii* abundance. Decreased SCFA production in IBD, especially CD, can thus be substantiated throughout the levels of -omics approaches (also see Fig. 13.6). Indeed, enemas of butyrate have been successfully used in UC treatment [43].

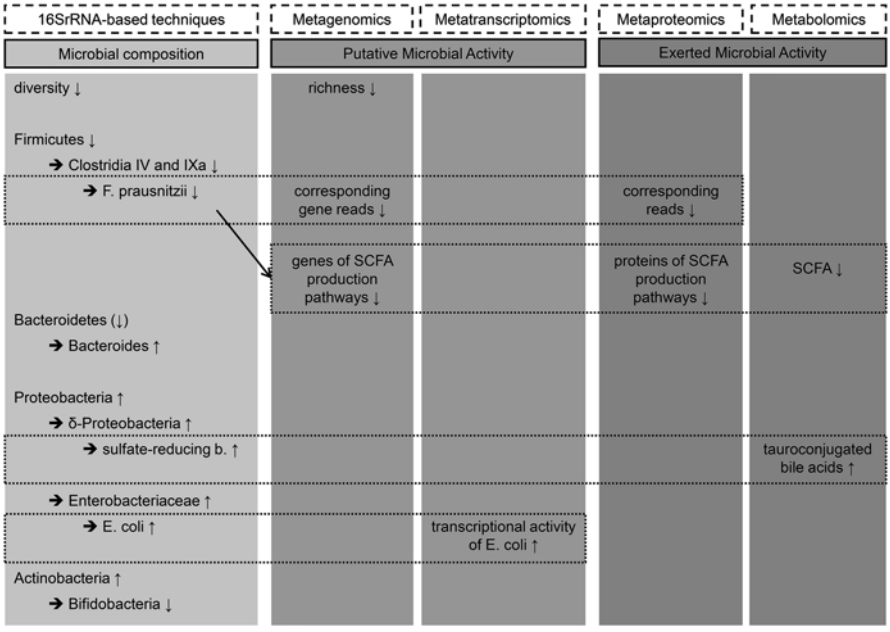

Fig. 13.6 IBD-related phenomena observed are partly interrelated across the different approaches of assessing total microbial functionality. *Dashed lines* indicate the approach used, *continuous lines* indicate the level of microbial functionality, and *dotted lines* indicate interrelations of findings on these different levels

Arachidonic Acid Metabolites

Metabonomic analyses on fecal samples also revealed arachidonic acid (AA) and its metabolites as implicated in IBD [182]. AA is converted by host enzymes into eicosanoids, mediators of inflammation. A regulation of eicosanoids was not convincingly reflected in fecal water, but this might be attributed to the spatial resolution, as these molecules exert their functions not in the lumen but at the basolateral side of the epithelium. This highlights the restriction of metabonomics using fecal water: it may be applied to study microbial metabolites but is limited to luminally secreted metabolites on the host side, or those low amounts of metabolites shed with epithelial cells.

13.3.3.2 Intestinal Tissue

In contrast to abundance in fecal water samples, tissue levels of both AA and eicosanoids were reportedly elevated in animal models [193, 194] and IBD patients [194], emphasizing the importance of metabolite compartmentalization. Untargeted metabonomic studies on intestinal biopsy tissues were effective in distinguishing healthy from UC [195] or CD samples [196] and also could discriminate between CD and UC [196]. Differences described within the metabolite profiles of healthy versus inflamed intestine lie especially within the group of glycerophospholipids, with decreases in glycerophosphocholine [195, 197] and lysophosphatidylcholines [193]. These findings are likely associated with the generation of inflammatory lipid mediators and altered tissue morphology, such as the infiltration of immune cells and changes in membrane fluidity. Tissue concentrations of myoinositol are decreased in UC patients [195, 197]. As myoinositol could be food or host derived [198], the meaning of this finding is not yet clear. It either reflects impaired absorption or could be related to alterations in membrane-incorporated phosphatidylinositol [199]. Lactate concentrations in intestinal tissue seem to be reduced in both UC and CD patients [197]. Despite the mechanistic inexplicability of this finding, this might serve as justification for the use of lactate-producing bacterial strains as probiotics in IBD therapy (see Sect. 13.13.2).

Although metabonomic studies of intestinal tissue have not revealed further microbiota-related alterations, these studies have elucidated molecular mechanisms during microbiota-driven pathogenesis.

13.3.3.3 Plasma

Up to now, there is no convincing description of IBD-associated alterations of microbial metabolites in human plasma samples. This may also be due to a limited number of studies analyzing plasma metabolites in IBD patients compared to fecal water or urinary metabolites. Inflammatory mediators including cytokines, eicosanoids, and prostaglandins are elevated in the plasma of IBD patients. In addition, many of the

plasma metabolites found implicated in murine IBD-like pathologies reflect changes in host energy homeostasis, such as lipoproteins, glycerophospholipids, lipids, or amino acids [200]. These changes probably result from inflammation-associated symptoms such as malabsorption, wasting, and protein catabolism.

13.3.3.4 Urine

As it is easy to collect and to process, urine seems a useful reservoir to apply metabonomic approaches. Indeed, several studies demonstrate that metabonomics can be applied on urinary metabolite profiles for the discrimination of healthy state from colitis in mice [201] and IBD in humans [170, 202]. Increased levels of amino acids probably reflect a catabolic state of the host. N-Methylhistamine, presumably deriving from mast cells, was found elevated in the urine of IBD patients [203]. The majority of the regulated metabolites described for urinary samples are host derived. It can be speculated that urinary samples may reflect the absorbed and excreted part of the microbial metabonome. Trimethylamine, a metabolite uniquely produced by the microbiota, was significantly elevated in the urine of colitic mice and correlated to disease progression [201]. But as IBD is also characterized by impaired barrier integrity, increased abundance of microbial metabolites could simply be an artifact of increased translocation and is not necessarily directly associated with IBD etiopathology.

13.3.4 Integration of Meta-omics Approaches Regarding Bacterial Functionality in IBD

In summary, -omics approaches allow the deduction of aspects of microbe-host interactions that can contribute major knowledge to the field of IBD research. The fact that metagenomics, metatranscriptomics, metaproteomics, and metabonomics (on fecal water at least) are able to similarly reflect changes in microbial functionality, as described above and summarized in Fig. 13.6, is auspicious. Nevertheless, these techniques need extensive data mining and are limited by the availability of the characterization of genes, transcripts, proteins, and metabolites, respectively.

13.3.5 Integrating Microbial and Host -Omics in Regard to IBD

An appealing approach to gain better insight into microbe-host interactions is the integration of data sets of both microbial composition or functionality and host molecular processes. Using microbe compositional data and host transcriptional profiles, it was described that there are correlations between colitis-associated

intestinal bacteria and potentially disease-relevant transcripts in the colonic mucosa [144]. In this context, reduced overall correlations and thus a loss of microbe-host cross talk in UC patients and unaffected twins of the same genotypic background as compared to unaffected healthy donors have been reported.

Furthermore, using metaproteomics on fecal samples gives the opportunity to detect both bacterial and human proteins, respectively. Unlike metabonomics, metaproteomic analyses can discriminate between bacterial and host sources. Using the fecal samples from a cohort of twins with healthy, concordant, or disconcordant status for CD, for which bacterial -omics data are available, and then characterizing the host proteome allowed drawing conclusions about the relationship between host status and observed microbial differences. The host proteome in stool of CD patients, predominantly ICD, was enriched in proteins associated with inflammatory response and host defense (e.g., α-defensin), as well as wound healing [181]. Proteins associated with barrier integrity were depleted in ICD patients (e.g., protocadherin). These findings go along with the notion that IBD patients suffer from a defective epithelial barrier and a compromised defense toward microbiota at the epithelial interface. Furthermore, there were high amounts of pancreatic enzymes found in ICD feces [181], indicating impaired absorption and correlating to the finding that luminal BA metabolites are increased in the same CD patients [182].

13.4 Dietary Manipulation of Intestinal Microbiota in the Context of IBD

The intestinal microbiota is not only a key driver of inflammation in the context of IBD [90] but also represents an interface between environment (diet) and host. The manipulation of the bacterial composition of the microbiota via diet might be a promising means of IBD treatment, especially in pediatrics where aggressive therapies are eschewed. It is crucial to discriminate between preventive and therapeutic strategies, as well as applications that intend to prolong phases of remission. Some alimentary strategies and food components have been reportedly successful regarding the latter. However, the effects of some approaches differ between the etiopathologies of CD, UC, and pouchitis, an IBD-like inflammation of an artificial rectum surgically formed out of ileal tissue during colectomy. Besides the nutritional therapies, research also focuses on the identification of dietary components that may affect the risk of IBD development.

13.4.1 Prebiotics

Given the potential of diet to modulate intestinal microbial composition and functionality, there are many approaches to implement nutritional intervention in IBD treatment strategies, in particular in the context of prebiotics. The concept of

prebiotics is the indirect modulation of microbial composition via the selective enhancement of favorable strains. When indigestible dietary fibers reach the colon, they are fermented by anaerobic bacteria, resulting in the production of SCFA. Importantly, SCFA not only serve as energy substrates for bacteria and thus enhance growth but can also alter intestinal microbial composition via a drop of the luminal pH and selective fostering of certain bacteria [204]. In humans, the prebiotic compounds inulin, lactulose, and fructo- and galacto-oligosaccharides have been shown to promote the growth of *Bifidobacteria*, *Eubacteria*, and *Lactobacilli* [205–209], which are considered to exhibit health benefits [210]. Randomized controlled trials using prebiotics in IBD patients are scarce, but small trials indicate reduced disease activity with concomitant alterations of bacterial composition upon inulin or fructo-oligosaccharide ingestion in the condition of CD [211] and of pouchitis [212]. Positive effects of prebiotics were also shown in animal studies on chemically induced colitis [213–216] and transgenic rodent models [217, 218], with increased amounts of *Lactobacilli* and *Bifidobacteria* and decreased macroscopic inflammation. Some of these studies in both humans and mouse models also report increased levels of butyrate [212–214].

13.4.2 Probiotics

Probiotics aim to actively alter microbial composition by introducing live bacteria. The prerequisite for a successful probiotic action therefore is the survival and sustaining of the bacteria in the complex and competitive microbial network. Originally derived from fermented food, especially dairy products, probiotic strains studied in regard to IBD include above all *Lactobacilli*, *Bifidobacteria*, and nonpathogenic *E. coli* Nissle 1917, as well as mixtures of these strains and a handful of other species. Human studies are limited in patient numbers and are focused on a variety of probiotic strains or combinations, different outcomes, and diverse kinds of cohorts. Meta-analyses try to estimate the overall beneficial effect of probiotics and indicated that in summary probiotics might be applicable in patients with colonic phenotype for the maintenance of remission rather than induction of remission or treatment of active disease [219].

The use of *Lactobacilli* has been studied in several mouse model experiments as well as clinical trials. Different strains (*GG*, *paracasei*, *plantarum*, *salivarius*) were found to exert a variety of effects on host immune response, such as induction of mucin expression [220] or suppression of cytokine production [221, 222] and T-cell proliferation [223]. Although there is a lack of trials in IBD patients that convincingly show a beneficial effect regarding disease activity or remission maintenance [224, 225], there is evidence for the amelioration of colitis in different mouse models in the case of *Lactobacilli* [221, 222, 226–228]. It became apparent that the probiotic strains tested for effects on IBD have to be chosen carefully, and also in regard to the experimental setup, when a study found *L. plantarum* and *LGG* to aggravate DSS-induced colitis, whereas *L. paracasei* exhibited protective effects [229]. In addition, *E. faecalis*, a member of the core gut microbiome historically

applied as probiotic strain, has been shown to compromise the intestinal barrier via the matrix metalloprotease GelE and to thus contribute to IBD pathogenesis in susceptible mouse models [230].

Probiotic *Bifidobacteria* of various strains (*infantis*, *breve*, *bifidum*) resulted in reduced inflammation in mouse models of colitis [221, 227, 231–234], and *Bifidobacterium*-fermented milk was found to ameliorate disease in UC patients [235, 236]. The mechanisms observed are similar to those reported for *Lactobacilli*, such as alterations of cytokine levels [231, 233]. As in the case of *Lactobacilli*, randomized placebo-controlled studies successfully using *Bifidobacteria* in IBD patients are scarce.

E. coli Nissle, a nonpathogenic *E. coli* strain, was effective in attaining as well as maintaining remission in UC patients [237–239]. This strain might exert beneficial effects by strengthening the intestinal barrier [240], similarly to the barrier-related effects of both *Lactobacilli* and *Bifidobacteria* [222, 241–243].

Also mixtures of probiotics are commonly used. The formula VSL#3 combines eight different probiotic lactic acid bacteria: four strains of *Lactobacilli* (*acidophilus*, *bulgaricus*, *casei*, *plantarum*), three strains of *Bifidobacteria* (*breve*, *infantis*, *longum*), and *Streptococcus thermophilus*, all of which are usually present in the human intestinal microbiota. This mixture has been shown to induce or maintain remission in pouchitis [244, 245], mild to moderate UC [246, 247], and pediatric UC patients [248]. In contrast to these promising results in pouchitis and UC, probiotic therapies failed to show effects in CD patients [249, 250]. The mechanism of action is poorly understood and the combination of the different strains impedes the study of molecular mechanisms. Animal models have been used to study the effects of VSL#3 concerning immune stimulation and regulation, some of which have reported a DNA-dependent effect of the probiotic mixture [251, 252]. One secreted component of VSL#3, the *L. paracasei*-derived prtP-encoded lactocepin, was shown to reduce inflammation in a murine colitis model [253], due to anti-inflammatory effects via the degradation of chemokines [254].

Noteworthy are also the effects observed for the use of synbiotics. The idea behind synbiotic approaches is to promote the survival and growth of the applied probiotics by simultaneous ingestion of prebiotics. Indeed, clinical trials revealed beneficial effects of synbiotics in UC patients [255, 256].

In summary, there is a need for more randomized, double-blind clinical trials focusing on the feasibility of probiotics in IBD therapy. In many cases, it is not clear if live bacteria, bacterial surface compounds, or secreted bacterial compounds are needed for the effects observed. Evidence for beneficial effects of probiotics, mainly in the case of *E. coli* Nissle and VSL#3, in both animal models and clinical trials is promising, though for colitis and pouchitis only and not for inflammation of the small intestine. The different etiologies of CD and UC might be responsible for this discrepancy. Recent studies indicate that eukaryotes such as *Saccharomyces boulardii* [257, 258] or helminths [259] might be effective in prolonging remission phases in CD, probably due to selective attenuation of Th1 antigenic responses (implicated in IBD) [260], and shift to Th2 (directed against parasites; also see Fig. 13.4) [261, 262]. However, the research on the use of these organisms in IBD is still in its infancy.

13.4.3 Enteral Nutrition and Elemental Diet

Enteral nutrition has been applied successfully in the treatment of IBD [263], especially in children with CD [264–266]. As IBD is characterized by a reduced bacterial diversity, it might appear contradictory that enteral nutrition seems to reduce bacterial diversity by depriving the microbiota of nutrients [267]. The reduction of diversity might correlate to a reduced antigenic load of the microbiota in this case. But a reduction of mucosal antigen exposure could also result from the nature of the feed, as semisynthetic enteral nutrition formulas are very low in bacterial antigens. In addition, a liquid diet results in faster intestinal transit, reducing the time of exposure to ingested putative antigens. Alternatively, enteral nutrition might also be able to modulate the metabolic activity of the microbiota [268].

A reduction of microbial diversity is also observed with the therapeutic use of elemental diets in IBD [269]. The term elemental diet describes easily digestible and peptide- or amino acid-based formulas. Some clinical trials support the efficaciousness of commercially available formulas in decreasing disease activity indices and relapse frequency comparably to steroid treatments [270–273].

The use of enteral nutrition or also certain elemental diets in IBD therapy seems promising, but still these treatment approaches do not always produce equally beneficial results as standard therapies using medication [274]. In addition, the mechanisms behind the amelioration of disease are not clearly understood. In general, as both nutritional approaches involve only easily digestible formulas, usually containing low amounts of fat, a general relief of the digestive systems in terms of enzyme production, chylomicron formation, and possibly mechanical stimulation is induced. In addition, non-polymeric diets are free of gluten: mouse studies indicated a potential aggravation of disease by gluten-containing diets [275], and gastrointestinal symptoms of non-celiac disease patients are ameliorated by gluten-free diet [276].

These possibly affected digestive processes concern the small intestine rather than the colon, which goes along with the finding that enteral and elemental diets are more effective in ileal than colonic IBD phenotypes [277]. Together with presumably altered composition or functionality of microbiota, there are numerous effects of these approaches on the intestinal tract, hampering the elucidation of molecular mechanisms.

13.4.4 Indications for Nutrients as Selection Factors for Pathobionts

In the search of dietary factors contributing to IBD development, various nutritional compounds and their putative molecular functions have been thoroughly discussed [278–280]. Attempts to link pre-illness diet to IBD risk did not provide consistent results [281–284].

Among the dietary factors suspected to trigger IBD are mainly carbohydrates and fat, in particular refined sugar, saturated fatty acids, and omega-6 fatty acids,

whereas dietary fibers and food groups such as fruit and vegetables may be protective [280]. The lack of unequivocal evidence from epidemiological studies for the implication of specific dietary factors might result from the complex etiology of IBD and also indicates that diet probably only affects IBD development in combination with host genetics and other environmental factors. However, there are accumulating indications for a role of dietary fat in IBD pathogenesis. A systematic literature review of retrospective epidemiologic data concluded that a high dietary intake of fat, adjusted for total energy intake and behavioral confounders, increases risk of IBD [280]. In contrast, body mass index or total energy intake did not correlate to the development of incident IBD in a prospective cohort study [285]. This indicates a specific role for dietary lipids in the pathogenesis of IBD. Indeed, high-fat diets (HFD) have been shown to impair intestinal health in chemical and genetic animal models of colitis [162, 286, 287] and CD-like ileitis [288]. There are indications from animal studies that HFD promotes intestinal inflammatory processes [287, 289, 290] and perturbs barrier function [189, 288, 291], but the mechanisms underlying the facilitation of disease onset or its aggravation remain not fully elucidated. HFD modulates the composition of the intestinal microbiota [287, 292]. The modulation of the bacterial composition by HFD ingestion can result in increased pathogenicity of the intestinal microbiota, especially by fostering the growth of pathobionts that induce inappropriate inflammatory response in a susceptible host [162]. There are indications that HFD exerts this modulation of the microbial ecosystem via shifts in BA composition [162, 186, 189]. Only 1 day after facing a diet high in animal fat, the microbial community starts to shape accordingly, with increased abundance of bile acid-tolerant species and reduction of plant carbohydrate-metabolizing species [293]. HFD-associated modification of the microbial composition, reduction of barrier function, and aggravation of pathology are reportedly independent of an obese phenotype [189, 288, 292]. In summary, diets rich in fat may aggravate IBD via the modulation of intestinal luminal factors. This mechanism could also contribute to the beneficial effects reported for enteral and elemental nutrition, as these forms of alimentation generally lead to decreased fat consumption compared to a typical Western diet.

Likewise, there is evidence for iron playing a crucial role in IBD pathogenesis from a mouse model with CD-like phenotype: dietary but not systemic iron aggravated disease compared to a low-iron diet, probably via the modulation of intestinal microbial composition, and in combination with increased cellular stress in IECs [294]. These findings might particularly impact on IBD treatment in the future, considering the fact that IBD patients are often anemic and treated with iron supplements.

13.4.5 Nutrients: Nutrigenetics in IBD

Besides the effects of diet on intestinal microbial composition and function, diet may also impact on IBD in a more direct way. Nutrigenetics in the context of IBD aims at identifying nutrient-gene interactions associated with disease-conditioning and disease-modulating situation. Thereby, two main aspects have come into

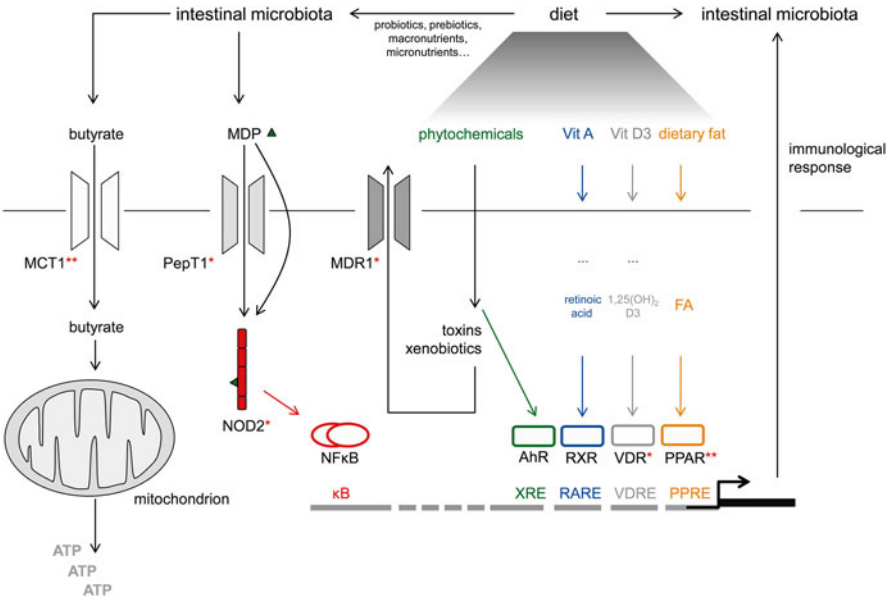

Fig. 13.7 Nutrigenetics in the context of IBD investigate nutrient-gene interactions associated with disease-conditioning and disease-modulating situation. The regulation of cellular energy demand, for example, via butyrate import, and the effect of transcription factors activated by nutrients are critical in IBD. *MCT1* monocarboxylate transporter 1, *ATP* adenosine triphosphate, *PepT1* peptide transporter 1, *MDP* muramyl dipeptide, *NOD2* nucleotide-binding oligomerization domain 2, *NFκB* nuclear factor κB, *MDR1* multidrug resistance protein 1, *AhR* aryl hydrocarbon receptor, *Vit A* vitamin A, *XRE* xenobiotic response element, *RXR* retinoid X receptor, *RARE* retinoic acid response element, *Vit D* vitamin D, *VDR* vitamin D receptor, *VDRE VDR* responsive element. *FA* fatty acid, *PPAR* peroxisome proliferator-activated receptor, *PRE* PPAR responsive element, * IBD susceptibility gene, ** regulated in IBD

play: (1) the general regulation of cellular energy demand and (2) transcription factors activated by nutrients as the closest interface of diet and regulation of gene expression. Major findings are summarized in Fig. 13.7.

13.4.5.1 Cellular Energy Demand

Meeting the cellular energy demand might be of particular importance in IECs that are challenged by energy-consuming inflammatory processes. Besides impaired β-oxidation resulting from polymorphisms in the carnitine transporter OCTN2 [105], there are reports of decreased butyrate oxidation in the inflamed mucosa of UC patients [295, 296] and in animal models of experimental colitis [297]. Additionally, the monocarboxylate transporter MCT1, responsible for butyrate uptake in colonocytes [298], is reportedly downregulated in IBD [299]. Beneficial modulation of the microbial composition could enhance butyrate supply and attenuate energy deficiency in IEC. Notably,

luminal levels of acetate and butyrate were reported to decrease rapidly with ingestion of diets high in fat [293].

13.4.5.2 Nutrient-Induced Signaling

Diet- and microbiota-derived xenobiotics are actively removed from IEC via the multidrug resistance protein (MDR1). Variants of MDR1 are associated with UC [300, 301]. Xenobiotic substances remaining in the host cell may activate the transcription factor aryl hydrocarbon receptor (Ahr) [302]. Derivatives of diet-derived vitamins A and D bind to transcription factors retinoid X receptor (RXR) and vitamin D receptor (VDR), respectively, whereas fatty acids activate peroxisome proliferator-activated receptors (PPARs). VDR polymorphisms are associated with IBD [303], and also contribution of PPARs is under debate [302, 304]. PepT1, a major brush border peptide transporter, is also implicated as IBD susceptibility gene [305]. It allows uptake of muramyl dipeptide which activates NOD2 and thus inflammatory response pathways via NFκB [306, 307]. Activated transcription factors such as NFκB, Ahr, RXR, VDR, or PPAR modulate immune response downstream and thus in turn also potentially impact on microbiota, emphasizing the close interrelations of host, dietary factors, and microbiota.

In summary, it seems likely that dietary components or alimentary strategies impact on IBD directly or through alterations of the composition or metabolic activity of the microbiota. Therefore, personalized nutrition strategies might profoundly improve disease activity or remission maintenance in the future.

13.5 Conclusions and Perspective

While the human intestinal microbiota is needed to build up and maintain an adequate immune system, a loss of homeostasis regarding the interactions between microbes and the host intestinal mucosal immune system can result in aberrant or disproportionate inflammatory responses, with IBD as an example with numerous cases. Given that the microbial ecology is a major modulator of gastrointestinal health, the manipulation of its composition and functionality seems a promising therapeutic strategy for acute infections as well as chronic diseases such as IBD. Metabonomics as well as other -omics techniques will be useful monitoring the degree of modulation and may help reveal functional interactions.

While dietary manipulation via nutrients, probiotics, or prebiotics is a rather mild way of interference, more dramatic changes can be achieved using antibiotics or fecal microbial transplantation (FMT). Transplanting donor microbiota has been successfully performed in cases of *Clostridium difficile* infections [308], but its application in IBD is still rather new and controversial. Mainly, case reports are available so far regarding the management of IBD by FMT, with many of them suggesting a beneficial effect as reviewed/summarized in [309, 310]. A pilot trial

reported the alleviation of symptoms in pediatric UC patients [311]. No serious adverse effects were reported in this study, whereas fever and an increase in plasma inflammatory markers were observed in a study involving five adult UC patients, while only one patient showed symptom improvement [312].

It is believed that FMT can restore antibiotic-related disruption intestinal of the microbial diversity [308]. However, not much is known about the mechanisms that render one microbial community of a certain shape harmless while another turns out deleterious. The research for defining optimal donor microbiota characteristics, donor-recipient match parameters, and pre-FMT conditioning is crucial for FMT to be further adapted and developed into an approved therapy and might at the same time provide more insight into mechanisms of microbe-host cross talk during intestinal health and disease.

References

1. Artis D. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nat Rev Immunol. 2008;8(6):411–20. Epub 2008/05/13.
2. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell. 2006;124(4):837–48. Epub 2006/02/25.
3. Zoetendal EG, von Wright A, Vilpponen-Salmela T, Ben-Amor K, Akkermans AD, de Vos WM. Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. Appl Environ Microbiol. 2002;68(7):3401–7. Epub 2002/06/29.
4. Dethlefsen L, McFall-Ngai M, Relman DA. An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature. 2007;449(7164):811–8. Epub 2007/10/19.
5. Scott KP, Gratz SW, Sheridan PO, Flint HJ, Duncan SH. The influence of diet on the gut microbiota. Pharmacol Res: Off J Ital Pharmacol Soc. 2013;69(1):52–60. Epub 2012/11/14.
6. Derrien M, Vaughan EE, Plugge CM, de Vos WM. Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. Int J Syst Evol Microbiol. 2004;54(Pt 5):1469–76.
7. Berry D, Stecher B, Schintlmeister A, Reichert J, Brugiroux S, Wild B, et al. Host-compound foraging by intestinal microbiota revealed by single-cell stable isotope probing. Proc Natl Acad Sci U S A. 2013;110(12):4720–5. Epub 2013/03/15.
8. Martens EC, Koropatkin NM, Smith TJ, Gordon JI. Complex glycan catabolism by the human gut microbiota: the Bacteroidetes Sus-like paradigm. J Biol Chem. 2009;284(37):24673–7. Epub 2009/06/26.
9. Sonnenburg JL, Xu J, Leip DD, Chen CH, Westover BP, Weatherford J, et al. Glycan foraging in vivo by an intestine-adapted bacterial symbiont. Science. 2005;307(5717):1955–9. Epub 2005/03/26.
10. Granato D, Bergonzelli GE, Pridmore RD, Marvin L, Rouvet M, Corthesy-Theulaz IE. Cell surface-associated elongation factor Tu mediates the attachment of Lactobacillus johnsonii NCC533 (La1) to human intestinal cells and mucins. Infect Immun. 2004;72(4):2160–9. Epub 2004/03/25.
11. Ruiz-Palacios GM, Cervantes LE, Ramos P, Chavez-Munguia B, Newburg DS. Campylobacter jejuni binds intestinal H(O) antigen (Fuc alpha 1, 2Gal beta 1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection. J Biol Chem. 2003;278(16):14112–20. Epub 2003/02/04.

12. Clavel T, Charrier C, Braune A, Wenning M, Blaut M, Haller D. Isolation of bacteria from the ileal mucosa of TNFdeltaARE mice and description of Enterorhabdus mucosicola gen. nov., sp. nov. Int J Syst Evol Microbiol. 2009;59(Pt 7):1805–12.
13. Begon M, Harper JL, Townsend CR. Ecology. Individuals, populations and communities. 3rd ed. Wiley-Blackwell; 1996. Oxford.
14. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. Nat Rev Immunol. 2004;4(6):478–85. Epub 2004/06/03.
15. Bouskra D, Brezillon C, Berard M, Werts C, Varona R, Boneca IG, et al. Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. Nature. 2008;456(7221):507–10. Epub 2008/11/07.
16. Tlaskalova-Hogenova H, Sterzl J, Stepankova R, Dlabac V, Veticka V, Rossmann P, et al. Development of immunological capacity under germfree and conventional conditions. Ann N Y Acad Sci. 1983;409:96–113. Epub 1983/06/30.
17. Moreau MC, Ducluzeau R, Guy-Grand D, Muller MC. Increase in the population of duodenal immunoglobulin A plasmocytes in axenic mice associated with different living or dead bacterial strains of intestinal origin. Infect Immun. 1978;21(2):532–9. Epub 1978/08/01.
18. Umesaki Y, Setoyama H, Matsumoto S, Okada Y. Expansion of alpha beta T-cell receptor-bearing intestinal intraepithelial lymphocytes after microbial colonization in germ-free mice and its independence from thymus. Immunology. 1993;79(1):32–7. Epub 1993/05/01.
19. Duan J, Chung H, Troy E, Kasper DL. Microbial colonization drives expansion of IL-1 receptor 1-expressing and IL-17-producing gamma/delta T cells. Cell Host Microbe. 2010;7(2):140–50. Epub 2010/02/18.
20. Williams AM, Probert CS, Stepankova R, Tlaskalova-Hogenova H, Phillips A, Bland PW. Effects of microflora on the neonatal development of gut mucosal T cells and myeloid cells in the mouse. Immunology. 2006;119(4):470–8. Epub 2006/09/26.
21. Niess JH, Brand S, Gu X, Landsman L, Jung S, McCormick BA, et al. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science. 2005;307(5707):254–8. Epub 2005/01/18.
22. Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, et al. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol. 2001;2(4):361–7. Epub 2001/03/29.
23. Iliev ID, Mileti E, Matteoli G, Chieppa M, Rescigno M. Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. Mucosal Immunol. 2009;2(4):340–50. Epub 2009/04/24.
24. Siddiqui KR, Powrie F. CD103+ GALT DCs promote Foxp3+ regulatory T cells. Mucosal Immunol. 2008;1 Suppl 1:S34–8. Epub 2008/12/23.
25. Schulz O, Jaensson E, Persson EK, Liu X, Worbs T, Agace WW, et al. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. J Exp Med. 2009;206(13):3101–14. Epub 2009/12/17.
26. Schlitzer A, McGovern N, Teo P, Zelante T, Atarashi K, Low D, et al. IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. Immunity. 2013;38(5):970–83. Epub 2013/05/28.
27. Watchmaker PB, Lahl K, Lee M, Baumjohann D, Morton J, Kim SJ, et al. Comparative transcriptional and functional profiling defines conserved programs of intestinal DC differentiation in humans and mice. Nat Immunol. 2013;8:22. Epub 2013/12/03.
28. Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. Nature. 2008;453(7195):620–5. Epub 2008/05/30.
29. Atarashi K, Nishimura J, Shima T, Umesaki Y, Yamamoto M, Onoue M, et al. ATP drives lamina propria T(H)17 cell differentiation. Nature. 2008;455(7214):808–12. Epub 2008/08/22.
30. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell. 2009;139(3):485–98. Epub 2009/10/20.
31. Gaboriau-Routhiau V, Rakotobe S, Lecuyer E, Mulder I, Lan A, Bridonneau C, et al. The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. Immunity. 2009;31(4):677–89. Epub 2009/10/17.

32. Uematsu S, Fujimoto K, Jang MH, Yang BG, Jung YJ, Nishiyama M, et al. Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. Nat Immunol. 2008;9(7):769–76. Epub 2008/06/03.
33. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, et al. Induction of colonic regulatory T cells by indigenous Clostridium species. Science. 2011;331(6015):337–41. Epub 2011/01/06.
34. Round JL, Lee SM, Li J, Tran G, Jabri B, Chatila TA, et al. The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. Science. 2011;332(6032):974–7. Epub 2011/04/23.
35. Chung DR, Kasper DL, Panzo RJ, Chitnis T, Grusby MJ, Sayegh MH, et al. CD4+ T cells mediate abscess formation in intra-abdominal sepsis by an IL-17-dependent mechanism. J Immunol. 2003;170(4):1958–63. Epub 2003/02/08.
36. Gibson 3rd FC, Onderdonk AB, Kasper DL, Tzianabos AO. Cellular mechanism of intraabdominal abscess formation by Bacteroides fragilis. J Immunol. 1998;160(10):5000–6. Epub 1998/05/20.
37. Bauer H, Horowitz RE, Levenson SM, Popper H. The response of the lymphatic tissue to the microbial flora. Studies on germfree mice. Am J Pathol. 1963;42:471–83. Epub 1963/04/01.
38. Hapfelmeier S, Lawson MA, Slack E, Kirundi JK, Stoel M, Heikenwalder M, et al. Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. Science. 2010;328(5986):1705–9. Epub 2010/06/26.
39. Durkin HG, Chice SM, Gaetjens E, Bazin H, Tarcsay L, Dukor P. Origin and fate of IgE-bearing lymphocytes. II Modulation of IgE isotype expression on Peyer's patch cells by feeding with certain bacteria and bacterial cell wall components or by thymectomy. J Immunol. 1989;143(6):1777–83. Epub 1989/09/15.
40. Segain JP, Raingeard, de la Bletiere D, Bourreille A, Leray V, Gervois N, Rosales C, et al. Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. Gut. 2000;47(3):397–403. Epub 2000/08/15.
41. Saemann MD, Bohmig GA, Osterreicher CH, Burtscher H, Parolini O, Diakos C, et al. Anti-inflammatory effects of sodium butyrate on human monocytes: potent inhibition of IL-12 and up-regulation of IL-10 production. FASEB J: Off Pub Fed Am Soc Experi Biol. 2000;14(15):2380–2. Epub 2000/10/12.
42. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, et al. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature. 2009;461(7268):1282–6. Epub 2009/10/30.
43. Scheppach W, Sommer H, Kirchner T, Paganelli GM, Bartram P, Christl S, et al. Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis. Gastroenterology. 1992;103(1):51–6. Epub 1992/07/01.
44. Chung H, Pamp SJ, Hill JA, Surana NK, Edelman SM, Troy EB, et al. Gut immune maturation depends on colonization with a host-specific microbiota. Cell. 2012;149(7):1578–93. Epub 2012/06/26.
45. Meyer-Hoffert U, Hornef MW, Henriques-Normark B, Axelsson LG, Midtvedt T, Putsep K, et al. Secreted enteric antimicrobial activity localises to the mucus surface layer. Gut. 2008;57(6):764–71. Epub 2008/02/06.
46. Vaishnava S, Behrendt CL, Ismail AS, Eckmann L, Hooper LV. Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. Proc Natl Acad Sci U S A. 2008;105(52):20858–63. Epub 2008/12/17.
47. Ayabe T, Satchell DP, Wilson CL, Parks WC, Selsted ME, Ouellette AJ. Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. Nat Immunol. 2000;1(2):113–8. Epub 2001/03/15.
48. Cash HL, Whitham CV, Behrendt CL, Hooper LV. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. Science. 2006;313(5790):1126–30. Epub 2006/08/26.
49. Hooper LV, Stappenbeck TS, Hong CV, Gordon JI. Angiogenins: a new class of microbicidal proteins involved in innate immunity. Nat Immunol. 2003;4(3):269–73. Epub 2003/01/28.

50. Kida Y, Shimizu T, Kuwano K. Sodium butyrate up-regulates cathelicidin gene expression via activator protein-1 and histone acetylation at the promoter region in a human lung epithelial cell line, EBC-1. Mol Immunol. 2006;43(12):1972–81. Epub 2006/01/21.
51. Burger-van Paassen N, Loonen LM, Witte-Bouma J, Korteland-van Male AM, de Bruijn AC, van der Sluis M, et al. Mucin Muc2 deficiency and weaning influences the expression of the innate defense genes Reg3beta, Reg3gamma and angiogenin-4. PLoS ONE. 2012;7(6):e38798. Epub 2012/06/23.
52. Menard S, Forster V, Lotz M, Gutle D, Duerr CU, Gallo RL, et al. Developmental switch of intestinal antimicrobial peptide expression. J Exp Med. 2008;205(1):183–93. Epub 2008/01/09.
53. Salzman NH, Hung K, Haribhai D, Chu H, Karlsson-Sjoberg J, Amir E, et al. Enteric defensins are essential regulators of intestinal microbial ecology. Nat Immunol. 2010;11(1):76–83. Epub 2009/10/27.
54. Peterson DA, McNulty NP, Guruge JL, Gordon JI. IgA response to symbiotic bacteria as a mediator of gut homeostasis. Cell Host Microbe. 2007;2(5):328–39. Epub 2007/11/17.
55. Pasquier B, Launay P, Kanamaru Y, Moura IC, Pfirsch S, Ruffie C, et al. Identification of FcalphaRI as an inhibitory receptor that controls inflammation: dual role of FcRgamma ITAM. Immunity. 2005;22(1):31–42. Epub 2005/01/25.
56. Macpherson AJ, Uhr T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science. 2004;303(5664):1662–5. Epub 2004/03/16.
57. Suzuki K, Meek B, Doi Y, Muramatsu M, Chiba T, Honjo T, et al. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. Proc Natl Acad Sci U S A. 2004;101(7):1981–6. Epub 2004/02/10.
58. Yanagibashi T, Hosono A, Oyama A, Tsuda M, Hachimura S, Takahashi Y, et al. Bacteroides induce higher IgA production than Lactobacillus by increasing activation-induced cytidine deaminase expression in B cells in murine Peyer's patches. Biosci Biotechnol Biochem. 2009;73(2):372–7. Epub 2009/02/10.
59. Trinchieri G, Sher A. Cooperation of Toll-like receptor signals in innate immune defence. Nat Rev Immunol. 2007;7(3):179–90. Epub 2007/02/24.
60. Rehman A, Sina C, Gavrilova O, Hasler R, Ott S, Baines JF, et al. Nod2 is essential for temporal development of intestinal microbial communities. Gut. 2011;60(10):1354–62. Epub 2011/03/23.
61. Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, et al. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem. 2003;278(11):8869–72. Epub 2003/01/16.
62. Petnicki-Ocwieja T, Hrncir T, Liu YJ, Biswas A, Hudcovic T, Tlaskalova-Hogenova H, et al. Nod2 is required for the regulation of commensal microbiota in the intestine. Proc Natl Acad Sci U S A. 2009;106(37):15813–8. Epub 2009/10/07.
63. Li E, Hamm CM, Gulati AS, Sartor RB, Chen H, Wu X, et al. Inflammatory bowel diseases phenotype, C. difficile and NOD2 genotype are associated with shifts in human ileum associated microbial composition. PLoS ONE. 2012;7(6):e26284.
64. Frank DN, Robertson CE, Hamm CM, Kpadeh Z, Zhang T, Chen H, et al. Disease phenotype and genotype are associated with shifts in intestinal-associated microbiota in inflammatory bowel diseases. Inflamm Bowel Dis. 2011;17(1):179–84. Epub 2010/09/15.
65. Couturier-Maillard A, Secher T, Rehman A, Normand S, De Arcangelis A, Haesler R, et al. NOD2-mediated dysbiosis predisposes mice to transmissible colitis and colorectal cancer. J Clin Invest. 2013;3(4):384–7. Epub 2013/01/03.
66. Nones K, Knoch B, Dommels YE, Paturi G, Butts C, McNabb WC, et al. Multidrug resistance gene deficient (mdr1a-/-) mice have an altered caecal microbiota that precedes the onset of intestinal inflammation. J Appl Microbiol. 2009;107(2):557–66. Epub 2009/03/24.
67. Garrett WS, Lord GM, Punit S, Lugo-Villarino G, Mazmanian SK, Ito S, et al. Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. Cell. 2007;131(1):33–45. Epub 2007/10/10.

68. Gulati AS, Shanahan MT, Arthur JC, Grossniklaus E, von Furstenberg RJ, Kreuk L, et al. Mouse background strain profoundly influences Paneth cell function and intestinal microbial composition. PLoS ONE. 2012;7(2):e32403. Epub 2012/03/03.
69. Rawls JF, Mahowald MA, Ley RE, Gordon JI. Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. Cell. 2006;127(2):423–33. Epub 2006/10/24.
70. Fonager K, Sorensen HT, Olsen J. Change in incidence of Crohn's disease and ulcerative colitis in Denmark. A study based on the National Registry of Patients, 1981–1992. Int J Epidemiol. 1997;26(5):1003–8.
71. Loftus CG, Loftus Jr EV, Harmsen WS, Zinsmeister AR, Tremaine WJ, Melton 3rd LJ, et al. Update on the incidence and prevalence of Crohn's disease and ulcerative colitis in Olmsted County, Minnesota, 1940–2000. Inflamm Bowel Dis. 2007;13(3):254–61. Epub 2007/01/09.
72. Bernstein CN, Blanchard JF, Rawsthorne P, Wajda A. Epidemiology of Crohn's disease and ulcerative colitis in a central Canadian province: a population-based study. Am J Epidemiol. 1999;149(10):916–24. Epub 1999/05/26.
73. Loftus Jr EV, Silverstein MD, Sandborn WJ, Tremaine WJ, Harmsen WS, Zinsmeister AR. Crohn's disease in Olmsted County, Minnesota, 1940–1993: incidence, prevalence, and survival. Gastroenterology. 1998;114(6):1161–8. Epub 1998/06/03.
74. Moum B, Vatn MH, Ekbom A, Aadland E, Fausa O, Lygren I, et al. Incidence of Crohn's disease in four counties in southeastern Norway, 1990–93. A prospective population-based study. The Inflammatory Bowel South-Eastern Norway (IBSEN) Study Group of Gastroenterologists. Scand J Gastroenterol. 1996;31(4):355–61.
75. Vind I, Riis L, Jess T, Knudsen E, Pedersen N, Elkjaer M, et al. Increasing incidences of inflammatory bowel disease and decreasing surgery rates in Copenhagen City and County, 2003–2005: a population-based study from the Danish Crohn colitis database. Am J Gastroenterol. 2006;101(6):1274–82. Epub 2006/06/15.
76. Manninen P, Karvonen AL, Huhtala H, Rasmussen M, Collin P. The epidemiology of inflammatory bowel diseases in Finland. Scand J Gastroenterol. 2010;45(9):1063–7. Epub 2010/05/07.
77. Jussila A, Virta LJ, Salomaa V, Maki J, Jula A, Farkkila MA. High and increasing prevalence of inflammatory bowel disease in Finland with a clear North-South difference. J Crohns Colitis. 2012;42(3):482–9. Epub 2012/11/13.
78. Nerich V, Monnet E, Etienne A, Louafi S, Ramee C, Rican S, et al. Geographical variations of inflammatory bowel disease in France: a study based on national health insurance data. Inflamm Bowel Dis. 2006;12(3):218–26. Epub 2006/03/15.
79. Shivananda S, Lennard-Jones J, Logan R, Fear N, Price A, Carpenter L, et al. Incidence of inflammatory bowel disease across Europe: is there a difference between north and south? Results of the European Collaborative Study on Inflammatory Bowel Disease (EC-IBD). Gut. 1996;39(5):690–7. Epub 1996/11/01.
80. Ahuja V, Tandon RK. Inflammatory bowel disease in the Asia-Pacific area: a comparison with developed countries and regional differences. J Dig Dis. 2010;11(3):134–47. Epub 2010/06/29.
81. Thia KT, Loftus Jr EV, Sandborn WJ, Yang SK. An update on the epidemiology of inflammatory bowel disease in Asia. Am J Gastroenterol. 2008;103(12):3167–82. Epub 2008/12/18.
82. Probert CS, Jayanthi V, Hughes AO, Thompson JR, Wicks AC, Mayberry JF. Prevalence and family risk of ulcerative colitis and Crohn's disease: an epidemiological study among Europeans and south Asians in Leicestershire. Gut. 1993;34(11):1547–51. Epub 1993/11/01.
83. Cheon JH. Genetics of inflammatory bowel diseases: a comparison between Western and Eastern perspectives. J Gastroenterol Hepatol. 2012;27:919–27. Epub 2012/11/30.
84. Halme L, Paavola-Sakki P, Turunen U, Lappalainen M, Farkkila M, Kontula K. Family and twin studies in inflammatory bowel disease. World J Gastroenterol: WJG. 2006;12(23):3668–72. Epub 2006/06/15.

85. Halfvarson J, Bodin L, Tysk C, Lindberg E, Jarnerot G. Inflammatory bowel disease in a Swedish twin cohort: a long-term follow-up of concordance and clinical characteristics. Gastroenterology. 2003;124(7):1767–73. Epub 2003/06/14.
86. Orholm M, Binder V, Sorensen TI, Rasmussen LP, Kyvik KO. Concordance of inflammatory bowel disease among Danish twins. Results of a nationwide study. Scandinavian J Gastroenterol. 2000;35(10):1075–81. Epub 2000/12/01.
87. Thompson NP, Driscoll R, Pounder RE, Wakefield AJ. Genetics versus environment in inflammatory bowel disease: results of a British twin study. BMJ. 1996;312(7023):95–6. Epub 1996/01/13.
88. Tysk C, Lindberg E, Jarnerot G, Floderus-Myrhed B. Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. Gut. 1988;29(7):990–6. Epub 1988/07/01.
89. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature. 2012;491(7422):119–24. Epub 2012/11/07.
90. Sartor RB. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nat Clin Pract Gastroenterol Hepatol. 2006;3(7):390–407. Epub 2006/07/05.
91. Clavel T, Haller D. Bacteria- and host-derived mechanisms to control intestinal epithelial cell homeostasis: implications for chronic inflammation. Inflamm Bowel Dis. 2007;13(9):1153–64. Epub 2007/05/04.
92. D'Haens GR, Geboes K, Peeters M, Baert F, Penninckx F, Rutgeerts P. Early lesions of recurrent Crohn's disease caused by infusion of intestinal contents in excluded ileum. Gastroenterology. 1998;114(2):262–7. Epub 1998/02/07.
93. Duchmann R, Kaiser I, Hermann E, Mayet W, Ewe K. Meyer zum Buschenfelde KH. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). Clin Exp Immunol. 1995;102(3):448–55.
94. Macpherson A, Khoo UY, Forgacs I, Philpott-Howard J, Bjarnason I. Mucosal antibodies in inflammatory bowel disease are directed against intestinal bacteria. Gut. 1996;38(3):365–75. Epub 1996/03/01.
95. van der Waaij LA, Kroese FG, Visser A, Nelis GF, Westerveld BD, Jansen PL, et al. Immunoglobulin coating of faecal bacteria in inflammatory bowel disease. Eur J Gastroenterol Hepatol. 2004;16(7):669–74. Epub 2004/06/18.
96. Greenbloom SL, Steinhart AH, Greenberg GR. Combination ciprofloxacin and metronidazole for active Crohn's disease. Can J Gastroenterol = J canadien de gastroenterologie. 1998;12(1):53–6.
97. Rutgeerts P, Van Assche G, Vermeire S, D'Haens G, Baert F, Noman M, et al. Ornidazole for prophylaxis of postoperative Crohn's disease recurrence: a randomized, double-blind, placebo-controlled trial. Gastroenterology. 2005;128(4):856–61. Epub 2005/04/13.
98. Arnold GL, Beaves MR, Pryjdun VO, Mook WJ. Preliminary study of ciprofloxacin in active Crohn's disease. Inflamm Bowel Dis. 2002;8(1):10–5. Epub 2002/02/12.
99. Yang SK, Jung Y, Hong M, Kim H, Ye BD, Lee I, et al. No association between TNFSF15 and IL23R with ulcerative colitis in Koreans. J Hum Genet. 2011;56(3):200–4. Epub 2011/01/14.
100. Silverberg MS, Duerr RH, Brant SR, Bromfield G, Datta LW, Jani N, et al. Refined genomic localization and ethnic differences observed for the IBD5 association with Crohn's disease. EJHG. 2007;15(3):328–35. Epub 2007/01/11.
101. Picornell Y, Mei L, Taylor K, Yang H, Targan SR, Rotter JI. TNFSF15 is an ethnic-specific IBD gene. Inflamm Bowel Dis. 2007;13(11):1333–8. Epub 2007/08/01.
102. Kenny EE, Pe'er I, Karban A, Ozelius L, Mitchell AA, Ng SM, et al. A genome-wide scan of Ashkenazi Jewish Crohn's disease suggests novel susceptibility loci. PLoS Genet. 2012;8(3):e1002559. Epub 2012/03/14.
103. Lees CW, Barrett JC, Parkes M, Satsangi J. New IBD genetics: common pathways with other diseases. Gut. 2011;60(12):1739–53. Epub 2011/02/09.

104. Muise AM, Walters TD, Glowacka WK, Griffiths AM, Ngan BY, Lan H, et al. Polymorphisms in E-cadherin (CDH1) result in a mis-localised cytoplasmic protein that is associated with Crohn's disease. Gut. 2009;58(8):1121–7. Epub 2009/04/29.
105. Barrett JC, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD, et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nat Genet. 2008;40(8):955–62. Epub 2008/07/01.
106. Rivas MA, Beaudoin M, Gardet A, Stevens C, Sharma Y, Zhang CK, et al. Deep resequencing of GWAS loci identifies independent rare variants associated with inflammatory bowel disease. Nat Genet. 2011;43(11):1066–73. Epub 2011/10/11.
107. Umeno J, Asano K, Matsushita T, Matsumoto T, Kiyohara Y, Iida M, et al. Meta-analysis of published studies identified eight additional common susceptibility loci for Crohn's disease and ulcerative colitis. Inflamm Bowel Dis. 2011;17(12):2407–15. Epub 2011/02/26.
108. Kaser A, Lee AH, Franke A, Glickman JN, Zeissig S, Tilg H, et al. XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. Cell. 2008;134(5):743–56. Epub 2008/09/09.
109. Spenle C, Hussenet T, Lacroute J, Lefebvre O, Kedinger M, Orend G, et al. Dysregulation of laminins in intestinal inflammation. Pathol Biol. 2012;60(1):41–7. Epub 2011/11/22.
110. Schmehl K, Florian S, Jacobasch G, Salomon A, Korber J. Deficiency of epithelial basement membrane laminin in ulcerative colitis affected human colonic mucosa. Int J Colorectal Dis. 2000;15(1):39–48. Epub 2000/04/15.
111. Bertin J, Wang L, Guo Y, Jacobson MD, Poyet JL, Srinivasula SM, et al. CARD11 and CARD14 are novel caspase recruitment domain (CARD)/membrane-associated guanylate kinase (MAGUK) family members that interact with BCL10 and activate NF-kappa B. J Biol Chem. 2001;276(15):11877–82. Epub 2001/03/30.
112. Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature. 2001;411(6837):599–603. Epub 2001/06/01.
113. Lesage S, Zouali H, Cezard JP, Colombel JF, Belaiche J, Almer S, et al. CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease. Am J Hum Genet. 2002;70(4):845–57. Epub 2002/03/05.
114. Consortium TWTCC. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature. 2007;447(7145):661–78. Epub 2007/06/08.
115. Wehkamp J, Harder J, Weichenthal M, Schwab M, Schaffeler E, Schlee M, et al. NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. Gut. 2004;53(11):1658–64. Epub 2004/10/14.
116. Kobayashi KS, Chamaillard M, Ogura Y, Henegariu O, Inohara N, Nunez G, et al. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. Science. 2005;307(5710):731–4. Epub 2005/02/05.
117. Biswas A, Liu YJ, Hao L, Mizoguchi A, Salzman NH, Bevins CL, et al. Induction and rescue of Nod2-dependent Th1-driven granulomatous inflammation of the ileum. Proc Natl Acad Sci U S A. 2010;107(33):14739–44. Epub 2010/08/04.
118. Saitoh T, Fujita N, Jang MH, Uematsu S, Yang BG, Satoh T, et al. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. Nature. 2008;456(7219):264–8. Epub 2008/10/14.
119. Travassos LH, Carneiro LA, Ramjeet M, Hussey S, Kim YG, Magalhaes JG, et al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. Nat Immunol. 2010;11(1):55–62. Epub 2009/11/10.
120. Wehkamp J, Salzman NH, Porter E, Nuding S, Weichenthal M, Petras RE, et al. Reduced Paneth cell alpha-defensins in ileal Crohn's disease. Proc Natl Acad Sci U S A. 2005;102(50):18129–34. Epub 2005/12/07.
121. Homer CR, Richmond AL, Rebert NA, Achkar JP, McDonald C. ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in Crohn's disease pathogenesis. Gastroenterology. 2010;139(5):1630–41. 41 e1-2. Epub 2010/07/20.

122. Glas J, Wagner J, Seiderer J, Olszak T, Wetzke M, Beigel F, et al. PTPN2 gene variants are associated with susceptibility to both Crohn's disease and ulcerative colitis supporting a common genetic disease background. PLoS ONE. 2012;7(3):e33682. Epub 2012/03/30.
123. Scharl M, Mwinyi J, Fischbeck A, Leucht K, Eloranta JJ, Arikkat J, et al. Crohn's disease-associated polymorphism within the PTPN2 gene affects muramyl-dipeptide-induced cytokine secretion and autophagy. Inflamm Bowel Dis. 2012;18(5):900–12. Epub 2011/10/25.
124. Parkes M, Barrett JC, Prescott NJ, Tremelling M, Anderson CA, Fisher SA, et al. Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. Nat Genet. 2007;39(7):830–2. Epub 2007/06/08.
125. McGovern DP, Gardet A, Torkvist L, Goyette P, Essers J, Taylor KD, et al. Genome-wide association identifies multiple ulcerative colitis susceptibility loci. Nat Genet. 2010;42(4):332–7. Epub 2010/03/17.
126. MacMicking JD, Taylor GA, McKinney JD. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. Science. 2003;302(5645):654–9. Epub 2003/10/25.
127. Wiede F, Shields BJ, Chew SH, Kyparissoudis K, van Vliet C, Galic S, et al. T cell protein tyrosine phosphatase attenuates T cell signaling to maintain tolerance in mice. J Clin Invest. 2011;121(12):4758–74. Epub 2011/11/15.
128. You-Ten KE, Muise ES, Itie A, Michaliszyn E, Wagner J, Jothy S, et al. Impaired bone marrow microenvironment and immune function in T cell protein tyrosine phosphatase-deficient mice. J Exp Med. 1997;186(5):683–93. Epub 1997/08/29.
129. Hassan SW, Doody KM, Hardy S, Uetani N, Cournoyer D, Tremblay ML. Increased susceptibility to dextran sulfate sodium induced colitis in the T cell protein tyrosine phosphatase heterozygous mouse. PLoS ONE. 2010;5(1):e8868. Epub 2010/01/30.
130. Murai M, Turovskaya O, Kim G, Madan R, Karp CL, Cheroutre H, et al. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. Nat Immunol. 2009;10(11):1178–84. Epub 2009/09/29.
131. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. Cell. 1993;75(2):263–74. Epub 1993/10/22.
132. Sellon RK, Tonkonogy S, Schultz M, Dieleman LA, Grenther W, Balish E, et al. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. Infect Immun. 1998;66(11):5224–31. Epub 1998/10/24.
133. Duerr RH, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly MJ, et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. Science. 2006;314(5804):1461–3. Epub 2006/10/28.
134. Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity. 2000;13(5):715–25. Epub 2000/12/15.
135. Barnich N, Darfeuille-Michaud A. Abnormal CEACAM6 expression in Crohn disease patients favors gut colonization and inflammation by adherent-invasive E. coli. Virulence. 2010;1(4):281–2. Epub 2010/12/24.
136. Carvalho FA, Barnich N, Sivignon A, Darcha C, Chan CH, Stanners CP, et al. Crohn's disease adherent-invasive Escherichia coli colonize and induce strong gut inflammation in transgenic mice expressing human CEACAM. J Exp Med. 2009;206(10):2179–89. Epub 2009/09/10.
137. Swidsinski A, Ladhoff A, Pernthaler A, Swidsinski S, Loening-Baucke V, Ortner M, et al. Mucosal flora in inflammatory bowel disease. Gastroenterology. 2002;122(1):44–54. Epub 2002/01/10.
138. Walker AW, Sanderson JD, Churcher C, Parkes GC, Hudspith BN, Rayment N, et al. High-throughput clone library analysis of the mucosa-associated microbiota reveals dysbiosis and differences between inflamed and non-inflamed regions of the intestine in inflammatory bowel disease. BMC Microbiol. 2011;11:7. Epub 2011/01/12.
139. Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, et al. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut. 2006;55(2):205–11. Epub 2005/09/29.

140. Ott SJ, Musfeldt M, Wenderoth DF, Hampe J, Brant O, Folsch UR, et al. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. Gut. 2004;53(5):685–93. Epub 2004/04/15.
141. Willing BP, Dicksved J, Halfvarson J, Andersson AF, Lucio M, Zheng Z, et al. A pyrosequencing study in twins shows that gastrointestinal microbial profiles vary with inflammatory bowel disease phenotypes. Gastroenterology. 2010;139(6):1844–54. e1. Epub 2010/09/08.
142. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci U S A. 2007;104(34):13780–5. Epub 2007/08/19.
143. Rehman A, Lepage P, Nolte A, Hellmig S, Schreiber S, Ott SJ. Transcriptional activity of the dominant gut mucosal microbiota in chronic inflammatory bowel disease patients. J Med Microbiol. 2010;59(Pt 9):1114–22. Epub 2010/06/05.
144. Lepage P, Hasler R, Spehlmann ME, Rehman A, Zvirbliene A, Begun A, et al. Twin study indicates loss of interaction between microbiota and mucosa of patients with ulcerative colitis. Gastroenterology. 2011;141(1):227–36. Epub 2011/05/31.
145. Willing B, Halfvarson J, Dicksved J, Rosenquist M, Jarnerot G, Engstrand L, et al. Twin studies reveal specific imbalances in the mucosa-associated microbiota of patients with ileal Crohn's disease. Inflamm Bowel Dis. 2009;15(5):653–60. Epub 2008/11/22.
146. Gophna U, Sommerfeld K, Gophna S, Doolittle WF, Veldhuyzen, van Zanten SJ. Differences between tissue-associated intestinal microfloras of patients with Crohn's disease and ulcerative colitis. J Clin Micro. 2006;44(11):4136–41. Epub 2006/09/22.
147. Baumgart M, Dogan B, Rishniw M, Weitzman G, Bosworth B, Yantiss R, et al. Culture independent analysis of ileal mucosa reveals a selective increase in invasive Escherichia coli of novel phylogeny relative to depletion of Clostridiales in Crohn's disease involving the ileum. ISME J. 2007;1(5):403–18. Epub 2007/11/29.
148. Mylonaki M, Rayment NB, Rampton DS, Hudspith BN, Brostoff J. Molecular characterization of rectal mucosa-associated bacterial flora in inflammatory bowel disease. Inflamm Bowel Dis. 2005;11(5):481–7. Epub 2005/05/04.
149. Fujimoto T, Imaeda H, Takahashi K, Kasumi E, Bamba S, Fujiyama Y, et al. Decreased abundance of Faecalibacterium prausnitzii in the gut microbiota of Crohn's disease. J Gastroenterol Hepatol. 2012. Epub 2012/12/12.
150. Sokol H, Seksik P, Furet JP, Firmesse O, Nion-Larmurier I, Beaugerie L, et al. Low counts of Faecalibacterium prausnitzii in colitis microbiota. Inflamm Bowel Dis. 2009;15(8):1183–9. Epub 2009/02/25.
151. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, Gratadoux JJ, et al. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A. 2008;105(43):16731–6. Epub 2008/10/22.
152. Martinez-Medina M, Aldeguer X, Gonzalez-Huix F, Acero D, Garcia-Gil LJ. Abnormal microbiota composition in the ileocolonic mucosa of Crohn's disease patients as revealed by polymerase chain reaction-denaturing gradient gel electrophoresis. Inflamm Bowel Dis. 2006;12(12):1136–45. Epub 2006/11/23.
153. Macfarlane S, Furrie E, Cummings JH, Macfarlane GT. Chemotaxonomic analysis of bacterial populations colonizing the rectal mucosa in patients with ulcerative colitis. Clin Infect Dis: Off Pub Infect Dis Soc Am. 2004;38(12):1690–9. Epub 2004/07/01.
154. Jalanka-Tuovinen J, Salonen A, Nikkila J, Immonen O, Kekkonen R, Lahti L, et al. Intestinal microbiota in healthy adults: temporal analysis reveals individual and common core and relation to intestinal symptoms. PLoS ONE. 2011;6(7):e23035. Epub 2011/08/11.
155. Loubinoux J, Bronowicki JP, Pereira IA, Mougenel JL, Faou AE. Sulfate-reducing bacteria in human feces and their association with inflammatory bowel diseases. FEMS Microbiol Ecol. 2002;40(2):107–12. Epub 2002/05/01.
156. Gibson GR, Macfarlane GT, Cummings JH. Sulphate reducing bacteria and hydrogen metabolism in the human large intestine. Gut. 1993;34(4):437–9. Epub 1993/04/01.

157. Pitcher MC, Beatty ER, Cummings JH. The contribution of sulphate reducing bacteria and 5-aminosalicylic acid to faecal sulphide in patients with ulcerative colitis. Gut. 2000;46(1):64–72. Epub 1999/12/22.
158. Muyzer G, Stams AJ. The ecology and biotechnology of sulphate-reducing bacteria. Nat Rev Microbiol. 2008;6(6):441–54. Epub 2008/05/08.
159. Roediger WE, Duncan A, Kapaniris O, Millard S. Reducing sulfur compounds of the colon impair colonocyte nutrition: implications for ulcerative colitis. Gastroenterology. 1993;104(3):802–9. Epub 1993/03/01.
160. Wu YC, Wang XJ, Yu L, Chan FK, Cheng AS, Yu J, et al. Hydrogen sulfide lowers proliferation and induces protective autophagy in colon epithelial cells. PLoS ONE. 2012;7(5):e37572. Epub 2012/06/09.
161. Laue H, Denger K, Cook AM. Taurine reduction in anaerobic respiration of Bilophila wadsworthia RZATAU. Appl Environ Microbiol. 1997;63(5):2016–21. Epub 1997/05/01.
162. Devkota S, Wang Y, Musch MW, Leone V, Fehlner-Peach H, Nadimpalli A, et al. Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10-/- mice. Nature. 2012;487(7405):104–8. Epub 2012/06/23.
163. Ishioka T, Kuwabara N, Oohashi Y, Wakabayashi K. Induction of colorectal tumors in rats by sulfated polysaccharides. Crit Rev Toxicol. 1987;17(3):215–44. Epub 1987/01/01.
164. Ohkusa T. Production of experimental ulcerative colitis in hamsters by dextran sulfate sodium and changes in intestinal microflora. Nihon Shokakibyo Gakkai zasshi: Japan J Gastroenterol. 1985;82(5):1327–36. Epub 1985/05/01.
165. Fava F, Danese S. Intestinal microbiota in inflammatory bowel disease: friend of foe? World J Gastroenterol: WJG. 2011;17(5):557–66. Epub 2011/02/26.
166. Nagalingam NA, Kao JY, Young VB. Microbial ecology of the murine gut associated with the development of dextran sodium sulfate-induced colitis. Inflamm Bowel Dis. 2011;17(4):917–26. Epub 2011/03/11.
167. Araki Y, Mukaisho K, Sugihara H, Fujiyama Y, Hattori T. Proteus mirabilis sp intestinal microflora grow in a dextran sulfate sodium-rich environment. Int J Mol Med. 2010;25(2):203–8. Epub 2010/01/01.
168. Bloom SM, Bijanki VN, Nava GM, Sun L, Malvin NP, Donermeyer DL, et al. Commensal Bacteroides species induce colitis in host-genotype-specific fashion in a mouse model of inflammatory bowel disease. Cell Host Microbe. 2011;9(5):390–403. Epub 2011/05/18.
169. Morgan XC, Tickle TL, Sokol H, Gevers D, Devaney KL, Ward DV, et al. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome Biol. 2012;13(9):R79. Epub 2012/09/28.
170. Stephens NS, Siffledeen J, Su X, Murdoch TB, Fedorak RN, Slupsky CM. Urinary NMR metabolomic profiles discriminate inflammatory bowel disease from healthy. J Crohns Colitis. 2013;7(2):e42–8. Epub 2012/05/26.
171. Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, et al. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host Microbe. 2007;2(2):119–29. Epub 2007/11/17.
172. Stecher B, Robbiani R, Walker AW, Westendorf AM, Barthel M, Kremer M, et al. Salmonella enterica serovar typhimurium exploits inflammation to compete with the intestinal microbiota. PLoS Biol. 2007;5(10):2177–89. Epub 2007/09/01.
173. Papa E, Docktor M, Smillie C, Weber S, Preheim SP, Gevers D, et al. Non-invasive mapping of the gastrointestinal microbiota identifies children with inflammatory bowel disease. PLoS ONE. 2012;7(6):e39242. Epub 2012/07/07.
174. Sokol H, Lepage P, Seksik P, Dore J, Marteau P. Temperature gradient gel electrophoresis of fecal 16S rRNA reveals active Escherichia coli in the microbiota of patients with ulcerative colitis. J Clin Microbiol. 2006;44(9):3172–7. Epub 2006/09/07.
175. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464(7285):59–65. Epub 2010/03/06.

176. Greenblum S, Turnbaugh PJ, Borenstein E. Metagenomic systems biology of the human gut microbiome reveals topological shifts associated with obesity and inflammatory bowel disease. Proc Natl Acad Sci U S A. 2012;109(2):594–9. Epub 2011/12/21.
177. Hansen JJ, Huang Y, Peterson DA, Goeser L, Fan TJ, Chang EB, et al. The colitis-associated transcriptional profile of commensal Bacteroides thetaiotaomicron enhances adaptive immune responses to a bacterial antigen. PLoS ONE. 2012;7(8):e42645. Epub 2012/08/11.
178. Patwa LG, Fan TJ, Tchaptchet S, Liu Y, Lussier YA, Sartor RB, et al. Chronic intestinal inflammation induces stress-response genes in commensal Escherichia coli. Gastroenterology. 2011;141(5):1842–51. e1-10. Epub 2011/07/06.
179. Verberkmoes NC, Russell AL, Shah M, Godzik A, Rosenquist M, Halfvarson J, et al. Shotgun metaproteomics of the human distal gut microbiota. ISME J. 2009;3(2):179–89. Epub 2008/10/31.
180. Kolmeder CA, de Been M, Nikkila J, Ritamo I, Matto J, Valmu L, et al. Comparative metaproteomics and diversity analysis of human intestinal microbiota testifies for its temporal stability and expression of core functions. PLoS ONE. 2012;7(1):e29913. Epub 2012/01/27.
181. Erickson AR, Cantarel BL, Lamendella R, Darzi Y, Mongodin EF, Pan C, et al. Integrated metagenomics/metaproteomics reveals human host-microbiota signatures of Crohn's disease. PLoS ONE. 2012;7(11):e49138. Epub 2012/12/05.
182. Jansson J, Willing B, Lucio M, Fekete A, Dicksved J, Halfvarson J, et al. Metabolomics reveals metabolic biomarkers of Crohn's disease. PLoS ONE. 2009;4(7):e6386. Epub 2009/07/29.
183. Marchesi JR, Holmes E, Khan F, Kochhar S, Scanlan P, Shanahan F, et al. Rapid and noninvasive metabonomic characterization of inflammatory bowel disease. J Proteome Res. 2007;6(2):546–51. Epub 2007/02/03.
184. Duboc H, Rainteau D, Rajca S, Humbert L, Farabos D, Maubert M, et al. Increase in fecal primary bile acids and dysbiosis in patients with diarrhea-predominant irritable bowel syndrome. Neurogastroenterol Motil: Off J Eur Gastrointestin Motility Soc. 2012;24(6):513–20. e246-7. Epub 2012/02/24.
185. Wohlgemuth S, Keller S, Kertscher R, Stadion M, Haller D, Kisling S, et al. Intestinal steroid profiles and microbiota composition in colitic mice. Gut Microbes. 2011;2(3):159–66. Epub 2011/08/27.
186. Islam KB, Fukiya S, Hagio M, Fujii N, Ishizuka S, Ooka T, et al. Bile acid is a host factor that regulates the composition of the cecal microbiota in rats. Gastroenterology. 2011;141(5):1773–81. Epub 2011/08/16.
187. Duboc H, Rajca S, Rainteau D, Benarous D, Maubert MA, Quervain E, et al. Connecting dysbiosis, bile-acid dysmetabolism and gut inflammation in inflammatory bowel diseases. Gut. 2012;40(18):9308–18. Epub 2012/09/21.
188. Stenman LK, Holma R, Korpela R. High-fat-induced intestinal permeability dysfunction associated with altered fecal bile acids. World J Gastroenterol: WJG. 2012;18(9):923–9. Epub 2012/03/13.
189. Suzuki T, Hara H. Dietary fat and bile juice, but not obesity, are responsible for the increase in small intestinal permeability induced through the suppression of tight junction protein expression in LETO and OLETF rats. Nutr Metab. 2010;7:19. Epub 2010/03/13.
190. Raimondi F, Santoro P, Barone MV, Pappacoda S, Barretta ML, Nanayakkara M, et al. Bile acids modulate tight junction structure and barrier function of Caco-2 monolayers via EGFR activation. Am J Physiol Gastrointest Liver Physiol. 2008;294(4):G906–13. Epub 2008/02/02.
191. Gadaleta RM, van Erpecum KJ, Oldenburg B, Willemsen EC, Renooij W, Murzilli S, et al. Farnesoid X receptor activation inhibits inflammation and preserves the intestinal barrier in inflammatory bowel disease. Gut. 2011;60(4):463–72. Epub 2011/01/19.
192. Takaishi H, Matsuki T, Nakazawa A, Takada T, Kado S, Asahara T, et al. Imbalance in intestinal microflora constitution could be involved in the pathogenesis of inflammatory bowel disease. IJMM. 2008;298(5–6):463–72. Epub 2007/09/28.
193. Baur P, Martin FP, Gruber L, Bosco N, Brahmbhatt V, Collino S, et al. Metabolic phenotyping of the Crohn's disease-like IBD etiopathology in the TNF(DeltaARE/WT) mouse model. J Proteome Res. 2011;10(12):5523–35. Epub 2011/10/28.

194. Fernandez-Banares F, Esteve-Comas M, Mane J, Navarro E, Bertran X, Cabre E, et al. Changes in mucosal fatty acid profile in inflammatorybowel disease and in experimental colitis: a common response to bowel inflammation. Clin Nutr. 1997;16(4):177–83. Epub 1997/08/01.
195. Bjerrum JT, Nielsen OH, Hao F, Tang H, Nicholson JK, Wang Y, et al. Metabonomics in ulcerative colitis: diagnostics, biomarker identification, and insight into the pathophysiology. J Proteome Res. 2010;9(2):954–62. Epub 2009/10/29.
196. Bezabeh T, Somorjai RL, Smith IC, Nikulin AE, Dolenko B, Bernstein CN. The use of 1H magnetic resonance spectroscopy in inflammatory bowel diseases: distinguishing ulcerative colitis from Crohn's disease. Am J Gastroenterol. 2001;96(2):442–8. Epub 2001/03/10.
197. Balasubramanian K, Kumar S, Singh RR, Sharma U, Ahuja V, Makharia GK, et al. Metabolism of the colonic mucosa in patients with inflammatory bowel diseases: an in vitro proton magnetic resonance spectroscopy study. Magn Reson Imaging. 2009;27(1):79–86. Epub 2008/07/05.
198. Holub BJ. The nutritional significance, metabolism, and function of myo-inositol and phosphatidylinositol in health and disease. Adv Nutr Res. 1982;4:107–41. Epub 1982/01/01.
199. Holub BJ. Metabolism and function of myo-inositol and inositol phospholipids. Annu Rev Nutr. 1986;6:563–97. Epub 1986/01/01.
200. Martin FP, Rezzi S, Philippe D, Tornier L, Messlik A, Holzlwimmer G, et al. Metabolic assessment of gradual development of moderate experimental colitis in IL-10 deficient mice. J Proteome Res. 2009;8(5):2376–87. Epub 2009/03/28.
201. Murdoch TB, Fu H, MacFarlane S, Sydora BC, Fedorak RN, Slupsky CM. Urinary metabolic profiles of inflammatory bowel disease in interleukin-10 gene-deficient mice. Anal Chem. 2008;80(14):5524–31. Epub 2008/06/19.
202. Williams HR, Cox IJ, Walker DG, North BV, Patel VM, Marshall SE, et al. Characterization of inflammatory bowel disease with urinary metabolic profiling. Am J Gastroenterol. 2009;104(6):1435–44. Epub 2009/06/06.
203. Winterkamp S, Weidenhiller M, Otte P, Stolper J, Schwab D, Hahn EG, et al. Urinary excretion of N-methylhistamine as a marker of disease activity in inflammatory bowel disease. Am J Gastroenterol. 2002;97(12):3071–7. Epub 2002/12/21.
204. Macfarlane S, Macfarlane GT, Cummings JH. Review article: prebiotics in the gastrointestinal tract. Aliment Pharmacol Ther. 2006;24(5):701–14. Epub 2006/08/22.
205. Langlands SJ, Hopkins MJ, Coleman N, Cummings JH. Prebiotic carbohydrates modify the mucosa associated microflora of the human large bowel. Gut. 2004;53(11):1610–6. Epub 2004/10/14.
206. Gibson GR, Beatty ER, Wang X, Cummings JH. Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. Gastroenterology. 1995;108(4):975–82. Epub 1995/04/01.
207. Gibson GR, Probert HM, Loo JV, Rastall RA, Roberfroid MB. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr Res Rev. 2004;17(2):259–75. Epub 2004/12/01.
208. Vulevic J, Drakoularakou A, Yaqoob P, Tzortzis G, Gibson GR. Modulation of the fecal microflora profile and immune function by a novel trans-galactooligosaccharide mixture (B-GOS) in healthy elderly volunteers. Am J Clin Nutr. 2008;88(5):1438–46. Epub 2008/11/11.
209. Depeint F, Tzortzis G, Vulevic J, I'Anson K, Gibson GR. Prebiotic evaluation of a novel galactooligosaccharide mixture produced by the enzymatic activity of Bifidobacterium bifidum NCIMB 41171, in healthy humans: a randomized, double-blind, crossover, placebo-controlled intervention study. Am J Clin Nutr. 2008;87(3):785–91. Epub 2008/03/11.
210. Hormannsperger G, Clavel T, Haller D. Gut matters: Microbe-host interactions in allergic diseases. J Allergy Clin Immunol. 2012;129(6):1452–9. Epub 2012/02/11.
211. Lindsay JO, Whelan K, Stagg AJ, Gobin P, Al-Hassi HO, Rayment N, et al. Clinical, microbiological, and immunological effects of fructo-oligosaccharide in patients with Crohn's disease. Gut. 2006;55(3):348–55. Epub 2005/09/16.

212. Welters CF, Heineman E, Thunnissen FB, van den Bogaard AE, Soeters PB, Baeten CG. Effect of dietary inulin supplementation on inflammation of pouch mucosa in patients with an ileal pouch-anal anastomosis. Dis Colon Rectum. 2002;45(5):621–7. Epub 2002/05/11.
213. Lara-Villoslada F, de Haro O, Camuesco D, Comalada M, Velasco J, Zarzuelo A, et al. Short-chain fructooligosaccharides, in spite of being fermented in the upper part of the large intestine, have anti-inflammatory activity in the TNBS model of colitis. Eur J Nutr. 2006;45(7):418–25. Epub 2006/07/28.
214. Cherbut C, Michel C, Lecannu G. The prebiotic characteristics of fructooligosaccharides are necessary for reduction of TNBS-induced colitis in rats. J Nutr. 2003;133(1):21–7. Epub 2003/01/07.
215. Camuesco D, Peran L, Comalada M, Nieto A, Di Stasi LC, Rodriguez-Cabezas ME, et al. Preventative effects of lactulose in the trinitrobenzenesulphonic acid model of rat colitis. Inflamm Bowel Dis. 2005;11(3):265–71. Epub 2005/03/01.
216. Rumi G, Tsubouchi R, Okayama M, Kato S, Mozsik G, Takeuchi K. Protective effect of lactulose on dextran sulfate sodium-induced colonic inflammation in rats. Dig Dis Sci. 2004;49(9):1466–72. Epub 2004/10/16.
217. Hoentjen F, Welling GW, Harmsen HJ, Zhang X, Snart J, Tannock GW, et al. Reduction of colitis by prebiotics in HLA-B27 transgenic rats is associated with microflora changes and immunomodulation. Inflamm Bowel Dis. 2005;11(11):977–85. Epub 2005/10/22.
218. Gopalakrishnan A, Clinthorne JF, Rondini EA, McCaskey SJ, Gurzell EA, Langohr IM, et al. Supplementation with galacto-oligosaccharides increases the percentage of NK cells and reduces colitis severity in Smad3-deficient mice. J Nutr. 2012;142(7):1336–42. Epub 2012/04/13.
219. Sang LX, Chang B, Zhang WL, Wu XM, Li XH, Jiang M. Remission induction and maintenance effect of probiotics on ulcerative colitis: a meta-analysis. World J Gastroenterol: WJG. 2010;16(15):1908–15. Epub 2010/04/17.
220. Mack DR, Ahrne S, Hyde L, Wei S, Hollingsworth MA. Extracellular MUC3 mucin secretion follows adherence of Lactobacillus strains to intestinal epithelial cells in vitro. Gut. 2003;52(6):827–33. Epub 2003/05/13.
221. Matsumoto S, Hara T, Hori T, Mitsuyama K, Nagaoka M, Tomiyasu N, et al. Probiotic Lactobacillus-induced improvement in murine chronic inflammatory bowel disease is associated with the down-regulation of pro-inflammatory cytokines in lamina propria mononuclear cells. Clin Exp Immunol. 2005;140(3):417–26. Epub 2005/06/04.
222. Liu Z, Zhang P, Ma Y, Chen H, Zhou Y, Zhang M, et al. Lactobacillus plantarum prevents the development of colitis in IL-10-deficient mouse by reducing the intestinal permeability. Mol Biol Rep. 2011;38(2):1353–61. Epub 2010/06/24.
223. Peluso I, Fina D, Caruso R, Stolfi C, Caprioli F, Fantini MC, et al. Lactobacillus paracasei subsp. paracasei B21060 suppresses human T-cell proliferation. Infect Immun. 2007;75(4):1730–7. Epub 2007/01/24.
224. Oliva S, Di Nardo G, Ferrari F, Mallardo S, Rossi P, Patrizi G, et al. Randomised clinical trial: the effectiveness of Lactobacillus reuteri ATCC 55730 rectal enema in children with active distal ulcerative colitis. Aliment Pharmacol Ther. 2012;35(3):327–34. Epub 2011/12/14.
225. Zocco MA, Dal Verme LZ, Cremonini F, Piscaglia AC, Nista EC, Candelli M, et al. Efficacy of Lactobacillus GG in maintaining remission of ulcerative colitis. Aliment Pharmacol Ther. 2006;23(11):1567–74. Epub 2006/05/16.
226. Dieleman LA, Goerres MS, Arends A, Sprengers D, Torrice C, Hoentjen F, et al. Lactobacillus GG prevents recurrence of colitis in HLA-B27 transgenic rats after antibiotic treatment. Gut. 2003;52(3):370–6. Epub 2003/02/14.
227. Osman N, Adawi D, Ahrne S, Jeppsson B, Molin G. Modulation of the effect of dextran sulfate sodium-induced acute colitis by the administration of different probiotic strains of Lactobacillus and Bifidobacterium. Dig Dis Sci. 2004;49(2):320–7. Epub 2004/04/24.
228. Schultz M, Veltkamp C, Dieleman LA, Grenther WB, Wyrick PB, Tonkonogy SL, et al. Lactobacillus plantarum 299V in the treatment and prevention of spontaneous colitis in interleukin-10-deficient mice. Inflamm Bowel Dis. 2002;8(2):71–80. Epub 2002/02/21.

229. Mileti E, Matteoli G, Iliev ID, Rescigno M. Comparison of the immunomodulatory properties of three probiotic strains of Lactobacilli using complex culture systems: prediction for in vivo efficacy. PLoS ONE. 2009;4(9):e7056. Epub 2009/09/17.
230. Steck N, Hoffmann M, Sava IG, Kim SC, Hahne H, Tonkonogy SL, et al. Enterococcus faecalis metalloprotease compromises epithelial barrier and contributes to intestinal inflammation. Gastroenterology. 2011;141(3):959–71. Epub 2011/06/28.
231. McCarthy J, O'Mahony L, O'Callaghan L, Sheil B, Vaughan EE, Fitzsimons N, et al. Double blind, placebo controlled trial of two probiotic strains in interleukin 10 knockout mice and mechanistic link with cytokine balance. Gut. 2003;52(7):975–80. Epub 2003/06/13.
232. Setoyama H, Imaoka A, Ishikawa H, Umesaki Y. Prevention of gut inflammation by Bifidobacterium in dextran sulfate-treated gnotobiotic mice associated with Bacteroides strains isolated from ulcerative colitis patients. Micro Infect/ Institut Pasteur. 2003;5(2):115–22. Epub 2003/03/26.
233. Kim N, Kunisawa J, Kweon MN, Eog Ji G, Kiyono H. Oral feeding of Bifidobacterium bifidum (BGN4) prevents CD4(+) CD45RB(high) T cell-mediated inflammatory bowel disease by inhibition of disordered T cell activation. Clin Immunol. 2007;123(1):30–9. Epub 2007/01/16.
234. Philippe D, Favre L, Foata F, Adolfsson O, Perruisseau-Carrier G, Vidal K, et al. Bifidobacterium lactis attenuates onset of inflammation in a murine model of colitis. World J Gastroenterol: WJG. 2011;17(4):459–69. Epub 2011/01/29.
235. Ishikawa H, Akedo I, Umesaki Y, Tanaka R, Imaoka A, Otani T. Randomized controlled trial of the effect of bifidobacteria-fermented milk on ulcerative colitis. J Am Coll Nutr. 2003;22(1):56–63. Epub 2003/02/06.
236. Kato K, Mizuno S, Umesaki Y, Ishii Y, Sugitani M, Imaoka A, et al. Randomized placebo-controlled trial assessing the effect of bifidobacteria-fermented milk on active ulcerative colitis. Aliment Pharmacol Ther. 2004;20(10):1133–41. Epub 2004/12/01.
237. Rembacken BJ, Snelling AM, Hawkey PM, Chalmers DM, Axon AT. Non-pathogenic Escherichia coli versus mesalazine for the treatment of ulcerative colitis: a randomised trial. Lancet. 1999;354(9179):635–9. Epub 1999/08/31.
238. Kruis W, Fric P, Pokrotnieks J, Lukas M, Fixa B, Kascak M, et al. Maintaining remission of ulcerative colitis with the probiotic Escherichia coli Nissle 1917 is as effective as with standard mesalazine. Gut. 2004;53(11):1617–23. Epub 2004/10/14.
239. Henker J, Muller S, Laass MW, Schreiner A, Schulze J. Probiotic Escherichia coli Nissle 1917 (EcN) for successful remission maintenance of ulcerative colitis in children and adolescents: an open-label pilot study. Z Gastroenterol. 2008;46(9):874–5. Epub 2008/09/24.
240. Ukena SN, Singh A, Dringenberg U, Engelhardt R, Seidler U, Hansen W, et al. Probiotic Escherichia coli Nissle 1917 inhibits leaky gut by enhancing mucosal integrity. PLoS ONE. 2007;2(12):e1308. Epub 2007/12/13.
241. Gotteland M, Cruchet S, Verbeke S. Effect of Lactobacillus ingestion on the gastrointestinal mucosal barrier alterations induced by indometacin in humans. Aliment Pharmacol Ther. 2001;15(1):11–7. Epub 2001/01/03.
242. Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, et al. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut. 2009;58(8):1091–103. Epub 2009/02/26.
243. Hummel S, Veltman K, Cichon C, Sonnenborn U, Schmidt MA. Differential targeting of the E-Cadherin/beta-Catenin complex by gram-positive probiotic lactobacilli improves epithelial barrier function. Appl Environ Microbiol. 2012;78(4):1140–7. Epub 2011/12/20.
244. Mimura T, Rizzello F, Helwig U, Poggioli G, Schreiber S, Talbot IC, et al. Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. Gut. 2004;53(1):108–14. Epub 2003/12/20.
245. Gionchetti P, Rizzello F, Venturi A, Brigidi P, Matteuzzi D, Bazzocchi G, et al. Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial. Gastroenterology. 2000;119(2):305–9. Epub 2000/08/10.

246. Bibiloni R, Fedorak RN, Tannock GW, Madsen KL, Gionchetti P, Campieri M, et al. VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. Am J Gastroenterol. 2005;100(7):1539–46. Epub 2005/06/30.
247. Sood A, Midha V, Makharia GK, Ahuja V, Singal D, Goswami P, et al. The probiotic preparation, VSL#3 induces remission in patients with mild-to-moderately active ulcerative colitis. Clin Gastroenterol Hepatol: Off Clin Prac J Am Gastroenterol Assoc. 2009;7(11):1202–9. 9 e1. Epub 2009/07/28.
248. Miele E, Pascarella F, Giannetti E, Quaglietta L, Baldassano RN, Staiano A. Effect of a probiotic preparation (VSL#3) on induction and maintenance of remission in children with ulcerative colitis. Am J Gastroenterol. 2009;104(2):437–43. Epub 2009/01/29.
249. Malchow HA. Crohn's disease and Escherichia coli. A new approach in therapy to maintain remission of colonic Crohn's disease? J Clin Gastroenterol. 1997;25(4):653–8.
250. Chermesh I, Tamir A, Reshef R, Chowers Y, Suissa A, Katz D, et al. Failure of Synbiotic 2000 to prevent postoperative recurrence of Crohn's disease. Dig Dis Sci. 2007;52(2):385–9. Epub 2007/01/11.
251. Rachmilewitz D, Katakura K, Karmeli F, Hayashi T, Reinus C, Rudensky B, et al. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. Gastroenterology. 2004;126(2):520–8. Epub 2004/02/06.
252. Jijon H, Backer J, Diaz H, Yeung H, Thiel D, McKaigney C, et al. DNA from probiotic bacteria modulates murine and human epithelial and immune function. Gastroenterology. 2004;126(5):1358–73. Epub 2004/05/08.
253. Hoermannsperger G, Clavel T, Hoffmann M, Reiff C, Kelly D, Loh G, et al. Post-translational inhibition of IP-10 secretion in IEC by probiotic bacteria: impact on chronic inflammation. PLoS ONE. 2009;4(2):e4365. Epub 2009/02/07.
254. von Schillde MA, Hormannsperger G, Weiher M, Alpert CA, Hahne H, Bauerl C, et al. Lactocepin secreted by Lactobacillus exerts anti-inflammatory effects by selectively degrading proinflammatory chemokines. Cell Host Microbe. 2012;11(4):387–96. Epub 2012/04/24.
255. Ishikawa H, Matsumoto S, Ohashi Y, Imaoka A, Setoyama H, Umesaki Y, et al. Beneficial effects of probiotic bifidobacterium and galacto-oligosaccharide in patients with ulcerative colitis: a randomized controlled study. Digestion. 2011;84(2):128–33. Epub 2011/04/29.
256. Furrie E, Macfarlane S, Kennedy A, Cummings JH, Walsh SV, O'Neil DA, et al. Synbiotic therapy (Bifidobacterium longum/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. Gut. 2005;54(2):242–9. Epub 2005/01/14.
257. Guslandi M. Treatment of irritable bowel syndrome with Saccharomyces boulardii. J Clin Gastroenterol. 2011;45(8):740–1. author reply 1-2. Epub 2011/05/11.
258. Guslandi M, Mezzi G, Sorghi M, Testoni PA. Saccharomyces boulardii in maintenance treatment of Crohn's disease. Dig Dis Sci. 2000;45(7):1462–4. Epub 2000/08/29.
259. Summers RW, Elliott DE, Urban Jr JF, Thompson RA, Weinstock JV. Trichuris suis therapy for active ulcerative colitis: a randomized controlled trial. Gastroenterology. 2005;128(4):825–32. Epub 2005/04/13.
260. Dalmasso G, Cottrez F, Imbert V, Lagadec P, Peyron JF, Rampal P, et al. Saccharomyces boulardii inhibits inflammatory bowel disease by trapping T cells in mesenteric lymph nodes. Gastroenterology. 2006;131(6):1812–25. Epub 2006/11/08.
261. Kullberg MC, Pearce EJ, Hieny SE, Sher A, Berzofsky JA. Infection with Schistosoma mansoni alters Th1/Th2 cytokine responses to a non-parasite antigen. J Immunol. 1992;148(10):3264–70. Epub 1992/05/15.
262. Pearlman E, Kazura JW, Hazlett Jr FE, Boom WH. Modulation of murine cytokine responses to mycobacterial antigens by helminth-induced T helper 2 cell responses. J Immunol. 1993;151(9):4857–64. Epub 1993/11/01.
263. Watanabe O, Ando T, Ishiguro K, Takahashi H, Ishikawa D, Miyake N, et al. Enteral nutrition decreases hospitalization rate in patients with Crohn's disease. J Gastroenterol Hepatol. 2010;25 Suppl 1:S134–7. Epub 2010/07/14.

264. Yamamoto T, Nakahigashi M, Saniabadi AR. Review article: diet and inflammatory bowel disease–epidemiology and treatment. Aliment Pharmacol Ther. 2009;30(2):99–112. Epub 2009/05/15.
265. Yamamoto T, Shiraki M, Nakahigashi M, Umegae S, Matsumoto K. Enteral nutrition to suppress postoperative Crohn's disease recurrence: a five-year prospective cohort study. Int J Colorectal Dis. 2012;65(10):866–73. Epub 2012/09/28.
266. Day AS, Whitten KE, Lemberg DA, Clarkson C, Vitug-Sales M, Jackson R, et al. Exclusive enteral feeding as primary therapy for Crohn's disease in Australian children and adolescents: a feasible and effective approach. J Gastroenterol Hepatol. 2006;21(10):1609–14. Epub 2006/08/25.
267. Leach ST, Mitchell HM, Eng WR, Zhang L, Day AS. Sustained modulation of intestinal bacteria by exclusive enteral nutrition used to treat children with Crohn's disease. Aliment Pharmacol Ther. 2008;28(6):724–33. Epub 2009/01/16.
268. Hunter JO. Is diet a factor in the pathogenesis of IBD? Inflamm Bowel Dis. 2008;14 Suppl 2:S35–6. Epub 2008/09/26.
269. Kajiura T, Takeda T, Sakata S, Sakamoto M, Hashimoto M, Suzuki H, et al. Change of intestinal microbiota with elemental diet and its impact on therapeutic effects in a murine model of chronic colitis. Dig Dis Sci. 2009;54(9):1892–900. Epub 2008/12/06.
270. Pereira SP, Cassell TB, Engelman JL, Sladen GE, Murphy GM, Dowling RH. Plasma arachidonic acid-rich phospholipids in Crohn's disease: response to treatment. Clin Sci (Lond). 1996;91(4):509–12. Epub 1996/10/01.
271. Zoli G, Care M, Parazza M, Spano C, Biagi PL, Bernardi M, et al. A randomized controlled study comparing elemental diet and steroid treatment in Crohn's disease. Aliment Pharmacol Ther. 1997;11(4):735–40. Epub 1997/08/01.
272. Hanai H, Iida T, Takeuchi K, Arai H, Arai O, Abe J, et al. Nutritional therapy versus 6-mercaptopurine as maintenance therapy in patients with Crohn's disease. Digest Liver Dis: Off J Ital Soc Gastroenterol Ital Assoc Stud Liver. 2012;44(8):649–54. Epub 2012/05/01.
273. Gorard DA, Hunt JB, Payne-James JJ, Palmer KR, Rees RG, Clark ML, et al. Initial response and subsequent course of Crohn's disease treated with elemental diet or prednisolone. Gut. 1993;34(9):1198–202. Epub 1993/09/01.
274. Zachos M, Tondeur M, Griffiths AM. Enteral nutritional therapy for induction of remission in Crohn's disease. Cochrane Database Syst Rev. 2007(1):CD000542. Epub 2007/01/27.
275. Wagner SJ, Schmidt A, Effenberger MJ, Gruber L, Danier J, Haller D. Semisynthetic diet ameliorates Crohn's disease-like ileitis in TNFDeltaARE/WT mice through antigen-independent mechanisms of gluten. Inflamm Bowel Dis. 2013;19(6):1285–94. Epub 2013/04/10.
276. Newnham ED. Does gluten cause gastrointestinal symptoms in subjects without coeliac disease? J Gastroenterol Hepatol. 2011;26 Suppl 3:132–4. Epub 2011/04/02.
277. Esaki M, Matsumoto T, Nakamura S, Yada S, Fujisawa K, Jo Y, et al. Factors affecting recurrence in patients with Crohn's disease under nutritional therapy. Dis Colon Rectum. 2006;49(10 Suppl):S68–74. Epub 2006/11/16.
278. Gentschew L, Ferguson LR. Role of nutrition and microbiota in susceptibility to inflammatory bowel diseases. Mol Nutr Food Res. 2012;56(4):524–35. Epub 2012/04/13.
279. Gruber L, Lichti P, Rath E, Haller D. Nutrigenomics and nutrigenetics in inflammatory bowel diseases. J Clin Gastroenterol. 2012;46(9):735–47. Epub 2012/09/04.
280. Hou JK, Abraham B, El-Serag H. Dietary intake and risk of developing inflammatory bowel disease: a systematic review of the literature. Am J Gastroenterol. 2011;106(4):563–73. Epub 2011/04/07.
281. Miller B, Fervers F, Rohbeck R, Strohmeyer G. [Sugar consumption in patients with Crohn's disease]. Verhandlungen der Deutschen Gesellschaft fur Innere Medizin. 1976;82 Pt 1:922–4. Epub 1976/01/01. Zuckerkonsum bei Patienten mit Morbus Crohn.
282. Mayberry JF, Rhodes J, Newcombe RG. Increased sugar consumption in Crohn's disease. Digestion. 1980;20(5):323–6. Epub 1980/01/01.
283. Sonnenberg A. Geographic and temporal variations of sugar and margarine consumption in relation to Crohn's disease. Digestion. 1988;41(3):161–71. Epub 1988/01/01.

284. Shoda R, Matsueda K, Yamato S, Umeda N. Epidemiologic analysis of Crohn disease in Japan: increased dietary intake of n-6 polyunsaturated fatty acids and animal protein relates to the increased incidence of Crohn disease in Japan. Am J Clin Nutr. 1996;63(5):741–5. Epub 1996/05/01.
285. Chan SS, Luben R, Olsen A, Tjonneland A, Kaaks R, Teucher B, et al. Body mass index and the risk for Crohn's disease and ulcerative colitis: data from a European prospective cohort study (The IBD in EPIC Study). Am J Gastroenterol. 2013;108(4):575–82. Epub 2013/01/16.
286. Teixeira LG, Leonel AJ, Aguilar EC, Batista NV, Alves AC, Coimbra CC, et al. The combination of high-fat diet-induced obesity and chronic ulcerative colitis reciprocally exacerbates adipose tissue and colon inflammation. Lipids Health Dis. 2011;10:204. Epub 2011/11/15.
287. Kim KA, Gu W, Lee IA, Joh EH, Kim DH. High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway. PLoS ONE. 2012;7(10):e47713. Epub 2012/10/24.
288. Gruber L, Kisling S, Lichti P, Martin FP, May S, Klingenspor M, et al. High fat diet accelerates pathogenesis of murine Crohn's disease-like ileitis independently of obesity. PLoS ONE. 2013;8(8):e71661. Epub 2013/08/27.
289. Ding S, Chi MM, Scull BP, Rigby R, Schwerbrock NM, Magness S, et al. High-fat diet: bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse. PLoS ONE. 2010;5(8):e12191. Epub 2010/09/03.
290. Erdelyi I, Levenkova N, Lin EY, Pinto JT, Lipkin M, Quimby FW, et al. Western-style diets induce oxidative stress and dysregulate immune responses in the colon in a mouse model of sporadic colon cancer. J Nutr. 2009;139(11):2072–8. Epub 2009/09/18.
291. Lam YY, Ha CW, Campbell CR, Mitchell AJ, Dinudom A, Oscarsson J, et al. Increased gut permeability and microbiota change associate with mesenteric fat inflammation and metabolic dysfunction in diet-induced obese mice. PLoS ONE. 2012;7(3):e34233. Epub 2012/03/30.
292. Hildebrandt MA, Hoffmann C, Sherrill-Mix SA, Keilbaugh SA, Hamady M, Chen YY, et al. High-fat diet determines the composition of the murine gut microbiome independently of obesity. Gastroenterology. 2009;137(5):1716–24. e1-2. Epub 2009/08/27.
293. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014;505(7484):559–63. Epub 2013/12/18.
294. Werner T, Wagner SJ, Martinez I, Walter J, Chang JS, Clavel T, et al. Depletion of luminal iron alters the gut microbiota and prevents Crohn's disease-like ileitis. Gut. 2011;60(3):325–33. Epub 2010/11/16.
295. Den Hond E, Hiele M, Evenepoel P, Peeters M, Ghoos Y, Rutgeerts P. In vivo butyrate metabolism and colonic permeability in extensive ulcerative colitis. Gastroenterology. 1998;115(3):584–90. Epub 1998/08/28.
296. Roediger WE. The colonic epithelium in ulcerative colitis: an energy-deficiency disease? Lancet. 1980;2(8197):712–5. Epub 1980/10/04.
297. Ahmad MS, Krishnan S, Ramakrishna BS, Mathan M, Pulimood AB, Murthy SN. Butyrate and glucose metabolism by colonocytes in experimental colitis in mice. Gut. 2000;46(4):493–9. Epub 2000/03/15.
298. Ritzhaupt A, Wood IS, Ellis A, Hosie KB, Shirazi-Beechey SP. Identification and characterization of a monocarboxylate transporter (MCT1) in pig and human colon: its potential to transport L-lactate as well as butyrate. J Physiol. 1998;513(Pt 3):719–32. Epub 1998/11/24.
299. Thibault R, De Coppet P, Daly K, Bourreille A, Cuff M, Bonnet C, et al. Down-regulation of the monocarboxylate transporter 1 is involved in butyrate deficiency during intestinal inflammation. Gastroenterology. 2007;133(6):1916–27. Epub 2007/12/07.
300. Annese V, Valvano MR, Palmieri O, Latiano A, Bossa F, Andriulli A. Multidrug resistance 1 gene in inflammatory bowel disease: a meta-analysis. World J Gastroenterol: WJG. 2006;12(23):3636–44. Epub 2006/06/15.
301. Onnie CM, Fisher SA, Pattni R, Sanderson J, Forbes A, Lewis CM, et al. Associations of allelic variants of the multidrug resistance gene (ABCB1 or MDR1) and inflammatory bowel

disease and their effects on disease behavior: a case-control and meta-analysis study. Inflamm Bowel Dis. 2006;12(4):263–71. Epub 2006/04/25.
302. Annese V, Rogai F, Settesoldi A, Bagnoli S. PPARgamma in inflammatory bowel disease. PPAR Res. 2012;2012:620839. Epub 2012/09/22.
303. Simmons JD, Mullighan C, Welsh KI, Jewell DP. Vitamin D receptor gene polymorphism: association with Crohn's disease susceptibility. Gut. 2000;47(2):211–4. Epub 2000/07/18.
304. Wu GD. Is there a role for PPAR gamma in IBD? Yes, no, maybe. Gastroenterology. 2003;124(5):1538–42. Epub 2003/05/06.
305. Zucchelli M, Torkvist L, Bresso F, Halfvarson J, Hellquist A, Anedda F, et al. PepT1 oligopeptide transporter (SLC15A1) gene polymorphism in inflammatory bowel disease. Inflamm Bowel Dis. 2009;15(10):1562–9. Epub 2009/05/23.
306. Dalmasso G, Nguyen HT, Ingersoll SA, Ayyadurai S, Laroui H, Charania MA, et al. The PepT1-NOD2 signaling pathway aggravates induced colitis in mice. Gastroenterology. 2011;141(4):1334–45. Epub 2011/07/19.
307. Merlin D, Si-Tahar M, Sitaraman SV, Eastburn K, Williams I, Liu X, et al. Colonic epithelial hPepT1 expression occurs in inflammatory bowel disease: transport of bacterial peptides influences expression of MHC class 1 molecules. Gastroenterology. 2001;120(7):1666–79. Epub 2001/05/29.
308. van Nood E, Vrieze A, Nieuwdorp M, Fuentes S, Zoetendal EG, de Vos WM, et al. Duodenal infusion of donor feces for recurrent Clostridium difficile. N Engl J Med. 2013;368(5):407–15. Epub 2013/01/18.
309. Anderson JL, Edney RJ, Whelan K. Systematic review: faecal microbiota transplantation in the management of inflammatory bowel disease. Aliment Pharmacol Ther. 2012;36(6):503–16. Epub 2012/07/26.
310. Damman CJ, Miller SI, Surawicz CM, Zisman TL. The microbiome and inflammatory bowel disease: is there a therapeutic role for fecal microbiota transplantation? Am J Gastroenterol. 2012;107(10):1452–9. Epub 2012/10/05.
311. Kunde S, Pham A, Bonczyk S, Crumb T, Duba M, Conrad Jr H, et al. Safety, tolerability, and clinical response after fecal transplantation in children and young adults with ulcerative colitis. J Pediatr Gastroenterol Nutr. 2013;56(6):597–601. Epub 2013/04/02.
312. Angelberger S, Reinisch W, Makristathis A, Lichtenberger C, Dejaco C, Papay P, et al. Temporal bacterial community dynamics vary among ulcerative colitis patients after fecal microbiota transplantation. Am J Gastroenterol. 2013;108(10):1620–30. Epub 2013/09/26.
313. Balish E, Warner T. Enterococcus faecalis induces inflammatory bowel disease in interleukin-10 knockout mice. Am J Pathol. 2002;160(6):2253–7. Epub 2002/06/12.
314. Madsen KL, Doyle JS, Tavernini MM, Jewell LD, Rennie RP, Fedorak RN. Antibiotic therapy attenuates colitis in interleukin 10 gene-deficient mice. Gastroenterology. 2000;118(6):1094–105. Epub 2000/06/02.
315. Hoentjen F, Harmsen HJ, Braat H, Torrice CD, Mann BA, Sartor RB, et al. Antibiotics with a selective aerobic or anaerobic spectrum have different therapeutic activities in various regions of the colon in interleukin 10 gene deficient mice. Gut. 2003;52(12):1721–7. Epub 2003/11/25.
316. Tamagawa H, Hiroi T, Mizushima T, Ito T, Matsuda H, Kiyono H. Therapeutic effects of roxithromycin in interleukin-10-deficient colitis. Inflamm Bowel Dis. 2007;13(5):547–56. Epub 2007/01/25.
317. Schultz M, Tonkonogy SL, Sellon RK, Veltkamp C, Godfrey VL, Kwon J, et al. IL-2-deficient mice raised under germfree conditions develop delayed mild focal intestinal inflammation. Am J Physiol. 1999;276(6 Pt 1):G1461–72. Epub 1999/06/11.
318. Taurog JD, Richardson JA, Croft JT, Simmons WA, Zhou M, Fernandez-Sueiro JL, et al. The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats. J Exp Med. 1994;180(6):2359–64. Epub 1994/12/01.
319. Dianda L, Hanby AM, Wright NA, Sebesteny A, Hayday AC, Owen MJ. T cell receptor-alpha beta-deficient mice fail to develop colitis in the absence of a microbial environment. Am J Pathol. 1997;150(1):91–7. Epub 1997/01/01.

320. Bamias G, Okazawa A, Rivera-Nieves J, Arseneau KO, De La Rue SA, Pizarro TT, et al. Commensal bacteria exacerbate intestinal inflammation but are not essential for the development of murine ileitis. J Immunol. 2007;178(3):1809–18. Epub 2007/01/24.
321. Bamias G, Marini M, Moskaluk CA, Odashima M, Ross WG, Rivera-Nieves J, et al. Down-regulation of intestinal lymphocyte activation and Th1 cytokine production by antibiotic therapy in a murine model of Crohn's disease. J Immunol. 2002;169(9):5308–14. Epub 2002/10/23.
322. Habtezion A, Toivola DM, Butcher EC, Omary MB. Keratin-8-deficient mice develop chronic spontaneous Th2 colitis amenable to antibiotic treatment. J Cell Sci. 2005;118(Pt 9):1971–80. Epub 2005/04/21.
323. Panwala CM, Jones JC, Viney JL. A novel model of inflammatory bowel disease: mice deficient for the multiple drug resistance gene, mdr1a, spontaneously develop colitis. J Immunol. 1998;161(10):5733–44. Epub 1998/11/20.
324. Kang SS, Bloom SM, Norian LA, Geske MJ, Flavell RA, Stappenbeck TS, et al. An antibiotic-responsive mouse model of fulminant ulcerative colitis. PLoS Med. 2008;5(3):e41. Epub 2008/03/06.
325. Deng L, Zhou JF, Sellers RS, Li JF, Nguyen AV, Wang Y, et al. A novel mouse model of inflammatory bowel disease links mammalian target of rapamycin-dependent hyperproliferation of colonic epithelium to inflammation-associated tumorigenesis. Am J Pathol. 2010;176(2):952–67. Epub 2010/01/01.
326. Morrissey PJ, Charrier K. Induction of wasting disease in SCID mice by the transfer of normal CD4+/CD45RBhi T cells and the regulation of this autoreactivity by CD4+/CD45RBlo T cells. Res Immunol. 1994;145(5):357–62. Epub 1994/06/01.

Chapter 14
Deciphering the Gut Microbial Contribution to the Etiology of Autism Development

Ivan K.S. Yap and François-Pierre Martin

Abstract Autistic spectrum disorder (ASD) is a spectrum of early-onset lifelong neurodevelopmental disorders that severely impact social and behavioral functioning. It is a debilitating disorder that affects 1 % of the global children population with increasing prevalence and presented huge economic burden to the family and the nation. Current diagnosis for ASD is very subjective mainly because of the multifactorial nature of the disorders. The etiology of ASD is highly complex and multifaceted involving the gene, environment, and diet and is associated with various abnormalities that include immunologic, metabolic, and, more recently, the host–gut microbiome stability (Fig. 14.1). The gut microbiota is a consortium of bacteria that coexisted and coevolved with the host from the time of birth. As such the gut microbiome–mammalian "superorganism" represents a level of biological evolutionary development where true symbiosis is characterized by extensive "transgenomic" modulation of metabolism and functions between the two entities. The gut microbiota is involved in various mammalian biological processes including defense against pathogens, immunity, intestinal microvilli development, and recovery of metabolic energy through fermentation of otherwise nondigestible dietary fiber. In addition, the gut microbiota has been shown to communicate with the brain via the gut–brain axis to modulate brain development, function, and behavior. Recent evidence indicated that the gut microbiota influenced central nervous system development and responses to stress. Current understanding on the potential and extend of gut microbe involvement in brain development and host metabolic signaling is still in its infancy. Coupled with ever-increasing awareness on the importance of the gut microbiome in health and disease particularly autism, understanding the fundamental mechanistic interaction between host brain development and gut microbiota is crucial for unraveling the mystery behind the etiopathology of autism.

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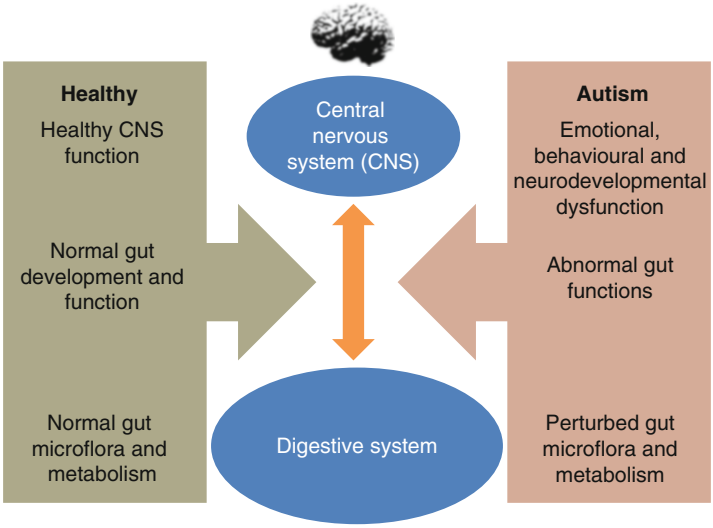

Fig. 14.1 Scheme summarizing the main factors related to gut–brain axis associated with brain development and autism

Keywords Autism • Disease • Gut–brain axis • Gut microbiota • Health • Mass spectrometry • Metabonomics • Metagenomics • Nuclear magnetic resonance spectroscopy • Nutrition • Systems biology

14.1 Gut–Brain Axis in Health and Disease

The term "gut–brain" or "brain–gut" axis is increasingly employed to define a bidirectional neurohumoral communication system. It comprised of neural pathways and humoral pathways, which include cytokines, hormones, and neuropeptides as signaling molecules [1]. The brain–gut–enteric microbiota axis includes the central nervous system, the neuroendocrine and neuroimmune systems, the sympathetic and parasympathetic arms of the autonomic nervous system, the enteric nervous system, and the intestinal microbiota [2]. Through this bidirectional communication network, signals from the brain can influence the motor, sensory, and secretory modalities of the GIT, and reciprocally, visceral messages from the GIT can influence brain function [2]. Recent evidences showed that gut microbiota communicates with the brain via the gut–brain axis to modulate brain development and behavioral phenotypes. In particular, this system provides the intestinal microbiota and its metabolites with a potential route towards the brain. Consequently, the gut microbiota could associate with brain functions as well as neurological diseases via the gut–brain axis [3], and further insights would require a better characterization of the composition and the metabolic activities of the gut microbiota. However,

identification of microbes constituting gut microbiota has been the main technological challenge currently due to massive amount of intestinal microbes and the difficulties in culture of gut microbes [3]. In parallel, many challenges remain to better assess, understand, and modulate gut microbial metabolic activities and their influence at panorganismal scale [4].

If recent studies have highlighted the depth of microbiota influence on the development and function of the host brain, one of the first observations resulted from the beneficial impact of orally administered antibiotics in reversing encephalopathy in patients with decompensated liver disease [5]. In addition, accumulating evidence further describes a relationship between psychiatric and gastrointestinal tract (GIT) disorders, such as irritable bowel syndrome (IBS) or inflammatory bowel disease (IBD), that are also associated with disturbances of the intestinal microbiota [6]. As highlighted in Chap. 14 of this book, there is compelling evidence that the brain may influence gastrointestinal functions (such as motility, secretion, and mucin production) as well as immune functions, and therefore being a direct vehicle for mediating the effects of emotional factors such as stress or depression influence [7–10].

These GIT disorders are also intimately related with gut dysbiosis [11], illustrating the potential brain influences on the microbial composition and activity along the GIT and reciprocally for the microbiota to modulate host metabolism [1]. Moreover, the gut microbiota, the intestinal mucosa, and the intestinal immune system issue multiple signals from the gut to the brain carried by sensory neurons, immune mediators, gut hormones, and microbiota-derived signaling molecules [12]. For instance, the influence of the gut microbiota on the development of the central nervous systems and stress responses was recently documented [13]. In this, two specific interactive systems are being highlighted, namely, the hypothalamus–pituitary–adrenal axis and the vagus nerve, as important means of communicating signals from gut microbes to the central nervous systems. Furthermore, recent efforts focused on the members of the neuropeptide Y (NPY) family of biologically active peptides, NPY, peptide YY (PYY), and pancreatic polypeptide (PP) [12]. PYY and PP are exclusively expressed by endocrine cells of the digestive system, whereas NPY is found at all levels of the gut–brain and brain–gut axis. Recent studies have extensively described how PYY is influenced by the intestinal microbiota, with particular interest in appetite regulation in the context of obesity pandemic. Due to its multilevel homeostatic mechanism, pharmacological manipulation of NPY–Y receptor system may have considerable therapeutic efficacy in many common metabolic and GIT disease in addition to psychiatric disorders.

Several lessons learned so far are mainly based and limited to preclinical studies [7], especially using gnotobiotic and germfree animal models. Such systems models have enabled rediscovering the multiple and complex facet of the interaction with the gut microbiota at multi-compartmental levels [4, 14–16]. The influence of the gut microbiota on the nervous system, brain development, and behavior, in particular during microbial colonization of the host, has recently been receiving profound interest [17]. In particular, the metabolic modulation of metabolites influencing functions of the nervous system, such as tryptophan and kynurenine levels, further illustrates the functional microbiota–neurohumoral relationship during gut colonization.

Moreover, novel evidence describes how gut microbiota type and presence can impact the cerebral biochemical profiles [18], including cerebral glycolytic metabolism.

14.2 Brain–Gut–Microbe Communication in Health and Disease

Autistic spectrum disorder (ASD) is a spectrum of neurological disorders characterized by a complex lifelong neurodevelopmental and sociological disorder with poorly defined etiology. ASDs are associated with an array of disabilities such as social withdrawal, speech impairment, and repetitive behavior [19] (Fig. 14.1). According to a recent estimate from the Centers for Disease Control and Prevention (CDC)'s Autism and Developmental Disabilities Monitoring (ADDM) Network, about 1 in 88 children, from the 14 communities of the network within the United States, have been identified with an ASD [20]. A recent global estimate by Elsabbagh *and coworkers* puts the global prevalence estimate to be about 62 in 10,000 [21] or 1 % of the global children population had an ASD [20]. More alarmingly, there has been a significant increase in incidence of ASD worldwide, and within the United States, between 2002 and 2008, there has been a 57 % increase in incidence of ASD [20]. ASD places a large economic burden on society with the cost of the disorder on the UK economy estimated to be £2.7 billion [22]. The lifetime cost for someone with ASD and intellectual disability is estimated at approximately £1.23 million and for someone with ASD without intellectual disability is approximately £0.80 million [22]. It has been reported that the ASDs affect all racial, ethnic, and socioeconomic group and that boys are five times more likely to have ASDs as compared to girls [20]. Studies have shown that among identical twins, if one child has an ASD, then the other will be affected about 36–95 % of the time. In nonidentical twins, if one child has an ASD, then the other is affected about 0–31 % of the time [23–25]. Parents who have a child with an ASD have a 2–18 % chance of having a second child who is also affected [26, 27]. ASDs tend to occur more often in people who have certain genetic or chromosomal conditions. About 10 % of children with autism are also identified as having Down syndrome, fragile X syndrome, tuberous sclerosis, and other genetic and chromosomal disorders [28–31]. A number of known disorders such as phenylketonuria and Smith–Lemli–Opitz syndrome have been shown to be associated with the ASD behavioral traits in children. These metabolic disorders are mainly autosomal recessive genetic disorders that present within the first 3 years of life. Current diagnosis for ASD is through a set of criteria defined in the Diagnostic and Statistical Manual of Mental Disorders 4th Edition (DSM-IV-TR) and behavioral observations made by clinician thus making diagnosis very subjective. The ongoing controversy about the precise definition of ASD stems from the current lack of understanding of the underlying causes of ASD and its multifactorial nature.

14.3 Gut Microbiota and ASD

Gastrointestinal dysfunction has been reported in ASD children [32], and studies have suggested that the condition may be associated with abnormal gut microbiota. Given the importance of the microbiome in mammalian metabolism, e.g., bile acid metabolism, there is a possibility of previously unrecognized etiologic connections between microbiome disorders and childhood developmental problems. Individuals with ASD are commonly exposed to repeated courses of multiple antibiotic therapies, and this may contribute to the complex relationships between gastrointestinal symbiosis and ASD by altering the composition or stability of their gut microbiota.

One of the very first suggestions of the potential involvement of bacteria in ASD was the publication by Bolte et al. where it was hypothesized that ASD may be linked to low-grade intestinal infection with *Clostridium tetani* [33]. It is well known that majority of children with ASD undergo extensive antibiotic therapy. Oral antibiotics can disrupt the stability and integrity of the "normal" gut microbiota thus resulting in an environment for opportunistic pathogens to colonize. One of this opportunistic pathogen is *C. tetani. Clostridium* belongs to the phylum of *Firmicutes*. They are rod-shaped obligate anaerobes that produce endospores [34]. Some of the most important biological pathogens belong to this genus of bacteria, namely, *C. botulinum*, *C. difficile*, and *C. tetani*, associated with botulism, antibiotic-associated diarrhea, and tetanus, respectively. *Clostridium* species such as *C. botulinum* and *C. tetani* are known to produce neurotoxins, which trigger the very clinical pathological manifestation that they are associated with. It has been shown that toxin produced by *C. tetani* in the intestine of experimental animals can be transported to the central nervous system via the vagus nerve resulting in the disruption of neurotransmitters release [35, 36]. It was suggested that such inhibition may lead to the myriads of behavioral deficits observed in children with ASD.

Sandler and colleagues conducted a small cohort study of oral vancomycin in autistic children which subsequently proved the hypotheses [37]. It was reported in the study that children receiving vancomycin treatment showed improvement in gastrointestinal problems such as abdominal pain, constipation, and/or diarrhea. In addition, behavioral improvements were observed with significant reduction in aggressive behavior, increased eye contact, and significant improvement in language and speech. However, such changes were dependent on vancomycin treatment and all children relapsed after discontinuation of the antibiotic. The work by Sandler et al. presented one of the first clear scientific evidences on the link between gut–brain axis and ASD. Several studies had subsequently indicated that children with ASD have perturbed gut microbiota as compared to typically developing children. To investigate further the potential involvement of *Clostridium* species (spp.) in ASD, Finegold et al. studied the feces of children with ASD and compared with healthy controls. It was found that children with ASD have higher levels of *Clostridium* spp. as well as greater species variation [38]. A subsequent study by the same group showed that children with ASD have elevated C. *bolteae* and *Clostridium*

clusters I and XI [39]. Around the same time, a study conducted by Parracho et al. comparing feces of children with ASD versus healthy siblings and healthy unrelated controls showed that children with ASD has higher levels of certain *Clostridium* spp. and, more interestingly, the healthy siblings had an intermediate level between their siblings with ASD and those of the healthy unrelated controls [40]. Further, a recent pyrosequencing study on fecal microbiota composition between typically developing controls versus ASD children and their normal functioning siblings showed that children with ASD had higher level of *Bacteroidetes* and lower level of *Firmicutes* as compared to the controls [41]. In addition, children with ASD also had lower level of several *Bifidobacterium* species while *Desulfovibrio* was higher [32, 41]. A study by Wang et al. also showed lower level of *Bifidobacterium* and *Akkermansia muciniphila* in ASD children [42].

In fact, the study by Finegold et al. showed that *Desulfovibrio* was present in half of the autistic subjects and in some siblings. More interestingly, none of the control subjects had *Desulfovibrio* [32]. Children with ASD are known to be sulfur deficient. Aldred et al. showed that individuals with autism have lower levels of plasma sulfate but considerably elevated levels of urinary sulfate as compared to normal individuals [43, 44]. These data suggest that autistic individuals may have impaired detoxification potential involving sulfation as evidenced by their inability to sulfate acetaminophen [44]. The presence of sulfate-reducing bacteria such as *Desulfovibrio* in ASD children could be one of the reasons behind the abnormality observed in sulfur metabolism. Moreover, the severity of ASD behavior is positively correlated with increased *Desulfovibrio* species [32].

14.4 Metabonomics in ASD

Finding the cause of ASD has proved challenging due to the multifactorial nature of the disorder, which also means that finding biochemical markers for ASD has remained elusive thus far. However, successful discovery of a set of specific and accurate biomarkers for ASD would not only help in understanding the pathophysiology of the condition but would, together with behavioral assessment, immensely help in the diagnosis of ASD thus allowing the possibility of early detection and thereby allowing early targeted intervention, which could possibly reduce severity of ASD [19]. Metabonomics or metabolic profiling approach is becoming increasingly important in identifying biomarkers of disease progression and drug intervention and can provide additional information to support or aid the interpretation of genomic and proteomic data. Since metabolic phenotypes are the results from the interaction between host genome [45] and the environment including diet and host microbiome [46, 47], perturbation in such complex interactions will lead to altered metabolic profiles, which can be studied using metabonomic approaches [48–52].

One of the earlier works on urinary phenotyping of autistic children was carried out by Lis et al. [53], where urine samples from autistic ($n = 19$) and normal (n = unknown) children were analyzed using anion exchange chromatography.

The study showed that autistic children have abnormal levels of urinary hippurate, 4-hydroxyhippurate, and *N*-methyl-2-pyridone-5-carboxamide (2PY) as compared to normal controls. It was postulated in the study that such observation in the urinary profiles could be due to several factors including involvement of gut microbiota and potential perturbation in endogenous metabolism. Hippurate is predominantly formed by hepatic glycine conjugation of dietary and gut microbial-derived benzoate, which is derived from plant phenolics [54]. Decreased urinary levels of hippurate could be an indication of reduced benzoic acid synthesis by the gut microbiota. The work by Lis et al. highlighted the potential of urine as a biochemical window into understanding ASD and a viable biomatrix for biomarker discovery and potentially diagnosis. The first metabonomic study on ASD was conducted by Yap et al. utilizing proton nuclear magnetic resonance (^{1}H NMR) spectroscopy-based metabonomic approach [55]. The study looked at the metabolic profiles of children diagnosed with ASD together with their non-autistic siblings and age-matched healthy volunteers. The main findings from the study showed alterations in nicotinic acid metabolism and gut microbe metabolism with increased urinary levels of 2PY, *N*-methyl nicotinic acid, and *N*-methyl nicotinamide and decreased levels of urinary gut microbe co-metabolites such as hippurate, phenylacetylglutamine, and 4-cresol sulfate. These observations were in agreement with the findings from Lis et al. [53] implicating the involvement of gut microbe in ASD and proved that metabonomic is an effective tool to aid understanding of the etiology of ASD and biomarker discovery. The study by Yap et al. also revealed differences in urinary amino acid levels such as glutamate, alanine, glycine, and taurine [55]. More intriguingly, the study also showed that the metabolic profiles of non-autistic siblings were quite different from their autistic siblings as well as the age-matched healthy volunteers. Such pattern was also observed by Parracho et al. when comparing the levels of *Clostridium* spp. between children with ASD versus healthy siblings and healthy unrelated controls, which showed that the healthy siblings had an intermediate level between their ASD siblings and the healthy unrelated controls. Such observation could indicate that the presence and levels of certain gut microbe could trigger the onset of ASD, which could lead to perturbation in the metabolic profiles and warrant further investigation.

Ming et al. utilized mass spectrometry-based metabonomic approach to further investigate metabolic perturbations in ASD children versus controls with the aim of identifying more specific biochemical disturbances linked to the pathogenesis of ASD [56]. The study showed that individuals with ASD showed differences in urinary amino acids such as glycine, alanine, and taurine as well as gut microbe co-metabolites such as propionic acid derivatives and bile acids. Results from this study validated the findings by Yap et al. [55], which showed perturbation in gut microbial co-metabolism. Furthermore, the study by Ming et al. also showed lower levels of urinary antioxidants carnosine and urate indicating potential increase in oxidative stress. More recently, Emond et al. evaluated the use of gas chromatography-coupled mass spectrometry metabonomic approach to study the urinary biochemical profiles of autistic versus healthy children [57]. The study

successfully differentiated autistic from healthy children, and several metabolites were identified to be significantly contributing the differences. Urinary metabolites succinate and glycolate were found to be higher in autistic children, whereas metabolites such as hippurate, 3-hydroxyphenylacetate, 3-hydroxyhippurate, and several other metabolites were found to be lower in autistic children. Interestingly, the urinary metabolites that were lower in autistic children were largely gut microbe co-metabolites.

Results from all four studies indicated a common factor that is the perturbation of gut microbe co-metabolites in ASD individuals [53, 55–57]. The later three studies [55–57] also demonstrated the potential of metabonomic as a noninvasive tool to study and understand the etiopathophysiology of ASD. In addition, the biochemical changes observed from these studies may provide novel biomarker information applicable for diagnostic and monitoring therapeutic interactions in the condition. The key findings on the role of the microbiome in brain development and the etiology and development of autism were summarized in Table 14.1.

Table 14.1 Role of the microbiome and microbial–host co-metabolites associated with brain development and autism: overview of key references

Biological compartment	Main findings	Refs
Microbiome	Certain bacteria produce neurotoxin that can be transported to the central nervous system via the vagus nerve. Bacteria implicated: *Clostridium* species; *Clostridium tetani*	[35, 36]
	Vancomycin-treatment leads to short-term improvement in gastrointestinal problems and behavioral improvements	[37]
	Higher levels and greater variation of *Clostridium* species were found in children with ASD, and normal functioning siblings of autistic children have intermediate level of *Clostridium* species between ASD and healthy unrelated controls. Bacteria implicated: *Clostridium* species	[38–40]
	Children with ASD were reported to have higher level of *Bacteroidetes* and lower level of *Firmicutes*. Bacteria implicated: *Bacteroidetes*; *Firmicutes*	[41]
Metabolome	Abnormal levels of urinary hippurate, 4-hydroxyhippurate, and *N*-methyl-2-pyridone-5-carboxamide (2PY) found in ASD children	[53]
	First metabonomics study on ASD. Reported increased urinary levels of 2PY, *N*-methyl nicotinic acid, and *N*-methyl nicotinamide and decreased levels of urinary gut microbe co-metabolites such as hippurate, phenylacetylglutamine, and 4-cresol sulfate in ASD children	[55]
	Metabolic profiling showed differences in urinary amino acids, i.e., glycine, alanine, and taurine, as well as gut microbe co-metabolites, i.e., propionic acid derivatives and bile acids, between normal and ASD children	[56]
	Found levels of urinary succinate and glycolate higher in ASD children and levels of urinary hippurate, 3-hydroxyphenylacetate, 3-hydroxyhippurate lower in ASD children	[57]

The growing ASD incidence attracts interest in better defining the role of recent changes in food intake and exposure in the etiology of the disease. For instance, essential fatty acids taken in diets mediate brain functions and structures during development and are involved in many brain-related disorders like autism [58]. Among the various lipid species, cell membrane components, including mainly phospholipids, are very rich in PUFAs in brain tissue, with AA and DHA representing up to 20 % of the dry brain weight [58]. Abnormalities in the fatty acid compositions of phospholipids have been implicated in several neurodevelopmental disorders that manifest with psychiatric symptoms. In particular, alteration in fatty acids and phospholipids, including not only reduced levels of n-3 PUFAs but also increased levels of saturated fatty acids in the red blood cell membrane [59] or in plasma [21], was described in autistic subjects. In particular, blood plasma of autistic patients showed an increase in most of the saturated fatty acids except for propionic acid and a decrease in most of polyunsaturated fatty acids, which could relate to multifactorial processes ranging from oxidative stress to mitochondrial dysfunction and lead to induced metabolic alterations in Saudi autistic patients [60]. The concomitant alteration in phospholipase activity associated with decreased levels of AA, docosatetraenoic acid, and DHA in red blood cell membranes from autistic subjects further supports a fundamental role of the phospholipid metabolic regulation in autism and the potential role of nutritional intervention for future prevention strategies [61]. This was recently exemplified by El-Ansary et al. [58] in a study comparing the relative concentrations of essential fatty acids (linoleic and alpha-linolenic), their long chain polyunsaturated fatty acids, and phospholipids in plasma of autistic patients from Saudi Arabia with age-matching controls. They reported a significant modulation of the metabolism of fatty acids, as assessed via an alteration of the ratio between essential fatty acids/long chain polyunsaturated fatty acids and omega-3/omega-6 fatty acids, and a decrease in circulating levels of phospholipids. The authors provide particular emphasis on phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine lipid species which could be used as potential biomarkers for future treatment or prevention strategies.

14.5 Therapeutic Perspectives

The potentiality of using metabolic and gut microbial metabolic markers for future therapeutic perspectives is significant but so far at their infancy, due to the yet limited definition and understanding of the processes leading to the gut–brain axis dysfunction in ASD. One should separate the nutritional approaches aiming at prevention from management of specific conditions or disease stages. Once additional and more consolidated phenotype characterizations of the human host–microbiome are available, more studies should be dedicated to investigate metabolic features associated with the gradual development of the ASD dysfunction. In particular, family at-risk subpopulations should be defined and studied in order to generate further hypotheses on environmental and nutritional strategies for prevention and management.

14.6 Conclusions

Comprehensive and long-term phenotyping of populations at risk is envisioned to provide some key and still missing insights into understanding mechanisms involved into the pandemic development of ASD. In this, novel methodologies, enabling to rediscover the intimate relationships with the gut functional ecology and interactions along the gut–brain axis, are foreseen as a fundamental cornerstone of the molecular mechanisms at play. The molecular hypotheses about etiology of the metabolic phenotype are still highly debated, but they suggest that patients should be screened for their microbiota for therapeutic strategies or preventive programs, which could benefit from novel and minimally invasive systems biology approaches.

References

1. Bercik P. The microbiota-gut-brain axis: learning from intestinal bacteria? Gut. 2011;60:288–9.
2. Grenham S, Clarke G, Cryan JF, et al. Brain-gut-microbe communication in health and disease. Front Physiol. 2011;2:94.
3. Chen X, D'Souza R, Hong ST. The role of gut microbiota in the gut-brain axis: current challenges and perspectives. Protein Cell. 2013;4:403–14.
4. Martin FP, Sprenger N, Yap IK, et al. Panorganismal gut microbiome-host metabolic crosstalk. J Proteome Res. 2009;8:2090–105.
5. Schiano TD. Treatment options for hepatic encephalopathy. Pharmacotherapy. 2010;30:16S–21.
6. Wu JC. Psychological co-morbidity in functional gastrointestinal disorders: epidemiology. Mech Manag J Neurogastroenterol Motil. 2012;18:13–8.
7. Collins SM, Surette M, Bercik P. The interplay between the intestinal microbiota and the brain. Nat Rev Microbiol. 2012;10:735–42.
8. Alonso C, Guilarte M, Vicario M, et al. Acute experimental stress evokes a differential gender-determined increase in human intestinal macromolecular permeability. Neurogastroenterol Motil Off J Eur Gastrointest Motil Soc. 2012;24(740–6):e348–9.
9. Rezzi S, Martin FP, Alonso C, et al. Metabotyping of biofluids reveals stress-based differences in gut permeability in healthy individuals. J Proteome Res. 2009;8:4799–809.
10. Santos J, Perdue MH. Stress and neuroimmune regulation of gut mucosal function. Gut. 2000;47 Suppl 4:iv49–51; discussion iv52.
11. Backhed F. Host responses to the human microbiome. Nutr Rev. 2012;70 Suppl 1:S14–7.
12. Holzer P, Reichmann F, Farzi A. Neuropeptide Y, peptide YY and pancreatic polypeptide in the gut-brain axis. Neuropeptides. 2012;46:261–74.
13. Forsythe P, Kunze WA, Bienenstock J. On communication between gut microbes and the brain. Curr Opin Gastroenterol. 2012;28:557–62.
14. Claus SP, Tsang TM, Wang Y, et al. Systemic multicompartmental effects of the gut microbiome on mouse metabolic phenotypes. Mol Syst Biol. 2008;4:219.
15. Martin FP, Dumas ME, Wang Y, et al. A top-down systems biology view of microbiome-mammalian metabolic interactions in a mouse model. Mol Syst Biol. 2007;3:112.
16. Claus SP, Ellero SL, Berger B, et al. Colonization-induced host-gut microbial metabolic interaction. mBio. 2011;2:e00271–10.
17. El Aidy S, Kunze W, Bienenstock J, et al. The microbiota and the gut-brain axis: insights from the temporal and spatial mucosal alterations during colonisation of the germfree mouse intestine. Benefic Microbes. 2012;3:251–9.

18. Matsumoto M, Kibe R, Ooga T, et al. Cerebral low-molecular metabolites influenced by intestinal microbiota: a pilot study. Front Syst Neurosci. 2013;7:9.
19. Walsh P, Elsabbagh M, Bolton P, et al. In search of biomarkers for autism: scientific, social and ethical challenges. Nat Rev Neurosci. 2011;12:603–12.
20. Autism and Developmental Disabilities Monitoring Network Surveillance Year Principal Investigators, Centers for Disease Control and Prevention, et al. Prevalence of autism spectrum disorders – autism and developmental disabilities monitoring network, 14 sites, United States, 2008. Morb Mortal Wkly Rep Surveill Summ. 2012;61:1–19.
21. Elsabbagh M, Divan G, Koh YJ, et al. Global prevalence of autism and other pervasive developmental disorders. Autism Res Off J Int Soc Autism Res. 2012;5:160–79.
22. Knapp M, Romeo R, Beecham J. Economic cost of autism in the UK. Autism Int J Res Practice. 2009;13:317–36.
23. Rosenberg RE, Law JK, Yenokyan G, et al. Characteristics and concordance of autism spectrum disorders among 277 twin pairs. Arch Pediatr Adolesc Med. 2009;163:907–14.
24. Hallmayer J, Cleveland S, Torres A, et al. Genetic heritability and shared environmental factors among twin pairs with autism. Arch Gen Psychiatry. 2011;68:1095–102.
25. Ronald A, Happe F, Bolton P, et al. Genetic heterogeneity between the three components of the autism spectrum: a twin study. J Am Acad Child Adolesc Psychiatry. 2006;45:691–9.
26. Ozonoff S, Young GS, Carter A, et al. Recurrence risk for autism spectrum disorders: a baby siblings research consortium study. Pediatrics. 2011;128:e488–95.
27. Sumi S, Taniai H, Miyachi T, et al. Sibling risk of pervasive developmental disorder estimated by means of an epidemiologic survey in Nagoya, Japan. J Hum Genet. 2006;51:518–22.
28. DiGuiseppi C, Hepburn S, Davis JM, et al. Screening for autism spectrum disorders in children with down syndrome: population prevalence and screening test characteristics. J Dev Behav Pediatr: JDBP. 2010;31:181–91.
29. Cohen D, Pichard N, Tordjman S, et al. Specific genetic disorders and autism: clinical contribution towards their identification. J Autism Dev Disord. 2005;35:103–16.
30. Hall SS, Lightbody AA, Reiss AL. Compulsive, self-injurious, and autistic behavior in children and adolescents with fragile X syndrome. Am J Ment Retard: AJMR. 2008;113:44–53.
31. Zecavati N, Spence SJ. Neurometabolic disorders and dysfunction in autism spectrum disorders. Curr Neurol Neurosci Rep. 2009;9:129–36.
32. Finegold SM. State of the art; microbiology in health and disease. Intestinal bacterial flora in autism. Anaerobe. 2011;17:367–8.
33. Bolte ER. Autism and clostridium tetani. Med Hypotheses. 1998;51:133–44.
34. Bruggemann H, Gottschalk G. Clostridia : molecular biology in the post-genomic era. Wymondham: Caister Academic; 2009.
35. Ahnert-Hilger G, Bigalke H. Molecular aspects of tetanus and botulinum neurotoxin poisoning. Prog Neurobiol. 1995;46:83–96.
36. Manning KA, Erichsen JT, Evinger C. Retrograde transneuronal transport properties of fragment c of tetanus toxin. Neuroscience. 1990;34:251–63.
37. Sandler RH, Finegold SM, Bolte ER, et al. Short-term benefit from oral vancomycin treatment of regressive-onset autism. J Child Neurol. 2000;15:429–35.
38. Finegold SM, Molitoris D, Song Y, et al. Gastrointestinal microbiota studies in late-onset autism. Clin Infect Dis Off Publ Infect Dis Soc Am. 2002;35:S6–16.
39. Song Y, Liu C, Finegold SM. Real-time PCR quantitation of clostridia in feces of autistic children. Appl Environ Microbiol. 2004;70:6459–65.
40. Parracho HM, Bingham MO, Gibson GR, et al. Differences between the gut microbiota of children with autistic spectrum disorders and that of healthy children. J Med Microbiol. 2005;54:987–91.
41. Finegold SM, Dowd SE, Gontcharova V, et al. Pyrosequencing study of fecal microbiota of autistic and control children. Anaerobe. 2010;16:444–53.
42. Wang L, Christophersen CT, Sorich MJ, et al. Low relative abundances of the mucolytic bacterium Akkermansia muciniphila and Bifidobacterium spp. in feces of children with autism. Appl Environ Microbiol. 2011;77:6718–21.

43. Aldred S, Moore KM, Fitzgerald M, et al. Plasma amino acid levels in children with autism and their families. J Autism Dev Disord. 2003;33:93–7.
44. Alberti A, Pirrone P, Elia M, et al. Sulphation deficit in "low-functioning" autistic children: a pilot study. Biol Psychiatry. 1999;46:420–4.
45. Sabeti PC, Varilly P, Fry B, et al. Genome-wide detection and characterization of positive selection in human populations. Nature. 2007;449:913–8.
46. Nicholson JK, Holmes E, Kinross J, et al. Host-gut microbiota metabolic interactions. Science. 2012;336:1262–7.
47. Holmes E, Loo RL, Stamler J, et al. Human metabolic phenotype diversity and its association with diet and blood pressure. Nature. 2008;453:396–400.
48. Kinross JM, Alkhamesi N, Barton RH, et al. Global metabolic phenotyping in an experimental laparotomy model of surgical trauma. J Proteome Res. 2011;10:277–87.
49. Saric J, Li JV, Wang Y, et al. Panorganismal metabolic response modeling of an experimental Echinostoma caproni infection in the mouse. J Proteome Res. 2009;8:3899–911.
50. Skordi E, Yap IK, Claus SP, et al. Analysis of time-related metabolic fluctuations induced by ethionine in the rat. J Proteome Res. 2007;6:4572–81.
51. Thomas EL, Parkinson JR, Hyde MJ, et al. Aberrant adiposity and ectopic lipid deposition characterize the adult phenotype of the preterm infant. Pediatr Res. 2011;70:507–12.
52. Yap IK, Brown IJ, Chan Q, et al. Metabolome-wide association study identifies multiple biomarkers that discriminate north and south Chinese populations at differing risks of cardiovascular disease: intermap study. J Proteome Res. 2010;9:6647–54.
53. Lis AW, McLaughlin I, Mpclaughlin RK, et al. Profiles of ultraviolet-absorbing components of urine from autistic children, as obtained by high-resolution ion-exchange chromatography. Clin Chem. 1976;22:1528–32.
54. Schwab AJ, Tao L, Yoshimura T, et al. Hepatic uptake and metabolism of benzoate: a multiple indicator dilution, perfused rat liver study. Am J Physiol Gastrointest Liver Physiol. 2001;280:G1124–36.
55. Yap IK, Angley M, Veselkov KA, et al. Urinary metabolic phenotyping differentiates children with autism from their unaffected siblings and age-matched controls. J Proteome Res. 2010;9:2996–3004.
56. Ming X, Stein TP, Barnes V, et al. Metabolic perturbance in autism spectrum disorders: a metabonomics study. J Proteome Res. 2012;11:5856–62.
57. Emond P, Mavel S, Aidoud N, et al. GC-MS-based urine metabolic profiling of autism spectrum disorders. Anal Bioanal Chem. 2013;405:5291–300
58. El-Ansary AK, Bacha AG, Al-Ayahdi LY. Impaired plasma phospholipids and relative amounts of essential polyunsaturated fatty acids in autistic patients from Saudi Arabia. Lipids Health Dis. 2011;10:63.
59. Bell JG, Sargent JR, Tocher DR, et al. Red blood cell fatty acid compositions in a patient with autistic spectrum disorder: a characteristic abnormality in neurodevelopmental disorders? Prostaglandins Leukot Essent Fatty Acids. 2000;63:21–5.
60. El-Ansary AK, Bacha AG, Al-Ayahdi LY. Plasma fatty acids as diagnostic markers in autistic patients from Saudi Arabia. Lipids Health Dis. 2011;10:62.
61. Bell JG, MacKinlay EE, Dick JR, et al. Essential fatty acids and phospholipase A2 in autistic spectrum disorders. Prostaglandins Leukot Essent Fatty Acids. 2004;71:201–4.

Chapter 15
The Modulation of Drug Efficacy and Toxicity by the Gut Microbiome

Ian D. Wilson and Jeremy K. Nicholson

Abstract The gut microbiota have been shown to have an important influence, direct and indirect, on the metabolism and toxicity of a wide range of drugs and xenobiotics. The major drug-metabolizing capability of the gut microbiota is reductive metabolism, but demethylation, dehydroxylation, deacylation, decarboxylation, and hydrolysis reactions have also been demonstrated as well as acetylation. Microbiome-driven drug metabolism can result positively in the activation of prodrugs to their pharmacologically active forms or alternatively result in adverse consequences such as toxicity. In addition, the gut microbiota can affect drug metabolism and toxicity indirectly via, e.g., competition of bacterial-derived metabolites for xenobiotic metabolism pathways or the modulation of host metabolic systems.

Keywords Deconjugation • Drug metabolism • Drugs • Gut bacteria • Metabolism • Microbiome • Prodrug activation • Toxicity • Xenobiotics

15.1 Introduction

With our expanding knowledge of the composition and role of the gut microbiota in human health and disease, interest has once more focused on the role of this important "external" organ in modifying the pharmacological effects of drugs, or their toxicity, and it currently forms an area of active research. In addition there is the interesting question of the modification of the microbiome using pharmaceuticals. This can be either intentional, in order to modify some of the activities of the gut microbiota beneficially, or unintentional, as a result of "collateral damage" caused by, e.g., exposure of the host to antibiotics, and cytotoxics etc. The latter poses the further question of how far one should go to mitigate this unintended damage to the gut ecosystem in the light of our increasing understanding of the importance of the

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gut microbiome to the host. It is now clearer than ever that these organisms are not merely passengers in the hosts digestive system, but important members of the crew.

As is now well accepted, in the adult human, the gut contains up to ca. a kilogram of bacteria, the bulk of which comprise species of obligate anaerobes from the genera *Bacteroides*, *Clostridium*, *Lactobacillus*, *Escherichia*, *Bifidobacteria*, and others, together with a variety of yeasts and other microorganisms. This results in a complex and still incompletely characterized ecology comprising ca. 1,000 species that coexist in a dynamic equilibrium. For the host the microbiome provides benefits via enhanced energy recovery from food, defense against pathogens, and interactions with the immune and nervous systems through a number of signaling molecules and metabolites. The composition of the gut microbiome is different between individuals and depends on many factors such as whether birth was conventional or by Cesarean section, diet, antibiotic treatment, environment, etc.

However, for the drug metabolism and toxicology communities, despite many early studies showing its importance in some instances of xenobiotic biotransformation, it is arguable that gut microbiota of animals and man has (at least until recently) become a "forgotten organ" for modulating drug metabolism, disposition, and toxicity (this is despite an early focus in vitro studies employing gut microbiota incubations where their influence of drug metabolism was investigated in depth (e.g., see Refs. [1, 2]). This lack of current awareness is potentially a serious oversight as the microbiota are not only capable of a very wide range of biotransformations but are also a source of physiological variability between individuals with clear potential to affect the disposition, and toxicity, of drugs and metabolites via secondary interactions mediated through, e.g., the metabolic exchange and the co-metabolism and processing of many diverse substrates [3]. Indeed, thanks to the gut microbiota, the host is continually exposed to bacterially derived metabolites and waste products that not only require processing and elimination but that may also modulate, to a greater or lesser extent, the host's drug-metabolizing systems via, e.g., the induction of drug-metabolizing enzymes (or their inhibition) or by the production of secondary metabolites that compete for particular drug-metabolizing pathways to give, in an analogous way to drug-drug interactions (DDIs), drug-microbiome interactions (DMIs). That this should be the case is unsurprising as undoubtedly an important driver for the development of the, so-called, phase I and II drug-metabolizing systems was the need to eliminate unwanted microbiota-derived metabolites such as, e.g., ethanol and benzoic acid. Such factors may be important in both personalized medicine and so-called idiosyncratic drug toxicity.

15.2 Drug-Metabolizing Capabilities of the Gut Microbiota

The metabolic capacity of the gut microbiota is large and is capable of undertaking a wide number of biotransformations on drugs and other xenobiotics. These metabolic capabilities include reductive and hydrolytic reactions as well as decarboxylation, dehydroxylation, dealkylation, de-halogenation, deamination, etc. (Fig. 15.1).

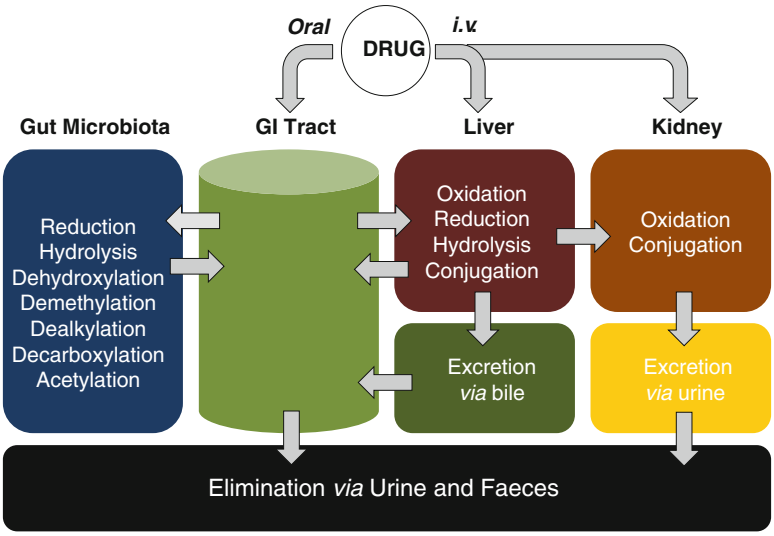

Fig. 15.1 A schematic highlighting the major metabolic reactions that are undertaken by the gut microbiota and illustrating the metabolic linkage with the host liver where biliary excretion and enterohepatic cycling of metabolites can result in host-microbiome co-metabolism

Another important set of reactions includes the modification of xenobiotic metabolites produced by the host but excreted into the gut (generally via the bile) such as glucuronides, sulfates, and glutathione conjugates. Examples, and potential consequences, of these types of drug metabolism are given below.

15.3 Microbiome-Based Metabolism of Drugs and Other Bioactive Compounds

15.3.1 Reductive Metabolism

The environment of the gut is well suited to non-oxidative metabolism, and the microbiome therefore provides the host with a metabolic organ of considerable capacity for the reductive biotransformation of drugs, and drug-like compounds, such as the polar azo dyes prontosil (*p*-(2,4-diaminophenylazo)benzenesulfonamide) [4] and neoprontosil (sodium 2-(*p*-sulphamyl-phenylazo)-7-acetamido-1-hydroxynaphthalene-3,6-disulfonate) [5]. These compounds are both prodrugs of sulfanilamide. Such reductions can therefore have important consequences resulting in the conversion of "prodrugs" into their active forms, the inactivation of compounds, or the toxification of otherwise innocuous compounds. Rafii and Cerniglia [6] demonstrated that a number of anaerobic species of bacteria present in the GI

tract of humans could reductively metabolize a range of azo dyes and nitropolycyclic aromatic hydrocarbons to aromatic amines. This activity resided in species of the genera *Clostridium* and *Eubacterium*. They studied the azo- and nitroreductases from three *Clostridium* strains and one *Eubacterium* strain, noting that these enzymes were produced constitutively in each of the bacteria, with the enzymes exhibiting different electrophoretic mobilities depending upon the bacteria from which they had been isolated. However, based on comparison of their electrophoretic mobilities, antibody affinity, and the fact that nitroaromatic compounds could act as competitive inhibitors against azoreductase activity, they considered that both azo- and nitroreductase activities resided in a single enzyme.

As well as prontosil and neoprontosil, important reductive bioactivations include those of, e.g., the 5-aminosalicylic acid prodrugs sulfasalazine (salicyl-azo-sulfapyridine) [7, 8], olsalazine [9], ipsalazide (5-Carboxymethylcarbamoyl-4-phenylazo)-salicylic acid), and balsalazide (5-(carboxyethtylcarbamoyl-4-phenylazo)-salicylic acid) [10] used in ulcerative colitis and inflammatory bowel conditions. Reductive metabolism of these compounds by the gut microbiota is essential to obtain the desired pharmacological activity in the gut via the release of the anti-inflammatory aminosalicylic acid moiety.

The consequences of reductive metabolism by the mictobiota can however, in addition to beneficial drug activation, potentially be quite serious. Thus the toxification of drugs via reductive metabolism has been implicated in the case of nitrazepam-induced teratogenicity in rats. In studies in the rat, Takeno and Sakai [11] found that incubation of nitrazepam in caecal content-derived bacterial suspensions resulted in extensive nitroreduction of the drug reduction to 7-aminonitrazepam (rat liver homogenates could also perform this biotransformation but only under anaerobic conditions). At an oral dose of 300 mg/kg nitrazepam, to pregnant rats, the nitro-reduced metabolites 7-aminonitrazepam and 7-acetylaminonitrazepam accounted for ca. 30 % of the drug-related material recovered in the excreta, falling to only 2 % following pretreatment with antibiotics. A direct relationship with antibiotic treatment and the decline in nitroreduction was confirmed when the nitroreductase activity of caecal contents was found to be almost completely eliminated by the antibiotic pretreatment (the activity in the liver homogenates was unaffected). In addition antibiotic pretreatment was observed to reduce the teratogenic effects of the drug. The combination of these experiments led the authors to conclude that the intestinal microbiota not only played an important role in the reductive metabolism of nitrazepam but that the teratogenic effects of the drug were not unrelated to this.

Similarly, when the in vitro and in vivo metabolism of clonazepam was investigated using germfree and ex-germfree rats, significant amounts of reduction of the drug clonazepam to 7-aminoclonazepam were seen that appeared to be related to the gut microbiota [12]. As with nitrazepam hepatic microsomes were also capable of this biotransformation but only under anaerobic conditions. When [^{14}C]-clonazepam was dosed orally to germfree rats, the reduced metabolites of the drug accounted for only 15 % of the radioactivity in the urine, with over 70 % of the dose as a phenolic metabolite. However, following colonization with an intestinal flora, some 77 % of

the metabolites observed resulted from nitroreduction with the major product identified as 7-acetamidoclonazepam.

The reductive cleavage of hydrazone linkages in drugs has also been observed. Thus, when the drug levosimendan was administered to dogs, Antila et al. [13] found evidence that the hydrazone linkage was cleaved by microbial action in the lower gastrointestinal tract. It was subsequently observed in humans that the cleavage product, (R)-6-(4-aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2H)-one (OR-1855), was then absorbed from the gut and subjected to further biotransformation to a pharmacologically active metabolite [14]. Similarly, following oral dosing to man, the drug eltrombopag was found to undergo extensive cleavage of its hydrazine linkage due to metabolism by gut microbiota [15]. This was evidenced by the production of the cleavage products following anaerobic incubation with rodent caecal contents or human fecal homogenate that was suppressed by antibiotics in both in vitro and in vivo experiments. However, on the basis of further in vivo studies, the authors concluded that concomitant antibiotic treatment in patients was unlikely to affect the pharmacokinetics of the drug in humans.

Reduction is, however, not limited to nitrogen-containing functional groups. Strong et al. [16] demonstrated the microbiota-driven reduction of the sulfoxide-containing drugs sulfinpyrazone and sulindac when these were incubated with human or rabbit feces. Sulfinpyrazone was reduced to a greater extent under anaerobic compared to aerobic conditions and it was noted that the reduction of sulindac was more extensive than that of sulfinpyrazone in human-derived feces. The presence of the antibiotics metronidazole and lincomycin in the growth medium markedly inhibited the reduction of sulfinpyrazone in human feces while tetracycline reduced sulfide production in rabbit feces/caecal contents. The formation of the sulfides of both sulindac and sulfinpyrazone ex vivo was found to be reduced for feces obtained from patients treated with metronidazole, and this antibiotic also decreased the in vivo reduction of sulfinpyrazone by the rabbit. More recently the H+/K+ATPase inhibitor omeprazole has also been shown in vitro to be reduced to its sulfide metabolite by the intestinal microbiota [17].

A particularly interesting example of reductive drug metabolism by the microbiota relates to the clinically important cardiac drug digoxin which is subject to quite extensive reductive metabolism to less pharmacologically active metabolites such as dihydrodigoxin and related compounds. Thus Lindenbaum et al. [18] determined the urinary excretion of the relatively cardioinactive reduced metabolites of digoxin (dihydrodigoxin and related compounds) in 131 normal subjects during studies of digoxin bioavailability. They found that in one-third of the subjects, reduced metabolites of the drug accounted for more than 5 % of the excretion of digoxin and its metabolites (after either single or multiple doses). This result was stable such that volunteers continued to excrete the same proportion of the reduced metabolites on repeated exposure to the drug digoxin. However, the exposure of some subjects to erythromycin resulted in a failure to excrete further quantities of the reduced metabolites following subsequent doses. In addition the presence of the

reduced metabolites in urine was lower if the drug was administered via the intravenous route (with oral administration the excretion of the reduced metabolites of digoxin was seen to vary inversely with bioavailability). On the basis of these findings, the authors hypothesized that this metabolic reduction was the result "of the activity of a variable component of the intestinal flora." Further studies [19, 20] implicated *Eubacterium lentum* as the sole organism forming these cardioinactive reduced metabolites of digoxin. However, the authors noted that the presence of this bacterium in the stools did not automatically mean that the host would excrete the reduced metabolite and further noted that there was an inverse relationship between the presence of increasing amounts of arginine in the growth medium and production of the reduced metabolites (observations that have only recently been explained as described below). Subsequently stool samples acquired from a group of 77 nursing home residents were analyzed to determine if there was a relationship between colonization with digoxin-reducing strains of *E. lentum* and infection with *Clostridium difficile* and the effects of previous antibiotic treatment, enteral feeding, and bowel movement habits [21]. This investigation found that colonization with the digoxin-reducing *E. lentum* was less prevalent in patients infected with *C. difficile*, previously treated with either antibiotics or enteral feedings. In addition normal bowel habits were more commonly associated with subjects who were not colonized with *C. difficile*.

As well as the factors described above, the same group also demonstrated that age was also important in the development of the ability of the gut microbiota to inactivate digoxin [22]. Studies in children showed that none of those less than 8 months of age produced reduced digoxin metabolites. A more adult pattern of digoxin metabolism was seen with age with one-third of the subjects studied producing reduced metabolites of the drug after 16 months of age. However, the levels of reductive digoxin metabolism seen in ca. 10 % of the adult populations were not observed in patients that were under 9 years of age. Bacterial cultures prepared from feces obtained from 73 babies aged less than 8 months were, however, shown to contain the appropriate digoxin-reducing bacteria, leading the authors to conclude that "maturation of the gut microbiota with respect to digoxin metabolism appears to be a protracted process. The relative digoxin resistance of infants and children is not due to bacterial inactivation" [22].

Differences in the production of reduced digoxin metabolites between populations have also been noted with one investigation showing that the drug was converted to reduced metabolites to a much greater extent by North American subjects (36 %) compared with a South Indian population (13.7 %). This difference was maintained when subjects from India went to live in the USA [23]. Within the Indian population there were also significant differences with the rural village population observed to produce ca. 5 % of the reduced metabolites as opposed to 23 % by urban dwellers.

Most recently Haiser et al. [24] have reinvestigated the reductive metabolism of digoxin using modern methods of molecular biology. Using a combination of transcriptional profiling, comparative genomics, and culture-based assays, they found a cytochrome-encoding operon that was upregulated by digoxin and inhibited by

arginine. This was not present in strains of *E. lenta* that did not metabolize the drug and was predictive of digoxin inactivation by the human gut microbiome. In vivo studies in gnotobiotic mice colonized with the digoxin-reducing strain of *E. lenta* revealed that dietary protein reduced the microbial metabolism of digoxin, resulting in changes in serum pharmacokinetics and urinary excretion (there was no such effect in mice colonized with the nonreducing strain of these bacteria). What then emerges from this most recent study is a more comprehensive picture of a microbiome-drug interaction that requires an appropriate strain of the bacteria involved to be present, with the resulting activity observed possibly also modulated by diet.

The gut reductive microbiota metabolism of metronidazole via the ring nitro group to its amino metabolite 1-(2-aminoimidazol-1-yl)-3 methoxypropanol-2-ol and acetamide (a known rat carcinogen) has been demonstrated both in vitro and in vivo in the rat [25, 26]. The suggested mechanism involved nucleophilic attack at carbons 2 and 4 of a partially reduced nitroimidazole ring, which was subsequently cleaved between positions 1 and 2 and 3 and 4 to yield both acetamide and *N*-(2-hydroxyethyl)-oxamic acid [27]. In vitro studies on cultures of rat caecal contents or *Clostridium perfringens* gave yields of 8–15 % of the metabolite (much higher than that of the oxamic acid metabolite). In vivo studies employing oral administration (200 mg/kg) of [^{14}C]-labeled metronidazole to rats resulted in 1.3–1.8 % of the dose being excreted in urine as acetamide with a further 0.9–2.4 % eliminated via the feces. However, no such excretion of acetamide was obtained with germfree rats.

A more recent example of the reductive metabolism of drugs involves the anticonvulsant zonisamide (1,2-benzisoxazole-3-methanesulfonamide) [28]. This drug has been shown to be mainly converted to 2-sulphamoylacetylphenol via the reduction of the benzisoxazole ring. While mammalian liver enzymes can perform this biotransformation, the role of the intestinal microbiota was also evaluated in vivo in the rat. In this study it was shown that antibiotic treatment significantly reduced the urinary and fecal excretion of the reduced metabolite following oral dosing with the drug. When the animals were recolonized, the presence of the microbiota restored the excretion of the metabolite. Further studies showed that the caecal contents obtained from control rats possessed zonisamide reductase activity that was absent from that of the antibiotic-treated rats. A number of strains of intestinal bacteria were tested for the ability to reduce zonisamide with *Clostridium sporogenes* showing the highest activity. On the basis of these studies, the authors concluded that, in vivo, the gut microbiota played a major role in the production of 2-sulphamoylacetylphenol. These studies contrasted with the conclusions of earlier in vitro studies that suggested that bacterial involvement in this metabolism, while it did occur, was not particularly important quantitatively compared to that observed in hepatic microsomes [29].

The in vitro reduction of the prodrug loperamide N-oxide to loperamide (used for the symptomatic treatment of diarrhea) was investigated using gut microbiota, gut contents, intestinal cells, and hepatocytes [30]. Efficient reduction was seen in the caecal contents from rat, dog, and human, and this activity was greatly reduced in the germfree rat.

Other losses of oxygen have been noted for the H_2 receptor antagonists ranitidine [31] and nitazidine [32], but not cimetidine or famotidine, as a result of the action of the gut microbiota in vitro [32].

As well as the toxicity associated with the reduction of nitrazepam described above, the reductive metabolism of the antibiotic chloramphenicol by the gut microbiota has been associated with often fatal, idiosyncratic, bone marrow aplasia [33]. The metabolite responsible was suggested as *p*-aminophenyl-2-amino-1,3-propanediol [34]. This metabolite is only generated by a small percentage of patients who take the drug orally and have a high percentage of the coliform bacteria that are capable of metabolizing chloramphenicol to this metabolite. However, other metabolites responsible for this toxicity have been proposed [32, 34] including *p*-nitrophenyl-*2*-dicloroacetamido-1,3-propanediol and 2-dichloroacetamid-3-hydroxypropio-*p*-nitrophenone [35].

15.4 Demethylations, Dehydroxylations, Deacylations, and Decarboxylations

Another well-known set of biotransformations of the gut microbiota is its ability to undertake metabolic demethylations, decarboxylations, and dehydroxylations. The demethylation of methamphetamine and 4′-hydroxymethamphetamine by the gut microbiota was shown by Caldwell and Hawksworth in the guinea pig [36]. The O- and N-demethylation of a range of compounds were explored by Smith and Griffiths for a range of compounds, incubated with rat-derived microbiota, who found that N-dealkylation did not occur for any of the drug molecules studied while O-dealkylation occurred only for relatively simple aromatic compounds [37]. A recent example of O-dealkylation is provided by studies in man on the metabolic fate of *N*4-(2,2-dimethyl-3-oxo-4-pyrid[1, 4]oxazin-6-yl)-5-fluoro-*N*2-(3,4,5-trimethyoxyphenyl)-2,4-pyrimidinediamine (R406) and its oral prodrug *N*4-(2,2-dimethyl-4-[(dihydrogenphosphonoxy)methyl]-3-oxo-5-pyrid[1, 4]oxazin-6-yl)-5-fluoro-*N*2-(3,4,5-trimethyoxyphenyl)-2,4-pyrimidinediamine disodium hexahydrate (fostamatinib), a spleen tyrosine kinase inhibitor [38]. Metabolic studies performed in vitro showed the prodrug to be rapidly converted to the active compound by human intestinal microsomes. The active species was also the major drug-related compound detected in human plasma following oral dosing. The bulk (ca. 80 %) of a [^{14}C]-dose of the drug was eliminated via the feces (with the remainder in urine) where the drug and a unique 3,5-benzene diol metabolite were detected. This 3,5-benzene diol metabolite appeared to result from the *O*-demethylation and dehydroxylation of one of the systemic metabolites of the drug (designated as R529) undertaken by anaerobic gut bacteria. This conclusion was based on the observation that R529 was converted to the 3,5-benzene diol on the in vitro incubation of this metabolite with human-derived feces. The experiments performed to investigate the overall metabolism of the drug suggested that production of the 3,5-benzene diol metabolite in man resulted from an initial hepatic cytochrome P450-mediated

p-*O*-demethylation of the drug followed by further gut microbiota *O*-demethylations and dehydroxylation.

As well as their studies on O- and N-dealkylations described above, Smith and Griffiths also investigated, in the same study [37], the in vitro gut microbial metabolism of N-acetylated compounds and found that phenacetin, acetanilide, and paracetamol, amongst others, all underwent significant amounts of N-deacylation. Such reactions, which unmask the aniline (phenetidine and p-aminophenol in the case of phenacetin and paracetamol, respectively), are clearly of interest with respect to potential toxicity.

Another interesting example of dehydroxylation/decarboxylation concerns L-DOPA (levodopa, L-3,4-dihydroxyphenylalanine) which is administered orally to treat Parkinson's disease. That the gut microbiota might be involved in the metabolism of the drug was first suggested by studies by Sandler et al. [39, 40] who noted, when treating patients suffering from Parkinson's disease with L-DOPA, that the urinary excretion of 3-hydroxyphenylacetic acid was increased. However, they also observed that concentrations this metabolite were significantly reduced in quantity by administration of neomycin suggesting that some microbial dehydroxylation of dopamine or L-DOPA occurs in man. Studies in rats [41] showed that 3-hydroxyphenylacetic acid was only seen in the urine of conventional, but not germfree, animals administered L-DOPA or dopamine. Both germfree and conventional animals were capable of converting *m*-tyramine to 3-hydroyphenylacetic acid suggesting that the conversion of dopamine to *m*-tyramine is bacterial in origin. In another study the microbial degradation of L-DOPA, ^{14}C-DL-DOPA (and potential phenolic metabolites) was investigated by means of incubations with rat caecal contents [42]. This work suggested that the biotransformation of DOPA was by way of 3,4-dihydroxyphenylacetic acid and decarboxylation or dehydroxylation to 4-methylcatechol or 3-hydroxyphenylacetic acid, respectively. In some incubations 3-hydroxyphenylacetic acid was further metabolized via decarboxylation to *m*-cresol. 3-Hydroxy-phenylpropionic acid was also detected as a metabolite. This is interesting as, to be effective, DOPA is decarboxylated following its passage across the blood-brain barrier to give dopamine, and Peppercorn and Goldman [43] suggested that this reaction could also be catalyzed by the gut microbiota which reduces the amount of dopamine reaching the brain. Results obtained by examining drug bioavailability after administration of L-DOPA to the dog gave average AUCs after hepatoportal and IV administration that were essentially the same [44]. However, when dosed duodenally, the AUC for L-DOPA was reduced, and this was combined with an increase in that of dopamine. When paromomycin and kanamycin were administered to suppress the gut microbiota, similar plasma and urinary excretion profiles were observed between control and antibiotic-treated animals. Further investigations of this decarboxylation determined the distribution of the decarboxylase activity of homogenates prepared from segments of the intestine. These showed that the jejunum had the highest enzyme activity, followed by the ileum and duodenum, leading the authors to conclude that, in the dog, the reduced bioavailability of orally administered levodopa occurred as a consequence L-DOPA decarboxylation in the gut wall rather than gut microbiota.

15.5 Effects of Bacterial Hydrolases on Drug Action and Toxicity

One of the more obvious and widely known effects that the gut microbiota exert on drug and xenobiotic metabolism, disposition, and toxicity relates to the effects of the hydrolytic enzymes responsible for the deconjugation of glucuronides, glucosides, etc. on both dietary pharmacologically active compounds and xenobiotic metabolites. Investigations have shown that the activities of the β-glucosidase and β-glucuronidase enzymes (as well as nitrate reductase and nitroreductase) determined in *Escherichia coli*, *Clostridium* sp., *Streptococcus* sp., *Bacteroides* sp., and *Lactobacillus salivarius* differ between strains of gut bacteria. Thus, *Clostridium* sp. showed the greatest enzymatic activity for β-glucosidase and β-glucuronidase (and nitroreductase, with *E. coli* the most active for nitrate reductase). The study was conducted both in vitro and in vivo and the authors found that, in general, the in vivo activity of the enzymes in was highest (although there were instances when this relationship was reversed) [45].

Effects on the bioavailability of natural products such as the flavone glucuronide baicalin (present in *Scutellariae radix*), where prior hydrolysis to the aglycone baicalein is followed by reconjugation following absorption [46], or effects on soy isoflavones to produce phytoestrogens such as equol are well known [47, 48]. In a similar way the effect of these gut microbiota-derived hydrolytic enzymes on the disposition of drugs and their metabolites is readily apparent in the case of conjugated metabolites (e.g., glucosides, glucuronides, and sulfates) that, following their production in the liver, are excreted in the bile. Hydrolysis by microbial enzymes results in their being resorbed, and this enterohepatic recycling can result in the modulation of the pharmacokinetic properties of the drug. In fact hydrolysis of glucuronide metabolites by bacterial enzymes can be responsible for considerable unwanted drug toxicity, and this has provided the impetus for the synthesis of specific inhibitors of bacterial glucuronidase to prevent this from occurring. The first use of this approach was applied to the DNA topoisomerase I inhibiting anticancer drug irinotecan by Wallace et al. [49]. The dose-limiting side effect of the drug, which is commonly used in the treatment of colon cancer, is the severe diarrhea that results from the hydrolysis of an inactive glucuronide metabolite by bacterial B-glucuronidases thereby reactivating the drug in the gut. By designing specific bacterial B-glucuronidase inhibitors, that had no effect on the mammalian B-glucuronidase, with crystal structures establishing that this selectivity was due to interaction with a bacterial B-glucuronidase-specific loop on the enzyme, the authors were able to eliminate irinotecan-induced toxicity in mice also orally administered the inhibitor. More recently the same approach was demonstrated to have similar effects on the small intestinal injury resulting from nonsteroidal anti-inflammatory drug (NSAID)-induced toxicity [50, 51]. Many of these NSAIDs contain a carboxylic acid and this represents a common site for glucuronidation, followed by excretion via the bile. In the first example of this approach [52], the bacteria-specific glucuronidase inhibitor (1-((6,8-dimethyl-2-oxo-1,2-dihydroquinolin-3-yl)-3-

(4-ethoxyphenyl)-1-(2-hydroxyethyl))thiourea) used with irinotecan was shown to inhibit the hydrolysis of diclofenac acyl glucuronide. Control or inhibitor-pretreated mice were administered an ulcerogenic dose of diclofenac and the effects noted. As expected, without pretreatment with the inhibitor, numerous large ulcers were detected in the small intestine while treated animals showed much reduced mucosal injury and enteropathy. This suggests that it is the liberation of the drug (and ulcerogenic metabolites) rather than the glucuronides themselves that is responsible for gut toxicity. Perhaps surprisingly, given the potential to reduce enterohepatic recycling, measurement of the pharmacokinetics of the drug with and without inhibitor pretreatment showed no differences in systemic drug exposure to diclofenac.

Subsequently the same authors showed that the inhibitor was equally effective at preventing NSAID-related damage when used when indomethacin, ketoprofen, and diclofenac were also administered to mice. Interestingly they demonstrated that the protective effects were maintained to some extent even if the inhibitor was administered several hours after the drug diclofenac [53], which undergoes extensive enterohepatic recirculation, had been dosed. These data are compatible with the hypothesis that pharmacological inhibition of bacterial β-glucuronidase-mediated cleavage of NSAID glucuronides in the small intestinal lumen can protect against NSAID-induced enteropathy caused by locally high concentrations of NSAID aglycones.

Another example of the potential of microbiome-driven hydrolysis to result in serious consequences for patients is provided by the antiviral drug sorivudine (1-β-D-arabinofuranosyl-5-(E)-(2-bromovinyl)uracil) used to combat infections by varicella-zoster virus and herpes simplex virus type 1 [54]. Recent studies have indicated that the drug should not be combined with anticancer drugs such as 5-fluorouracil (5-FU) or prodrugs such as tegafur because a metabolite, (E)-5-(2-bromovinyl)uracil (BVU), has been shown to cause the accumulation of 5-FU in the systemic circulation by inhibiting its degradation, resulting in enhanced, sometimes fatal, toxicity. The mechanism behind this toxicity seems to result from the inactivation of the hepatic dihydropyrimidine dehydrogenase (DPD) enzyme by BVU. The production of BVU from sorivudine was ascribed to bacterial phosphorolytic enzymes with high enzymatic activity detected in the caecal and large intestinal contents of the rat [54]. The measurement of phosphorylase activity performed on the cell-free extracts obtained from some 40 species of microbes showed that high activity to convert sorivudine to BVU was present in the *Bacteroides* species *B. vulgatus*, *B. thetaiotaomicron*, *B. fragilis*, *B. uniformis*, and *B. eggerthii*. Further studies showed that in rats treated with ampicillin or a mixture of bacitracin, neomycin and streptomycin, or metronidazole, only low concentrations of BVU were detectable in serum. In contrast when the amounts of BVU in serum were determined after the administration of kanamycin (used to selectively decrease the number of aerobes, rather than anaerobes), they were found to be higher in untreated animals. From these results the authors concluded that BVU is produced in vivo from sorivudine by intestinal anaerobic bacteria especially *Bacteroides* species.

15.6 Bacterial Acetylation

While many microbiome-driven biotransformations result in reduction or hydrolysis of drugs and their metabolites, an interesting exception is bacterial acetylation via bacterial N-acetyl transferases (NATs). Both N- and O-acetylation reactions have been shown to occur, and these reactions have been highlighted as being important in the bioactivation of genotoxic aromatic amines [55, 56]. In addition they are involved in the metabolism of drugs such as 5-aminosalyclic acid. Thus following oral dosing of 5-aminosalyclic acid, significant amounts of N-acetylated 5-aminosalycylic acid are excreted via the feces. When the drug was incubated with feces, using both aerobic and anaerobic conditions, this conjugation reaction was evident and this activity was demonstrated in a number of species [57]. Indeed, the acetylation of the aminosalicylate isomers 5-aminosalicylic acid (5-ASA) and 4-aminosalicylic acid (4-ASA), together with that of *p*-aminobenzoic acid, has been investigated in some detail, examining steady-state kinetics, time-dependent inhibition, and DNA hybridization in some 40 bacterial species, mostly from the human intestinal microbiota. This study showed *N*-acetyltransferase activity in 11 species of *Proteobacteriaceae* from seven genera (the *Citrobacter amalonaticus*, *Citrobacter farmeri*, *Citrobacter freundii*, *Klebsiella ozaenae*, *Klebsiella oxytoca*, *Klebsiella rhinoscleromatis*, *Morganella morganii*, *Serratia marcescens*, *Shigella flexneri*, *Plesiomonas shigelloides*, and *Vibrio cholerae*). The authors found that 5-aminosalicylic acid was acetylated much more efficiently than the 4-amino isomer (27–645 times), while *p*-aminobenzoic acid was only poorly acetylated. *Pseudomonas aeruginosa* was found to be the best acetylator with regard to both substrate spectrum and catalytic efficiency [57].

There have been suggestions that the adverse side effects of drugs that form 5-aminosalyclic acid such as olsalazine and sulfasalazine, which can result in pancreatitis in children, may be a result of the toxicity of the acetylated metabolite [58].

15.7 Microbial Metabolism of Glutathione Conjugates

While not direct metabolism of the xenobiotics themselves, the role of the microbiota in further processing glutathione conjugates formed in the liver from reactive metabolites, and then excreted in the bile, should be noted. Extensive metabolism of such conjugates, of a range of agrochemicals, was reviewed by Bakke and Gustafsson [59]. These studies showed the production of a large number of further metabolites via the degradation of the glutathione moiety for the conjugates of, e.g., 2-chloro-*N*-isopropylacetanilide (propachlor) and naphthalene, phenanthrene, etc., involving the bacterial C-S-lyases. Such metabolism has been shown to have the potential to result in the almost complete removal of the glutathione part of the conjugate to produce a free thiol group. This type of reaction has also been shown for

drugs such as paracetamol with the thiol subsequently further metabolized to give the methylthio metabolite of the drug [60]. The complete removal of the glutathione conjugate to regenerate the parent compound has also been observed [59].

15.8 Miscellaneous Biotransformations

The microbiota have demonstrated a range of other biotransformation capabilities, ether in vivo or in vitro, with regard to drug metabolism. For example, in the case of levamisole, used extensively as an anthelmintic drug, in both animals and humans, in vitro incubations under anaerobic conditions produced a number of ring-opened metabolites of the thiazole ring system [61]. The metabolites were mainly produced by the *Bacteroides* and *Clostridia*, and one of them, levametabol I, has been proposed as active for anti-colon tumor activity [61]. Another example of ring opening has been seen for the antipsychotic drug risperidone where cleavage of the benzisoxazole ring, in both parent compound and various hydroxylated metabolites, has been demonstrated both in vitro (caecal contents) and in vivo in the rat and in vivo in the dog [62].

15.9 Microbiome-Conditional Effects and Consequences

In the preceding text we have summarized the direct effects that the bacteria that comprise the gut microbiota can exert on the metabolism and toxicity of drugs, their metabolites, and related xenobiotics. In all likelihood these reported effects represent the tip of the iceberg as gut microbiota metabolism of drugs is not routinely assessed. However, the gut microbiota is not limited to direct effects and there are a number of indirect mechanisms whereby the microbiome can affect the metabolism, disposition, and toxicity of xenobiotics. Such effects include the modulation of host metabolic enzymes/transporters, competition for metabolism via particular host metabolic routes/enzymes, and enhancement of toxicity as a result of other effects on host biochemistry.

So, effects on the complement of the various xenobiotic metabolizing capabilities of important detoxification organs such as the gut and liver have been noted with the modulation of the levels of both cytochrome P450s and conjugating enzyme systems (e.g., [63–66]).

This includes the induction of P450s involved in the bioactivation of mutagens including the heterocyclic aromatic amine [63] 2-amino-3-methylimidazo[4,5-f] quinolone, amongst others. To date much of the information that we have on the effects of the microbiome on drug-metabolizing systems comes from a comparison of germfree animals with microbiome-competent controls. In a study examining the effects of the colonization of germfree mice with either individual strains of bacteria or complete ileal/caecal microbiota from conventionally raised mice,

DNA microarray analysis was used to determine the response of the intestine [64] revealing a number of effects on xenobiotic metabolizing capabilities. Thus colonization with *Bacteroides thetaiotaomicron* resulted in, amongst a range of other changes, decreases in glutathione *S*-transferase (GST), CYP2D2 (also known as debrisoquine hydroxylase), and the transporter "multidrug resistance protein 1a" (Mdr 1a). However, colonization with other species of gut-dwelling microbes, such as *Escherichia coli* and *Bifidobacterium infantis*, or indeed complete microbiota resulted in different outcomes with either no change (conventional gut microbiota) or increased expression of these enzymes. The authors concluded that these commensal bacteria were capable of modulating the expression of a range of host genes involved in a variety of "diverse and fundamental physiological functions" with the selective effects seen as a result of the type of bacteria used for colonization revealing how changes in the composition of the gut microbiome could have significant physiological effects.

In another study [65] the amounts of a number of enzymes involved in the Phase II conjugation of drugs and other xenobiotics were determined in livers and the small intestine, caecum, and colon of both germfree rats and those inoculated with microbiota from normal rats. Effects were noted on enzymes involved in glutathione metabolism such as GST glutathione peroxidase (GPX2). In addition differences in the amounts of epoxide hydrolases (EPHXs) and N-acetyltransferases (NAT) 1 and sulfotransferases (SULTs) were seen. Some of these enzymes were expressed in both liver and gut, while others showed regional differences (e.g., the SULTs were found in liver and large, but not the small intestine) and, in addition, gender effects were noted for the liver, but not gut, for a number of conjugating enzymes. Thus hepatic SULT1A1, SULT1C1, and SULT1C2 were seen to be elevated in germfree animals in both male and females (1.5- to 2.6-fold) while hepatic EPHX2 was 1.6-fold higher in female rats. The colonic germfree rats showed large differences compared to normal animals with GSTA1/2 4.0- and 5.0-fold higher in males and females, respectively, GSTA4 between 1.5 and 1.9-fold higher, and GSTM1 elevated by 1.1/1.5-fold. The epoxide hydrolases, EPHX1 and EPHX2, were 3.5/2.4- and 1.4/2.1-fold higher in male and female germfree rats, respectively. In the case of the sulfotransferases SULT1B1 and SULT1C2, the increases were 0.4/0.6- and 1.3/1.6-fold and for NAT2 amounted to 1.4/1.5-fold for male and female germfree rats, respectively.

When human gut microbiota was used for recolonization, the effects on the expression of such enzymes in the colon were smaller than those seen for recolonization with rat gut microbiota.

Further literature examples also show that germfree rats, and those inoculated with human gut microbiota, can exhibit differences in glucuronidating (UGT) and GST enzymes when dosed with (+)-catechin or (-)-epicatechin. In addition, animals with human gut microbiota had reduced CYP2C11 induction compared to the germfree animals [66].

Other gut microbial-conditional effects resulting from exposure to soy-derived phytoestrogens on host endogenous steroid (and thereby potentially xenobiotic) metabolism via a reduction in the excretion of 4-hydroxyestrogen and increased

2-hydroxyestrogen have been noted in postmenopausal women [67]. The authors suggested but did not actually demonstrate that this might result from changes in the expression of the CYPs involved in estrogen hydroxylation. Were this the case it would also be expected that there would also be knock on effects on the metabolism of drugs and other xenobiotics as well drug transporters.

Other indirect effects could easily result from competition between xenobiotics and microbial metabolites for metabolic pathways such as sulfation, often preferentially used for the conjugation of phenolic hydroxyls, but where capacity is limited. This appears to be the case for metabolism of compounds such as paracetamol [68]. For example, a study in human subjects administered 1 g of paracetamol indicated that the ratio of glucuronide to sulfate metabolites excreted in the urine, which varies considerably between individuals, was affected by competition for sulfation by microbially generated *p*-cresol (produced by bacterial metabolism of tyrosine and phenylalanine). If large amounts of *p*-cresol are present that compete with paracetamol for sulfation, there is the potential to reduce the ability of the host to detoxify the drug with potentially adverse consequences by directing metabolism toward the pathways that result in the production of the reactive quinone imine. Effects on the pharmacokinetics of orally administered paracetamol have been noted in a recent study in animals treated with antibiotics to eliminate the gut microbiota using a cocktail of bacitracin, streptomycin, and neomycin [69]. Paracetamol was dosed to both control and antibiotic-treated rats, with plasma concentrations of paracetamol and six metabolites determined via LC-MS/MS. The authors noted that the *AUC*s of the drug and its glutathione conjugate were higher in the antibiotic-treated animals while the metabolic efficiency of sulfate conjugation, as indicated by the ratio of the *AUCs* of paracetamol sulfate to paracetamol, was lower in antibiotic-treated compared to control rats (surprising given the induction of SULTs seen in the germfree animals described above) [65]. Irrespective of the reasons for the decreased sulfation seen in these pseudo-germfree animals, this study provides another example of the gut microbiota, or its absence, affecting drug conjugation.

Effects on toxicity have also been noted between normal and germfree rats, even when metabolism seems relatively unaffected, for the model hepatotoxin hydrazine. Thus, when administered to germfree rats at what was effectively a no effect dose level in controls, hydrazine resulted in profound effects, with some of the test animals becoming moribund [70]. Clearly, although an extreme example, this study demonstrates the value of a functional gut microbiome with respect to toxicity and suggests that different microbiomes might provide more subtle modulations of toxicity.

Such effects have obvious implications for drug toxicity testing, where differences in outcome may reflect not only strain but microbiome. Clearly, such effects also have the potential to produce unexpected, and potentially unwelcome, variability in response to the administration of drugs and exposure to toxins between individual patients and populations (and indeed some limited evidence suggests that there are microbially driven differences in drug metabolism between populations e.g., [23]).

However, despite a resurgence in interest in this "forgotten organ" (evidenced by a number of recent reviews, e.g., [71–76]) there is currently little, if any, real consideration of the potential of this to affect the various phases of drug absorption,

disposition, metabolism, excretion, pharmacology, or toxicity in either discovery or drug development programs. Similarly there is little evidence that regulatory bodies are aware of the potential importance of the gut microbiome. This is, potentially, a serious oversight and in our view a better understanding of these complex interactions could provide novel insights for drug discovery and development, and significant benefits for personalized medicine. The microbiome undoubtedly represents a "druggable target," and there is no doubt that it is possible to modulate both its composition and metabolic activity. It clearly deserves more attention from the drug metabolism community.

References

1. Illing HPA. Techniques for microfloral and associated metabolic studies in relation to the absorption and enterohepatic circulation of drugs. Xenobiotica. 1981;11:815–30.
2. Boxenbaum HG, Bekersky I, Jack MJ, Kaplan SA. Influence of gut microflora on bioavailability. Drug Metab Rev. 1979;9:259–79.
3. Nicholson JK, Wilson ID. Understanding 'global' systems biology: metabonomics and the continuum of metabolism. Nat Rev Drug Discov. 2003;2:668–76.
4. Gingel R, Bridges JW. Intestinal azo-reduction and glucuronide conjugation of prontosil. Xenobiotica. 1973;9:599–604.
5. Gingel R, Bridges JW, Williams RT. The role of the gut flora in the metabolism of prontosil and neoprontosil in the rat. Xenobiotica. 1971;1:143–56.
6. Rafii F, Cerniglia CE. Reduction of azo dyes and nitroaromatic compounds by bacterial enzymes from the human intestinal tract. Environ Health Perspect. 1995;103 Suppl 5:17–9.
7. Peppercorn MA, Goldman P. The role of intestinal bacteria in the metabolism of salicylazosulfapyridine. J Pharmacol Exp Ther. 1972;181:151–62.
8. Schroder H, Gustafsson BE. Azo reduction of salicyl-azosulphapyridine in germ free and conventional rats. Xenobiotica. 1973;3:225–31.
9. Truelove SC. Evolution of olsalazine. Scand J Gastroenterol. 1988;23(s148):3–6.
10. Chan RP, Pope DJ, Gilbert AP, Sacra PJ, Baron JH, Lennard-Jones JE. Studies of two novel sulfasalazine analogs, ipsalazide and balsalazide. Dig Dis Sci. 1983;28:609–15.
11. Takeno S, Sakai T. Involvement of the intestinal microflora in nitrazepam-induced teratogenicity in rats and its relationship to nitroreduction. Teratology. 1991;44:209–14.
12. Elmer GW, Remmel RP. Role of the intestinal microflora in clonazepam metabolism in the rat. Xenobiotica. 1984;14:829–40.
13. Antila S, Huuskonen H, Nevalainen T, Kanerva H, Vanninen P, Lehtonen L. Site dependent bioavailability and metabolism of levosimendan in dogs. Eur J Pharm Sci. 1999;9:85–91.
14. Antila S, Pesonen U, Lehtonen L, Tapanainen P, Nikkanen H, Vaahtera K, Scheinin H. Pharmacokinetics of levosimendan and its active metabolite OR-1896 in rapid and slow acetylators. Eur J Pharm Sci. 2004;23:213–22.
15. Deng Y, Rogers M, Sychterz C, Talley K, Qian Y, Bershas D, Ho M, Chen EP, Shi W, Chen EP, Serabjit-Singh C, Goryckj PD. Investigations of hydrazine cleavage of eltrombopag in humans. Investigations of hydrazine cleavage of eltrombopag in humans. Drug Metab Dispos. 2011;39:1747–54.
16. Strong HA, Renwick AG, George CF, Liu YF, Hill MJ. The reduction of sulphinpyrazone and sulindac by intestinal bacteria. Xenobiotica. 1987;17:685–96.
17. Watanabe K, Yamashita S, Furruno K, Kawasaki H, Gomita Y. Metabolism of omeprazole by gut flora in rats. J Pharm Sci. 1995;84:516–7.

18. Lindenbaum J, Tse-Eng D, Butler Jr VP, Rund DG. Urinary excretion of reduced metabolites of digoxin. Am J Med. 1981;71(1):67–74.
19. Dobkin JF, Saha JR, Butler Jr VP, Neu HC, Lindenbaum J. Inactivation of digoxin by Eubacterium lentum, an anaerobe of the human gut flora. Trans Assoc Am Physicians. 1982;95:22–9.
20. Saha JR, Butler Jr VP, Neu HC, Lindenbaum J. Digoxin-inactivating bacteria: identification in human gut flora. Science. 1983;220:325–7.
21. Bennet RG, Beamer BA, Greenough WB, Lindenbaum J, Bartlett JG. Colonisation with digoxin reducing strains of *Eubacterium lentum* and *Clostridium difficile* infection in nursing home patients. J Diarrhoeal Dis Res. 1992;10:87–92.
22. Linday L, Dobkin JF, Wang TC, Butler Jr VP, Saha JR, Lindenbaum J. Digoxin inactivation by the gut flora in infancy and childhood. Pediatrics. 1987;79:544–8.
23. Mathan VI, Wiederman J, Dobkin JF, Lindeenbaum J. Geographic differences in digoxin inactivation, a metabolic activity of the human anaerobic gut flora. Gut. 1989;30:971–7.
24. Haiser HJ, Gootenberg DB, Chatman K, Sirasani G, Balskus EP, Turnbaugh PJ. Predicting and manipulating cardiac drug inactivation by the human gut bacterium *Eggerthella lenta*. Science. 2013;341:295–8.
25. Koch RL, Chrystal EJT, Beaulieu Jr BB, Goldman P. Acetamide a metabolite of metronidazole formed by the intestinal flora. Biochem Pharmacol. 1979;28:3611–5.
26. Koch RL, Beaulieu BB, Goldman P. Role of the intestinal flora in the metabolism of misonidazole. Biochem Pharmacol. 1980;29:3281–4.
27. Koch RL, Goldman P. The anaerobic metabolism of metronidazole forms n-(2-hydroxyethyl)-oxamic acid. J Pharmacol Exp Ther. 1979;208:406–10.
28. Kitamura S, Sugihara K, Kuwasako M, Tatsumi K. The role of mammalian intestinal bacteria in the reductive metabolism of zonisamide. J Pharm Pharmacol. 1997;49:253–6.
29. Stiff DD, Robicheau JT. Reductive metabolism of the anticonvulsant agent zonisamide, a 1,2-benzisoxazole derivative. Xenobiotica. 1992;22:1–11.
30. Lavrijsen K, Van Dyck D, Van Houdt J, Hendrickx J, Monbaliu J, Woestenborghs R, Meuldermans W, Heykants J. Reduction of the prodrug loperamide oxide to its active drug loperamide in the gut of rats, dogs, and humans. Drug Metab Dispos. 1995;23:354–62.
31. Basit AW, Lacey LF. Colonic metabolism of ranitidine; implications for its delivery and absorption. Int J Pharm. 2001;227:157–65.
32. Basit AW, Newton JM, Lacey LF. Susceptibility of the H_2-receptor antagonists cimetidine, famotidine and nizatidine, to metabolism by the gastrointestinal microflora. Int J Pharm. 2002;237:23–33.
33. Yunis AA. Chloramphenicol toxicity: 25 years of research. Am J Med. 1989;87(3N):44N–8.
34. Holt R. The bacterial degradation of chloramphenicol. Lancet. 1967;289(7502):1259–1260.
35. Jimenez JJ, Arimura GK, Abou-Khalil WH, Isildar M, Junis AA. Chloramphenicol-induced bone marrow injury: potential role of bacterial metabolites of chloramphenicol. Blood. 1987;70:1180–5.
36. Caldwell J, Hawksworth GM. The demethylation of methamphetamine by intestinal microflora. J Pharm Pharmacol. 1973;25:422–4.
37. Smith GE, Griffiths LA. Metabolism of N-acylated and O-alkylated drugs by the intestinal microflora during anaerobic incubation in vitro. Xenobiotica. 1974;4:477–87.
38. Sweeny DJ, Li W, Clough J, Bhamidipati S, Singh R, Park G, Baluom M, Elliott G, Lau DT-W. Metabolism of fostamatinib, the oral methylene phosphate prodrug of the spleen tyrosine kinase inhibitor R406 in humans: contribution of hepatic and gut bacterial processes to the overall biotransformation. Drug Metab Dispos. 2010;38:1166–76.
39. Calne DB, Karoum F, Ruthven CRJ, Sandler M. The metabolism of orally administered L-DOPA in Parkinsonism. Br J Pharmacol. 1969;37:57–68.
40. Sandler M, Goodwin BL, Ruthven CRJ, Calne DB. Therapeutic implications in Parkinsonism of *m*-tyramine formation from L-Dopa in man. Nature. 1971;229:414–6.

41. Bakke OM. Degradation of DOPA by intestinal microorganisms in vitro. Acta Pharmacol Toxicol. 1971;30:115–21.
42. Goldin BR, Peppercorn MA, Goldman P. Contribution of host and intestinal microflora in the metabolism of L-Dopa by the rat. J Pharmacol Exp Ther. 1973;186:160–6.
43. Peppercorn MA, Goldman P. Drug-bacteria interaction. Rev Drug Metab Drug Interact. 1976;11:75–88.
44. Sasahara K, Nitanai T, Habara T, Kojima T, Kawahara Y, Morioka T, Nakajima E. Dosage form design for improvement of bioavailability of levodopa IV: possible causes of low bioavailability of oral levodopa in dogs. J Pharm Sci. 1981;70:730–73.
45. Cole CB, Fuller R, Mallet AK, Rowland IR. The influence of the host on expression of intestinal microbial enzyme activities involved in metabolism of foreign compounds. J Appl Bacteriol. 1985;59:549–53.
46. Akao T, Kawabata K, Yanagisawa E, Ishiahara K, Mizuhara Y, Wakuia Y, Sakashita Y, Koashi K. Baicalin, the predominant flavone glucuronide of scutellariae radix, is absorbed from the rat gastrointestinal tract as the aglycone and restored to its original form. J Pharm Pharmacol. 2000;52:1563–8.
47. Bowey E, Adlercreutz H, Rowland I. Metabolism of isoflavones and lignans by the gut microflora: a study in germ-free and human flora associated rats. Food Chem Toxicol. 2003;41:631–6.
48. Turner NJ, Thomson BM, Shaw IC. Bioactive isoflavones in functional foods: the importance of gut microflora and bioavailability. Nutr Rev. 2003;61:204–13.
49. Wallace BD, Wang H, Lane KT, Scott JE, Orans J, Koo JS, Venkatesh M, Jobin C, Yeh L-AL, Mani S, Redinbo MR. Alleviating cancer drug toxicity by inhibiting a bacterial enzyme. Science. 2010;330:831–5.
50. LoGuidice A, Wallace BD, Bendel L, Redinbo MR, Boelsterli UA. Pharmacologic targeting of bacteria-glucuronidase alleviates nonsteroidal anti-inflammatory drug-induced enteropathy in mice. J Pharmacol Exp Ther. 2012;341:447–54.
51. Saitta KS, Carmen Z, Lee KK, Fujimoto K, Redinbo MR, Boelsterli UA. Bacterial β-glucuronidase inhibition protects mice against enteropathy induced by indomethacin, ketoprofen or diclofenac: mode of action and pharmacokinetics. Xenobiotica. 2014;44:28–35.
52. Nakayama H, Kinouchi T, Kataoka K, Akimoto S, Matsuda Y, Ohnishi Y. Intestinal anaerobic bacteria hydrolyse sorivudine, producing the high blood concentration of 5-(*E*)-(2-bromovinyl) uracil that increases the level and toxicity of 5-fluorouracil. Pharmacogenetics. 1997;7:35–43.
53. Okuda H, Ogura K, Kato A, Takubo H, Watabe T. A possible mechanism of eighteen patient deaths caused by interactions of sorivudine, a new antiviral drug, with oral 5-fluorouracil prodrugs. J Pharmacol Exp Ther. 1998;287:791–9.
54. Grant DM, Josephy PD, Lord HL, Morrison LD. *Salmonella typhimurium* strains expressing human arylamine *N*-acetyltransferases: metabolism and mutagenic activation of aromatic amines. Cancer Res. 1992;52:3961–4.
55. Hein DW, Doll MA, Rustan TD, Gray K, Feng Y, Ferguson RJ, Grant DM. Metabolic activation and deactivation of arylamine carcinogens by recombinant human NAT1 and NAT2 acetyltransferases. Carcinogenesis (Land). 1993;14:1633–8.
56. van Hogezand RA, Kennis HM, van Schaik A, Koopman JP, van Hees PA, van Tongeren JH. Bacterial acetylation of 5-aminosalicylic acid in faecal suspensions cultured under aerobic and anaerobic conditions. Eur J Clin Pharmacol. 1992;43:189–92.
57. Delome'nie C, Fouix S, Longuemaux S, Brahimi N, Bizet C, Picard B, Denamur F, Dupreet J-M. Identification and functional characterization of arylamine *N*-acetyltransferases in eubacteria: evidence for highly selective acetylation of 5-aminosalicylic acid. J Bacteriol. 2001;183:3417–27.
58. Garau P, Orenstein SR, Neigut DA, Kocoshis SA. Pancreatitis associated with olsalazine and sulfasalazine in children with ulcerative colitis. J Pediatr Gastroenterol Nutr. 1994;18:481–5.

59. Bakke JE, Gustafsson JA. Role of intestinal flora in metabolism of agrochemicals conjugated with glutathione. Xenobiotica. 1986;16:1047–56.
60. Mikov M, Caldwell J, Dolphin CT, Smith RL. The role of intestinal microflora in the formation of the methylthio adduct metabolites of paracetamol. Studies in neomycin-pretreated and germ-free mice. Biochem Pharmacol. 1988;37:1445–9.
61. Shu YZ, Kingston DGI, Van Tassall RL, Wilkins TD. Metabolism of levamisole, an anti-colon cancer drug by human intestinal bacteria. Xenobiotica. 1992;21:737–50.
62. Meuldermans W, Hendrickx J, Mannens G, Lavrijsen K, Janssen C, Bracke J, Le Jeune L, Lauwers W, Heykants J. The metabolism and excretion of risperidone after oral administration to rats and dogs. Drug Metab Dispos. 1994;22:129–38.
63. Rowland IR, Mallet AK, Cole CB, Fuller R. Mutagen activation by hepatic fractions from conventional, germ free and monoassociated rats. Arch Toxicol. 1987;11(Suppl):261–3.
64. Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI. Molecular analysis of commensal host-microbial relationships in the intestine. Science. 2001;291:881–3.
65. Meinl W, Sczesny S, Brigelius-Flohé R, Blaut M, Glatt H. Impact of gut microbiota on intestinal and hepatic levels of phase 2 xenobiotic-metabolizing enzymes in the rat. Drug Metab Dispos. 2009;37:1179–86.
66. Lhoste EF, Ouriet V, Bruel S, Flinois J-P, Brezillon C, Magdalou J, Cheze C, Nugon-Baudon L. The human colonic microbiota influences the alterations of xenobiotic-metabolizing enzymes by catechins in male F344 rats. Food Chem Toxicol. 2003;41:695–703.
67. Xu X, Duncan AM, Wangen KE, Kurzer MS. Soy consumption alters endogenous estrogen metabolism in postmenopausal women. Cancer Epidemiol Biomark Prev. 2000;9:781–6.
68. Clayton TA, Baker D, Lindon JC, Everett JR, Nicholson JK. Pharmacometabonomic identification of a significant host-microbiome metabolic interaction affecting human drug metabolism. Proc Natl Acad Sci U S A. 2009;106:14728–33.
69. Lee SH, An JH, Lee HJ, Jung BH. Evaluation of pharmacokinetic differences of acetaminophen in pseudo germ-free rats. Biopharm Drug Dispos. 2012;33:292–303.
70. Swann J, Wang Y, Abecia L, Costabile A, Tuohy K, Gibson G, Roberts D, Sidaway J, Jones H, Wilson ID, Nicholson J, Holmes E. Gut microbiome modulates the toxicity of hydrazine: a metabonomic study. Mol Biosyst. 2009;5:351–5.
71. Kang MJ, Kim HG, Kim JS, Oh DG, Um YJ, Seo CS, Han JW, Cho HJ, Kim GH, Jeong TC, Jeong HG. The effect of gut microbiota on drug metabolism. Expert Opin Drug Metab Toxicol. 2013;0:1–14.
72. Haiser HJ, Turnbaugh PJ. Is it time for a metagenomic basis of therapeutics? Science. 2012;336:1253–5.
73. Ursell LK, Knight R. Xenobiotics and the human gut microbiome: metatranscriptomics reveal the active players. Cell Metab. 2013;17:317–8.
74. Li H, Jia W. Cometabolism of microbes and host: implications for drug metabolism and drug-induced toxicity. Clin Pharmacol Ther. 2013;94:574–81.
75. Saad R, Rizkallah MR, Aziz RK. Gut Pharmacomicrobiomics: the tip of an iceberg of complex interactions between drugs and gut-associated microbes. Gut Pathog. 2012;4:16.
76. Jeong HG, Kang MJ, Kim HG, Oh DG, Kim JS, Lee SK, Jeong TC. Role of intestinal microflora in xenobiotic-induced toxicity. Mol Nutr Food Res. 2013;57:84–99.

Chapter 16
Understanding the Benefits of Bariatric Surgery on Gut Physiology: Implications for Obesity, Type 2 Diabetes, and Cardiovascular Disease

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Abstract Seminal discoveries in the bariatric surgery field have revealed a remarkable link between gastrointestinal physiology and obesity, a link that extends to remission of many metabolic diseases including type 2 diabetes and to risk of cardiovascular disease. Much of the beneficial health effects of bariatric surgery can be ascribed to weight loss. However, in the case of type 2 diabetes, resolution is so acute that it appears to be independent of the weight loss. These observations have created the intriguing scenario whereby altering gastrointestinal anatomy creates rapid physiological adaptations that manifest in normalization of glucose homeostasis. The cellular and molecular mechanisms that produce these favorable health changes are an area of intense scientific investigation. One leading hypothesis suggests that rerouting nutrient flow to the gut alters enteroendocrine signals and bile acid secretion that favors appetite suppression; increased energy expenditure and insulin action; and decreased inflammation, blood lipids, and hypertension. In addition, the gut microbiome has emerged as an area of particular interest with a focus on bacteria and metabolites that interact to influence weight regulation and metabolic health.

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The collective evidence presented herein provides strong support for an essential role for the gastrointestinal tract as a modulator of obesity-related disease. The gut is now considered a novel endocrine organ and a therapeutic target for prevention and treatment strategies that will benefit human health.

Keywords Appetite-hormones • Bariatric surgery • Bile acids • Cardiovascular disease • Diabetes • Glucose • Gut microbiome • Incretins • Inflammation • Insulin resistance • Microbiome • Nutrition • Obesity • Weight loss

16.1 Overview of Obesity

Obesity is a major public health concern because it is associated with increased morbidity and mortality. The World Health Organization defines overweight as a body mass index (BMI) of 25 kg/m^2 or more and obesity as a BMI of >30 kg/m^2. Obese patients are further characterized into class I (BMI 30–34.9 kg/m^2), class II (BMI 35–39.9 kg/m^2), and class III (BMI >40 kg/m^2). These subcategories are particularly important when considering disease risk, since chronic metabolic disease risk increases progressively from a BMI >20 kg/m^2 [1].

According to the National Health and Nutrition Examination Surveys (NHANES) in the United States, nearly 78 million (or 35.7 %) adults and 12.5 million (16.9 %) children/adolescents are obese. Although the prevalence of obesity in adults aged 20–74 years has more than doubled over the last 40 years (13.4 % in 1960–1962 vs. 35.1 % in 2005–2006), it appears to have plateaued in the last several years [2]. Unfortunately, it has become clear that the distribution among obesity status in adults has shifted, such that a change in prevalence of superobesity (>50 BMI kg/m^2 or class IV) has occurred from 0.9 % in 1960–1962 to 6.2 % in 2005–2006 [3].

Obesity is responsible for more than 2.8 million deaths worldwide per year, owing to an increased prevalence of related comorbidities, including hypertension, heart disease, stroke, back and lower extremity weight-bearing degenerative problems, cancer, and type 2 diabetes [4]. Moreover, obesity is an independent risk factor for death, and some reports indicate that there is a 20–40 % increase in mortality in those who are overweight and upward of 300 % increased risk among those who are obese [5]. Lifestyle modification, consisting of a combination of nutrition, physical activity, and behavioral modification, is the first-line approach to promote weight loss. Although these weight loss interventions typically show initial promise, the long-term ability to maintain the desired weight benefit is typically lost even with use of pharmacotherapy. In fact, most patients who lose weight via behavioral changes and anti-obesity drugs tend to gain the weight back. The only known medical treatment for severe obesity that produces durable effects on body weight is bariatric surgery. In 1991, the National Institutes of Health established guidelines for surgical therapy for morbid obesity (BMI >40 kg/m^2 or BMI >35 kg/m^2 in the presence of two or more comorbidities) [6]. In fact, bariatric surgery has proven efficacy in not only ameliorating type 2 diabetes and cardiovascular disease

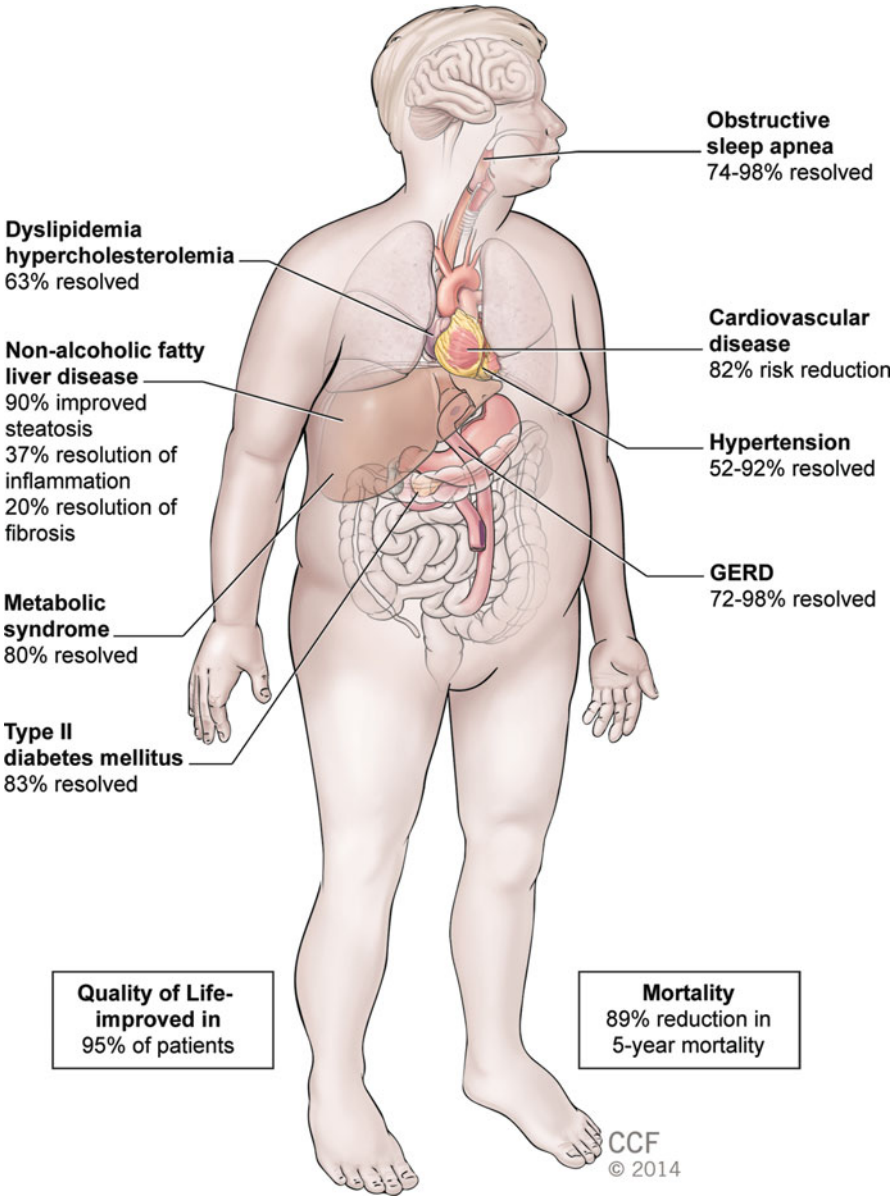

Fig. 16.1 Effects of bariatric surgery on physiological systems and disease states in obesity

risk factors but also mitigates non-alcoholic fatty liver disease, sleep apnea, and gastroesophageal reflux disorder (GERD) as well as reducing mortality (Fig. 16.1). These effects on multiple clinical conditions make bariatric surgery an important treatment option for many obese individuals.

This chapter examines the impact of bariatric surgery on gut physiology. There is increasing evidence that alterations in the gut after bariatric surgery not only contribute to the long-term weight loss maintenance but also to the treatment of type 2 diabetes and cardiovascular disease risk factors in patients with a BMI >35 kg/m^2. Central to the improvement in body weight is the increase in gut hormones known to suppress appetite. Special attention is also given to gut microbiome-related mechanisms that appear to be altered by bariatric surgery and may contribute to weight loss. The chapter also discusses efficacy of the bariatric surgery as a therapeutic modality to improve glycemic control and cardiometabolic health in relation to altered gut physiology and low surgical risk.

16.2 Bariatric Surgery Overview

Bariatric surgery is an effective therapy improving weight loss and metabolic health and is currently recommended for adults with a BMI of at least 40 kg/m^2 or 35 kg/m^2 with comorbidities. Although bariatric procedures are commonly referred to as restrictive and/or malabsorptive (Fig. 16.2) based on the presumed mechanism of weight loss [7], it is worth noting that not all bariatric surgeries produce the same effect on body weight, diabetes remission, and cardiometabolic resolution (Table 16.1).

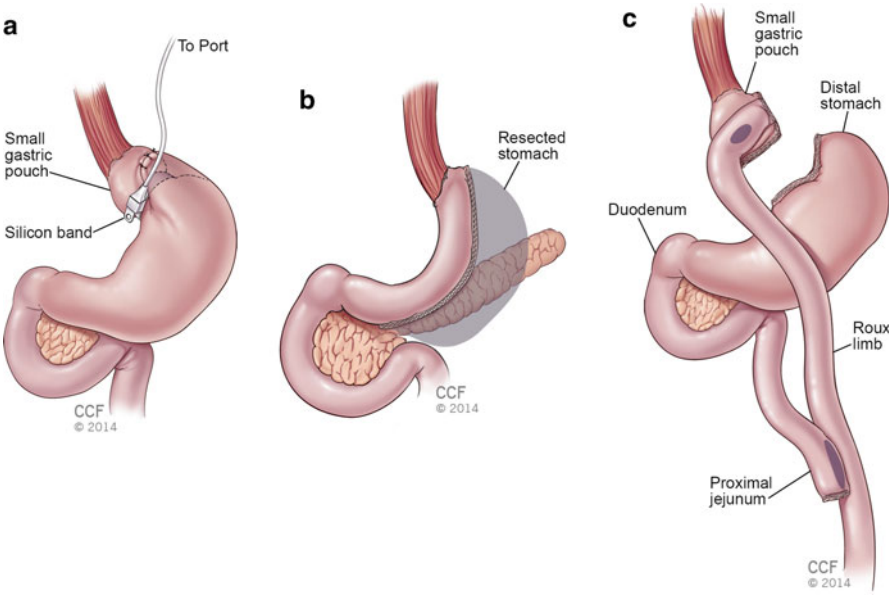

Fig. 16.2 Conventional bariatric surgery operations. (**a**) Laparoscopic adjustable gastric banding. (**b**) Sleeve Gastrectomy. (**c**) Roux-en-Y gastric bypass

Table 16.1 Metabolic effects of conventional bariatric techniques [19]

	Rates of improvement after surgery (%)		
Improvement	LAGB	RYGB	BPD
Excess weight loss	46.2	59.5	63.3
Resolution of type 2 diabetes	56.7	80.3	95.1
Remission of dyslipidemia	59	97	99
Resolution of hypertension	43	68	83
Operative mortality	0.1	0.5	1.1

LAGB laparoscopic adjustable gastric banding, *RYGB* Roux-en-Y gastric bypass, *BPD* biliopancreatic diversion

Restrictive procedures decrease the functional volume of the stomach, thereby increasing satiety due to limiting the intake of calories. These procedures often involve some form of material or "band" (i.e., laparoscopic adjustable gastric banding (LAGB)) and/or surgically resizing of the stomach with a stapler to create a small pouch (i.e., vertical gastroplasty (VBG) or sleeve gastrectomy (SG)) [8]. In LAGB, an adjustable plastic and silicone band is placed around the upper stomach to reduce the size of the channel between the proximal and distal stomach to reduce food consumption [9]. In VBG, a procedure not performed routinely any longer, part of the stomach is permanently stapled to create a smaller pouch along the lesser curvature of the stomach. Although the risk of death and major morbidity is low following LAGB or VBG, the amount of excess weight loss obtained is inferior compared to SG or malabsorptive procedures. After LAGB or VGB, patients lose nearly 46 % of their excess body weight, while over half experience type 2 diabetes remission.

Alternatively, SG is a relatively new surgical approach for obesity management. This nonreversible procedure involves resection of the greater curvature of the stomach by stapling it over a sizing tube 11–20 mm in diameter [10]. Although the effectiveness of SG with respect to weight loss and resolution of comorbidities is less than that of Roux-en-Y gastric bypass (RYGB), but greater than LAGB, SG is being used with increasing frequency due to fewer complications and reduced risk (SG accounted for 7.8 % of primary bariatric operations in 2010) [11]. Current advancements in restrictive procedures performed endoscopically have also the potential to expand bariatric intervention for weight loss management. In fact, endoluminal sleeves and intragastric balloons have demonstrated short-term benefit for weight loss and comorbidity improvements, although long-term, randomized trials are lacking [12].

Malabsorptive procedures are designed to reduce the area of intestinal mucosa available for nutrient absorption and restrict caloric intake similar to LAGB or SG. However, because the small intestine is shortened, they have added the component of malabsorption of fat and nutrients. Afterward, more patients experience remission of type 2 diabetes (82–99 %) compared with restrictive operations, even in patients with longer duration of disease, including those treated with insulin (Table 16.1). In biliopancreatic diversion with duodenal switch (BPD-DS), part of the stomach is resected, and the duodenum is cut just distal to the pylorus and

Table 16.2 Changes in digestive and gut physiology after bariatric surgery [73]

	LAGB	SG	RYGB
Masticating time	Higher	Higher	Higher
Food intake amount	Lower	Lower	Lower
Food transit time	Slowed	No change	Faster
Food preferences	More pureed/ less fiber	No change	Decrease high fat/sugary food
Acid production	No change	Lower	Decreased
Ghrelin	No change	Lower	Lower
GLP-1 and PYY	No change	No change	Increased

LAGB laparoscopic adjustable gastric banding, *RYGB* Roux-en-Y gastric bypass, *SG* sleeve gastrectomy

reattached to the ileum, bypassing the duodenum and jejunum (i.e., digestive limb) [8]. The bypassed duodenum and jejunum (i.e., biliopancreatic limb) only pass bile and pancreatic juices. These loops converge at a common channel at the end of the small intestine, and the contents then pass normally through the large intestine. Theoretically, although BPD provides a more physiologic digestive behavior and diminishes the risk of dumping syndrome, ulcerogenicity, and hypocalcaemia, the procedure is labor intensive and places the patient at high risk [13]. On the other hand, RYGB is considered the gold standard for bariatric surgery and is the most commonly performed operation [7, 13]. The procedure involves creating a gastric pouch, Roux limb (jejunum and ileum), and biliary limb. The small gastric pouch is connected to the mid-jejunum, bypassing the majority of the stomach, the entire duodenum, and part of the proximal jejunum. Similar to the BPD-DS, the biliary limb is reconnected approximately 150 cm distal relative to the pylorus so that bilo-pancreatic juices can facilitate digestion. After RYGB, the size of the pouch greatly limits the amount of food that can be eaten [7, 13].

Collectively, the efficacy of weight loss will differ depending upon the type of surgery. Further, the speed at which type 2 diabetes remits also varies with restrictive versus malabsorptive procedures (Table 16.2). For instance, after RYGB and BPD-DS, diabetes remits within days, even before the patients have lost much weight, while this does not occur after restrictive procedures [14]. Subsequently, newer restrictive techniques are currently under investigation to improve personalized approaches that best fit patients' weight loss and comorbidity resolution needs. The current short-term research in assessing endoluminal sleeves and intragastric balloons on weight regulation and metabolic health improvements appears promising, although longer-term follow-up data are needed [15].

16.2.1 Effects of Bariatric Surgery on Obesity

Weight loss success following bariatric surgery has been described by 50–75 % excess weight loss (EWL), 20–30 % initial weight loss, and achieving a BMI <35 kg/m^2 [16]. The largest, prospective interventional-based trial that examined

the effects of bariatric surgery (i.e., LAGB vs. VBG vs. RYGB) was on 4,047 obese patients with healthy matched treated control and is known as the Swedish Obesity Study [17]. The results demonstrated that, while the control group gained weight over time, the surgical groups lost on average 23 %, 17 %, and 18 % body weight at 2, 10, and 20 years, respectively [18]. Buchwald and colleagues conducted a meta-analysis on the effects of bariatric surgery-induced weight loss and obesity-related comorbidities. It was reported that at 2 years post-surgery, the overall excess weight loss for 10,172 patients was 61.2 % [19]. Patients typically lose less weight after LAGB than RYGB, and the peak excess weight loss typically occurs at 2–3 years with LAGB compared with 1–1.5 years with RYGB. The success of LAGB at inducing weight loss, however, at 4 years is comparable to RYGB [20]. Further, superobese (BMI >50 kg/m^2) individuals have less excess weight loss than patients with lower BMIs after RYGB, suggesting that bariatric surgery is successful at inducing weight loss, but the magnitude of this response varies among patients depending on surgical type and preoperative BMI [21].

It is important to recognize that while many patients experience successful weight loss, up to 20–25 % of patients experience weight regain [16]. These statistics do not negate the benefit of surgery, but rather suggest the need for all individuals to modify behavior to focus on food tolerance, proper energy requirements, eating triggers, beverage selection, and patient nutritional knowledge in conjunction with appropriate amounts of exercise.

16.2.2 Effects of Bariatric Surgery on Blood Glucose

Obesity is a major risk factor for type 2 diabetes and contributes to its development by inducing insulin resistance and inflammation, which in turn reduce beta-cell function [22–24]. The notion that bariatric surgery "cures" diabetes was recognized over 20 years ago. Pories et al. [25] demonstrated in 141 patients with type 2 diabetes or impaired glucose tolerance that all but two individuals had normalized glucose tolerance within 10 days after RYGB. At 7.6 years after surgery, 83 % of the diabetic patients were off their antidiabetic drugs, and 99 % of those with impaired glucose tolerance were normoglycemic with a normal fasting glucose and hemoglobin A1c [26]. In the Swedish Obesity Study, at 2 years post-surgery with an average weight loss of nearly 28 kg, 72 % of patients had complete resolution of type 2 diabetes compared with 21 % of controls [17]. Many of these patients had been able to stop taking oral hypoglycemic drugs or insulin, which is in contrast to the control group who had an increased need for these agents. These results are similar to those of Scopinaro et al. [27, 28] who reported long-term follow-up data on 312 patients with type 2 diabetes undergoing BPD and indicated that 99 % of patients achieved normal glucose concentrations by 1 year after surgery. At 10 years after surgery, 98 % of the patients were still in complete remission of diabetes (i.e., normal blood glucose without antidiabetic medication use). However, not all surgical approaches induce comparable glycemic benefit. Diabetes resolution was observed in approximately 98 % of patients who underwent BPD (with or without DS),

84 % who underwent RYGB, 72 % who underwent VBG, and 48 % who underwent adjustable gastric banding [19]. In addition, it is also worth considering that more recent guidelines for diabetes remission have been established, and current work suggests that approximately 50–70 % of individuals undergoing RYGB or SG may not meet remission criteria at 5 years post-operation [29]. This would suggest that biological factors, such as weight regain or insulin resistance, may contribute to diabetes relapse. Indeed, resolution of type 2 diabetes is likely to occur in those with the shortest duration of diabetes (<5 years) or milder forms of diabetes (diet-controlled), lower central obesity, and/or the greatest weight loss after surgery [30]. Conversely, patients who do not resolve diabetes post-surgery are usually older or have a more prolonged surgical disease course [25, 31, 32]. Thus, further work understanding how to optimize diabetes remission rates is needed following surgery. Nevertheless, the majority of these observational studies have been supported by randomized control trials (RCTs) in obese cohorts with type 2 diabetes [33–35]. For instance, the Surgical Treatment and Medications Potentially Eradicate Diabetes Efficiently (STAMPEDE) trial recently demonstrated the effects of bariatric surgery on controlling glycemia in obese individuals with type 2 diabetes. In the STAMPEDE trial, Schauer et al. [36] compared the effects of RYGB and SG versus intensive medical therapy in 150 obese patients with uncontrolled type 2 diabetes at 1 year post-operation. People were randomly assigned to surgical or medical therapy groups, and the primary end point was an HbA1c <6.0 %. The results indicated that RYGB and SG each produced significant improvements in HbA1c in 42 % and 37 % of patients, respectively, meeting glycemic control criteria. Taken together, bariatric surgery appears to result in dramatic glycemic control and weight loss improvements in obese patients with type 2 diabetes.

16.2.3 Effects of Bariatric Surgery on Cardiovascular Disease

In addition to glycemic control benefits, bariatric surgery reduces cardiovascular disease risk by, in part, improving dyslipidemia and hypertension. Obesity is strongly associated with atherogenic dyslipidemia, which is often defined as elevated triglycerides, high apolipoprotein B, small low-density lipoprotein (LDL) particles, and low high-density lipoprotein (HDL) cholesterol. Results of a meta-analysis showed marked decreases in levels of total cholesterol, LDL, and triglycerides after bariatric procedures [37]. In fact, approximately 70 % of patients experience an improvement in hyperlipidemia with optimal improvements typically derived after BPD and RYGB [19]. In the Swedish Obesity Study, significant improvements were observed in triglyceride and HDL concentrations at 2 and 10 years in the surgical versus the control group [17]. In recent RCTs, including the Diabetes Surgery Study and STAMPEDE, bariatric surgery decreased plasma triglycerides and increased circulating HDL more than medical therapy alone [34–36]. Collectively, these data demonstrate that bariatric surgery is not only effective at regulating blood glucose levels and sustaining weight loss but also an effective treatment option for improving

blood lipid profiles in obese people. These findings are likely to contribute to the overall reduction in cardiovascular disease events seen 20 years post-surgery [18].

Hypertension is also highly associated with obesity, and there is good evidence that weight loss reduces blood pressure [38]. In general, a decrease of 1 % body weight leads to a 1 mmHg decrease in systolic blood pressure and a 2 mmHg decrease in diastolic blood pressure [39, 40]. Similar to effects on dyslipidemia, hyperglycemia, and weight loss, bariatric surgery across all procedures has good effects on reducing blood pressure. In particular, approximately 61 and 79 % of the total population with hypertension had it either resolved or improved for up to 2 years post-surgery [19]. The Swedish Obesity Study examined the effect of obesity on hypertension by investigating the 8-year incidence of hypertension in obese patients treated with bariatric surgery (VGB, GB, and RYGB) versus matched obese controls [41]. Although there was no overall difference in systolic blood pressure and an increase in diastolic blood pressure at 8 years compared to the control group, it is worth noting that RYGB did appear to be the more favorable surgical procedure for decreasing systolic and diastolic blood pressure at 10 years (by 4.7 and 10.4 %, respectively, $P<0.10$) [17]. To understand why systolic blood pressure was unchanged, an examination of weight loss and age was analyzed [41]. Despite rapid improvements in body weight and blood pressure by 1 year, the slight increase in systolic and diastolic blood pressure over subsequent years was linked to the rate of weight gain and age. In fact, in the surgical group, the effect of blood pressure of 1 year (time between baseline and last observation in the study) was up to four times greater than the effect of 1 kg regained. Together, these results suggest that the direction of weight change is more closely linked to blood pressure than the initial weight loss, but age is an important factor. Bariatric surgery did not decrease diastolic blood pressure. In fact, diastolic blood pressure increased post-surgery. Given that pulse pressure is associated with increased risk for coronary artery disease [42], Sjostrom et al. examined whether surgery could lower pulse pressure compared to a control group [41]. The results indicated that the weight reduction post-surgery lowered the rate of increase in pulse pressure seen in obese patients. Taken together, the result of bariatric surgery on blood pressure is not a simple relationship, but there does seem to be some protective effects on risk for future coronary heart disease.

16.3 Role of Bariatric Surgery Linking Gut Physiology to Obesity-Related Disease

Although weight loss is likely important for gains in insulin sensitivity and beta-cell function following bariatric surgery [43], restrictive procedures do not induce comparable diabetes resolution rates as compared to BPD or RYGB. Moreover, despite malabsorption explaining to some extent reductions in reactive oxygen species and inflammation derived from excess glucose and lipid digestion in obese individuals, nutrient malabsorption does not occur after standard RYGB [44],

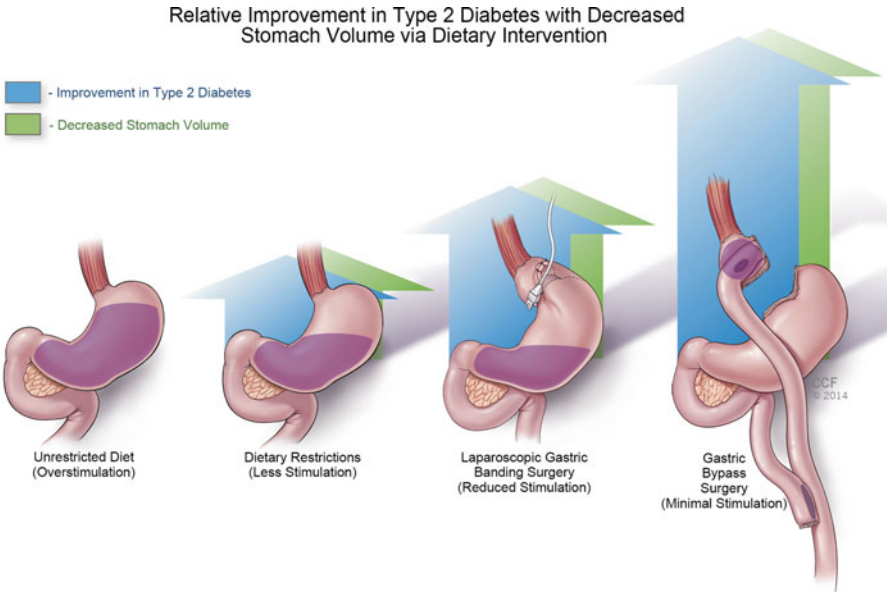

Fig. 16.3 Hypothetical model of nutrient-stimulated gastrointestinal dysfunction in type 2 diabetes

implying that additional factors likely drive the improvements in glycemic control post bariatric surgery [45]. Altered gut physiology is currently the leading candidate as the major mechanism to explain improved weight regulation, type 2 diabetes remission, and reduced risk of cardiovascular disease following bariatric surgery (Table 16.2).

Rubino and colleagues have hypothesized that overeating causes excessive stimulation of the gastrointestinal tract leading to metabolic disturbances that promote hyperglycemia, whereas restricting food contact with the gastrointestinal tract improves these conditions [43] (Fig. 16.3). Thus, rerouting of food through the gut may provide a way to alter the secretion of enteroendocrine factors that regulate insulin sensitivity and/or beta-cell function [46]. Surgical exclusion of the duodenum in the RYGB procedure and exclusion of the duodenum and jejunum in BPD result in altered sites – or at least altered relative distribution – of carbohydrate and fat absorption. This in turn is associated with an increase in anorectic hormones that induce satiety (e.g., GLP-1, PYY, OXY) and a decrease in the orexigenic hormone ghrelin that promotes hunger. These observations have led to the establishment of overlapping and discrete mechanisms that have been termed the "hindgut," "midgut," and "foregut" hypothesis. The hindgut hypothesis suggests that diabetes remission occurs because of accelerated delivery of nutrients to the distal intestine, which then augments an insulinotropic signal (e.g., GLP-1) that improves glucose homeostasis via enhanced insulin action [47]. Indeed, augmentation of GLP-1 secretion increases the insulin response to nutrient intake and, at least in animal

models, induces beta-cell proliferation [48], which together contribute to lowering blood glucose to normal levels. In addition to a rapid delivery of nutrients to the distal intestine and increased GLP-1 secretion, the "midgut" hypothesis suggests increased intestinal gluconeogenesis activating a neuro-hepato-portal sensor that regulates food intake and lowers hepatic glucose production [49]. In contrast, the "foregut hypothesis" suggests that nutrient interactions in the duodenum and proximal jejunum are diabetogenic and, hence, bypassing the duodenum, alleviate the intestinal factor that induces insulin resistance and beta-cell dysfunction [50, 51]. However, this later hypothesis has been questioned because a diabetic intestinal factor has not been identified and SG, which does not bypass the duodenum, also improves glycemic control. It is important to note that although the "hindgut," "midgut," and "foregut" hypotheses are often explained in terms of hormonal changes, they are not exclusive of altered nutrient flow that affects neural signaling. Interestingly, patients following surgery often report a reduction in snack numbers and/or portion size as well as food preference. In fact, these individuals often have reduced preference for sweet and fat-tasting foods [52]. In addition, although the gut hypotheses are often presented as mutually exclusive theories, no data actually exist excluding portions of the upper or lower intestine. Moreover, the exact molecular mechanism underlying the improvement in metabolism following RYGB is unknown, and it is likely that a number of gut hormones and neural signals produced at various sites of the gastrointestinal tract elicit unique mechanisms of action. Indeed, RYGB was reported to enhance intestinal glucose uptake and utilization, leading to overall improvements in systemic glucose control [53].

16.3.1 Modulation of Diabetes Remission via Altered Gut Hormones

Gastrointestinal hormones that augment insulin secretion following meal intake are known as incretins. Note, this effect is only observed when glucose or nutrients are ingested, not when they are given by an intravenous route [54]. GLP-1 (glucagon-like polypeptide 1) and GIP (glucose-dependent insulinotropic peptide) account for approximately 60 % of nutrient-related insulin secretion. In addition, GLP-1 suppresses glucagon and ghrelin and delays gastric emptying, which delays digestion and reduces postprandial hyperglycemia [55]. GLP-1 also acts on the brain to induce satiety, although the mechanism(s) remains largely unknown. Laferrere et al. [56] and others reported an increase in postprandial GLP-1 within 4 weeks following RYGB, whereas levels of GLP-1 did not rise with comparable weight loss induced by diet. These findings are consistent with data in patients with type 2 diabetes 1 year following RYGB in which elevated GLP-1 was significantly associated with insulin action [57]. Moreover, in the STAMPEDE trial, RYGB was shown to augment GLP-1 stimulation and acylated ghrelin suppression in association with beta-cell function to a greater extent than either SG or intensive medical therapy 2 years post-surgery [58, 59]. In general, RYGB is reported to enhance insulin secretion,

whereas gastric restrictive procedures reduce the need to secrete insulin [60]. On the other hand, GIP is secreted in the K cells located mainly in the duodenum and proximal jejunum and released in response to nutrients (mainly lipid). Unlike, GLP-1, GIP is more involved in lipid metabolism (storage) and is thus thought to play a more direct role in the pathogenesis of obesity. The effect of bariatric surgery on GIP is more controversial than the findings on GLP-1 such that the role of GIP is less clear in the regulation of lower fat mass and/or weight maintenance [52].

Non-insulinotropic gut hormones are altered after RYGB and include polypeptide tyrosine-tyrosine (PYY), oxyntomodulin (OXY), ghrelin, and cholecystokinin (CCK). Like GLP-1, PYY and OXY are co-secreted by the L cells of the distal small intestine and are responsible for reducing hunger, decreasing food intake, and delaying gastric emptying after meals. Moreover, while both OXY and PYY inhibit gastric acid secretion, only PYY reduces pancreatic and intestinal secretions [61]. While several studies have consistently documented increases in postprandial PYY and GLP-1 after gastric bypass [62–64], with some also reporting enhanced OXY [65], SG and BPD have also been shown to elevate PYY and GLP-1 [61]. Fewer studies have examined the role of CCK following bariatric surgery, but in general, CCK levels increase following RYGB, SG, and LAGB [61]. Ghrelin is a gastric hormone produced primarily in the stomach with secondary secretion emanating from the proximal small intestine. Ghrelin is best known as an appetite-stimulating hormone, but it also has additional effects on impairing insulin sensitivity and reducing glucose-stimulated insulin secretion [66]. Ghrelin suppression is usually improved following RYGB or SG, suggesting that suppression of hunger signals helps sustain weight loss. In contrast, ghrelin levels typically rise following diet-induced weight loss [67]. It is important to recognize, however, that the effect of bariatric surgery on ghrelin is controversial [57, 68].

16.4 Link Between Gut Microbiota, Excess Body Weight, and Metabolic Disease Risk

The gut microbiome has emerged as an important regulator of obesity, metabolism, and inflammation (Fig. 16.4). In the human intestine, approximately 400 bacterial species are present and together resemble a multicellular organ that has evolved to provide complex nutrient signaling and metabolic functions [69]. The vast majority of these microorganisms belong to three main groups *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* (comprising ~95 % of total intestinal bacteria) and reside in the distal portions of the intestine. The gut microbiome is a dynamic organ that changes in response to the environment. In animals provided a high-fat diet, the gut microbiota resulted in increased levels of *Firmicutes* prior to the development of obesity [70]. This renders the microbiota to be more obesogenic and may result in increased energy harvest from the diet. In line with this observation, Ley et al. demonstrated that food restriction, not macronutrient content per se (low-carbohydrate vs. low-fat), was linked to decreased levels of *Firmicutes* and elevated levels of *Bacteroidetes* [71],

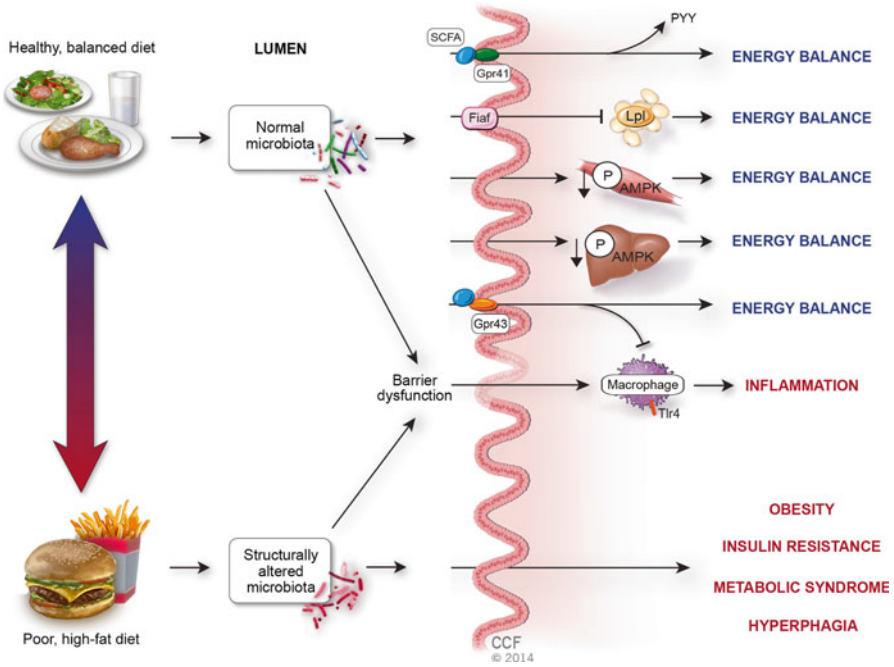

Fig. 16.4 Summary of the effects of the gut microbiome on weight regulation and energy metabolism

suggesting that nutrient overload is important for gut bacteria modifications. For instance, altered gut microbiome by high-fat feeding has been linked to intestinal inflammation and systemic insulin resistance [72]. Further evidence for the role of the gut microbiome in metabolic health is apparent using germfree rodents, which are protected from developing diet-induced obesity. In fact, germfree mice have lower adiposity and have higher food consumption when compared with control mice. But when germfree mice are colonized with cecal content from control mice, weight is rapidly gained and food consumption decreases, suggesting that the gut microbiome regulates fat mass and energy stores [73]. Further, genetically identical mice fed high-fat diets differ in their metabolic phenotype due to variation in gut microbiota composition [69], indicating that the cause of metabolic disease may be due to a complex interaction of environmental factors as well as gut microbiota profiles. In either event, germfree mice have reduced expression of SGLT-1 and CD36, which are important for fatty acid and glucose transport in the intestine [74]. These data together highlight gut bacteria as important modulators of nutrient absorption and suggest that at least two mechanisms are likely involved in explaining the link between obesity and gut microbiome: first, increased capacity to process otherwise indigestible polysaccharides, leading to subsequent rises in nutrients, and second, increased gene expression promoting fat storage in adipose

tissue [73]. Thus, the current working hypothesis is that obese animals are better able to extract energy from dietary intake and store those calories as fat. Indeed, there are similar observations from obese human studies [75].

16.4.1 Role of Bariatric Surgery on the Gut Microbiota

Bariatric surgery changes gut microbiota to reflect more of a lean phenotype. Zhang et al. [76] showed that microbiota functional differences occurred in obese individuals post-RYGB surgery. In fact, RYGB markedly altered *Gammaproteobacteria* (member of *Enterobacteriaceae*), decreased *Firmicutes*, and lowered methanogens (key for energy harvest). Similarly, Furet et al. [77] reported that gut microbiota adapted to RYGB surgery by increasing the *Bacteroides/Prevotella* ratio, which was correlated with reduced body fat. Moreover, the rise in *Escherichia coli* species also increased in individuals undergoing RYGB, and this change was significantly correlated with fat mass and leptin, independent of caloric intake restriction. Changes in adipose tissue mass are strongly linked to systemic inflammation, and consistent with weight loss following RYGB, the rise in *Faecalibacterium prausnitzii* species in type 2 diabetes was related to lower inflammation (hs-CRP and IL-6). Together, these findings strengthen the view that obesity and gut microbiota are intimately involved in the regulation of metabolic health. In fact, Kong et al. [78] demonstrated that RYGB increased gut microbiota richness, and this change in gut microbiota was directly correlated with genes encoding white adipose tissue mass, metabolism, and inflammation. Interestingly, approximately 50 % of these relationships were independent of caloric intake, suggesting that RYGB uniquely alters gut physiology in favor of weight reduction maintenance. Although distal portions of the small intestine contribute to the majority of nutrient absorption and gut microbiome in humans, the upper portions include gut bacteria that have metabolic function. Indeed, when microbiota from lean individuals are administered into the duodenum of humans with metabolic syndrome, insulin resistance declines independent of weight loss [79], suggesting that exclusion of the duodenum contributes to the regulation of glucose metabolism.

The cause for this altered gut flora milieu is presently an area of intense research, and several proposed mechanisms are currently being investigated. First, as demonstrated in rodent studies, the surgically induced restriction in food intake and/or change in food preference, including lower sugary foods and dietary fat, may explain modifications in the gut microbiome composition because of the smaller stomach size and shorter intestinal length [73]. Next, by diverting nutrients away from the proximal intestine, gut microbiota are exposed to more rapid food delivery and adapt accordingly. For instance, intestinal cells are exposed to more oxygen than usual due to the shorter intestinal lengths, and facultative anaerobes develop. Lastly, from an anatomical perspective, pH levels rise after RYGB surgery in the stomach and upper intestine. Although a pH <4 is potentially “deadly” for many microorganisms, some reports suggest that pH modification affects the overall gut

microbiota composition. Further, modification of acid secretion following RYGB not only lowers distal small intestine pH and influences the production of deconjugated primary bile acids but also increases acidification in the distal small intestine thereby increasing secondary bile acid levels via gut bacteria. Indeed, elevated secondary bile acid levels are reported to decrease hepatic fatty acid uptake, which may contribute to improvements in hepatic triglyceride metabolism and hepatic steatosis [80]. Alterations in gut microbiome in the colon have also been linked to lower pH, and these altered gut bacteria favor the rise in short-chain fatty acid production, which may help regulate lipid metabolism (see below: *Inflammation and Innate Immune Response Related to Gut Physiology*) [81]. Taken together, these findings following RYGB surgery support the gut microbiome as a key physiologic player involved in fostering nutrient sensing for both weight regulation and glucose homeostasis [78].

16.4.2 Inflammation and Innate Immune Response Related to Gut Physiology

Low-grade inflammation is a common comorbidity of type 2 diabetes and cardiovascular disease. Locally, the gut microbiome is directly linked to intestinal inflammation via changes in bacterial fragments and/or metabolites known to increase innate immune system responses. Subsequently, these bacterial components and metabolites have implicated the gut microbiome as a key factor in the development of obesity and metabolic disease [82] (Fig. 16.5). The innate immune system has the capacity to sense various bacterial components via pattern recognition receptors (PRRs). In general, there are two types of PRRs: Toll-like receptors (TLRs) and Nod-like receptors (NLRs). Although NLRs have important physiologic roles in gut health [83], TLRs have been well characterized and are the focus of the bacterial components discussed in this chapter.

16.4.2.1 Role of Lipopolysaccharides

Lipopolysaccharide (LPS) originating from Gram-negative bacteria in the gut induces low-grade inflammation and insulin resistance, thereby contributing to disturbances in energy metabolism that promote metabolic disease. LPS is sensed by TLR4, which is also recognized by nonmicrobial compounds such as saturated fatty acids [83]. LPS entry into the general circulation is elevated following high-fat diets (via a “leaky gut” phenomena or chylomicron transport mechanism) [84], and treatment with prebiotics significantly mitigates the development of glucose intolerance and inflammation derived from the liver and adipose of mice [84]. The mechanism by which prebiotics reduce LPS is unclear, but animal work suggests intestinal permeability improves via glucagon-like peptide-2 and cannabinoid-receptor-1 receptor-mediated pathways [85, 86]. These observations may be of particular

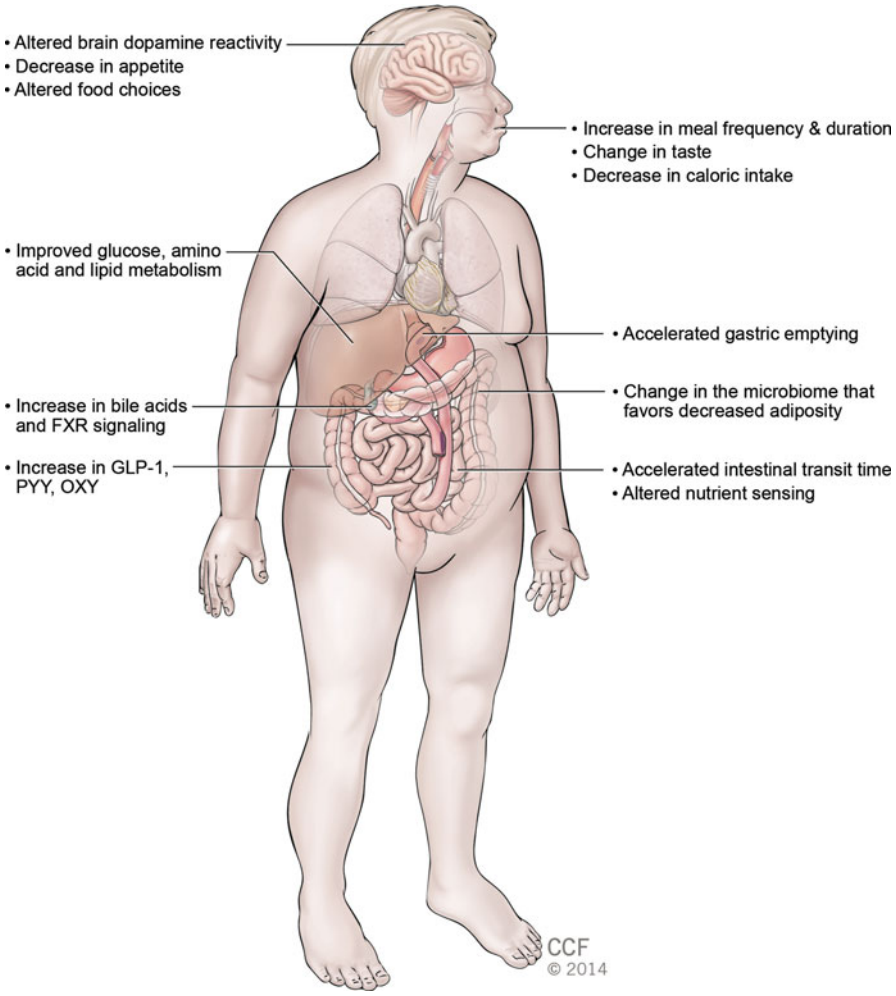

Fig. 16.5 Mechanisms that underpin the effects of RYGB on gut physiology

relevance for humans, as obesity is linked to elevated circulating LPS levels when compared with healthy controls [87]. Human work also supports the LPS linkage to hyperglycemia, as treatment with insulin-sensitizing agents in individuals with type 2 diabetes lowered LPS in line with greater rises in insulin action [88]. Further, elevated LPS in type 1 diabetic and vascular kidney disease was highly associated with serum triglycerides, diastolic blood pressure, and inflammation markers (e.g., MCP-1) [89], suggesting that metabolic LPS is linked to cardiovascular disease. Indeed, LPS levels are elevated in individuals with acute heart failure as compared to stable heart failure or controls, although clinical trials showing that reduced LPS leads to lower cardiovascular disease are lacking. Given that bariatric surgery

alters gut microbiome composition, it would seem reasonable to suspect changes in LPS. The limited evidence suggests that not only does bariatric surgery reduce LPS but that this reduction is also directly linked to lower adipose mass and improved HbA1c levels [87]. Taken together, alterations in gut microbiome appear to contribute to reductions in LPS that reduce metabolic disease risk.

16.4.2.2 Role of Short-Chain Fatty Acids

Short-chain fatty acids (SCFAs) are produced in the colon by gut microbes that ferment nondigestible polysaccharides (e.g., inulin) [85, 86]. The rise in SCFA (i.e., acetate, butyrate, and propionate) levels is important for weight regulation as they are implicated in satiety and decreased food intake, although SCFAs may act as substrate for lipogenesis. As mentioned previously (see above: *Modulation of Diabetes Remission* via *Altered Gut Physiology*), improved appetite regulation is in part related to elevated GLP-1 and PYY as well as lower ghrelin, which collectively contribute to reduced hunger and food intake. The effects of bariatric surgery on gut hormones and the gut microbiome would by inference suggest that the changes in SCFA contribute to the overall regulation of appetite. In fact, SCFAs have been shown to influence gut peptides, appetite, and energy expenditure [84]. For example, intracolonic and ileum infusion of mixed SCFA increased PYY secretion in rats and pigs, and supplementation of rat diets with acetate or butyrate lowered body weight [90]. Although studies in humans are limited, Arora et al. [91] suggested that propionate may reduce appetite. In addition to the effects on gut peptides, butyrate and propionate may increase leptin secretion from adipocytes [92]. This later mechanism may provide an alternative by which the gut directly communicates with adipose tissue to regulate body weight and feeding behavior. Interestingly, RYGB surgery enhanced propionate and lowered acetate levels in rodents, and this alteration in SCFA levels was attributed to the compositional change in gut microbiota. Increased propionate levels could contribute to improved weight regulation by blocking the conversion of acetate to triglyceride synthesis in the liver and blood, thereby reducing ectopic lipid depots. These findings are consistent with germfree animal work showing that transferring gut microbiota increases insulin and glucose-stimulated hepatic triglyceride synthesis via sterol response element binding protein (SREBP-1) and carbohydrate response element binding protein (ChREBP), respectively [90]. Moreover, decreased acetate levels per se may contribute to lower central and peripheral adiposity following bariatric surgery by reducing substrate availability for lipogenesis [93]. Concomitantly, bariatric surgery is reported to increase energy expenditure, and this change in thermogenesis may be attributable to gut microbial-derived changes in SCFA production. Interestingly, AMPK phosphorylation in the skeletal muscle and liver is increased in germfree mice on a Western diet [84]. Therefore, the gut microbiota through an unknown mechanism appear capable of altering peripheral and hepatic fatty acid oxidation and hepatic insulin sensitivity [94], an observation that is further strengthened by the fact that RYGB in rats increases hepatic AMPK activity [95]. In addition, the overall change in energy status of

adipose, hepatic, and skeletal muscle following surgery may be partially related to changes in fasting-induced adipose factor (FIAF). Indeed, colonization of germ-free mice is characterized by downregulation of FIAF expression, which in turn leads to increased lipoprotein lipase activity, enhanced lipid storage in adipocytes, and downregulation of PGC-1 α-related genes in peripheral tissue [90]. These later findings are consistent with evidence suggesting that propionate activates sympathetic nerve activity and has high binding affinity for GPR41 (G protein-coupled receptor), which is important for overall energy expenditure [93]. In fact, the effect of SCFA on GPR41 (as well as GPR43) may be relevant for modulating lipolysis, as infusion of acetate reduced circulating free fatty acids. Moreover, GPR43 is expressed in PYY containing L cells of the small intestine. Since L cells are also responsible for GLP-1 secretion, it is reasonable that SCFA may influence insulin secretion, and this is supported by rodent work [96]. Overall, these data highlight SCFA as an important factor that reduces appetite and/or increases energy metabolism to promote weight regulation.

16.4.2.3 Role of Trimethylamine-N-Oxide

Gut microbiota release choline from dietary phosphatidylcholine to form trimethylamine (TMA). TMA is transported to the liver via the portal vein and is oxidized to trimethylamine-N-oxide (TMAO). Elevated levels of plasma TMAO, choline, and betaine have dose-dependent associations with the presence of cardiovascular disease independent of conventional risk factors (e.g., blood pressure, triglycerides, etc.) and medication use [97]. Rats fed high choline diets or TMAO diets had elevated circulating TMAO levels and greater aortic root atherosclerotic plaque without alterations in plasma glucose or blood lipids. Although obesity may influence circulating TMAO levels, dietary manipulation appears important for modulating TMAO, such that high-fat and increased meat consumption lead to greater elevated TMAO compared with low-fat and vegetarian style meals. Data on TMAO following bariatric surgery is sparse. There are some urinary data to suggest that trimethylamine is elevated in rodents following bariatric surgery [98]. However, more work is needed to determine if the use of gut microbiota therapies (prebiotics and/or bariatric surgery) influences TMAO since this metabolite appears to be independently associated with cardiovascular disease risk [99].

16.4.2.4 Role of Hippurate

Intestinal bacteria metabolize low-weight aromatic compounds and polyphenols from the diet resulting in benzoic acid. In the liver, benzoic acid is conjugated with glycine to form hippurate, which is excreted in the urine [100]. Hippurate levels are lower in obese insulin-resistant rodents compared with wild-type controls [101]. In comparison to lean humans, morbidly obese individuals have low levels of

hippurate, which may be clinically relevant since low hippurate is linked with elevated blood pressure [102]. In animals, bariatric surgery increases urinary hippurate in parallel with weight loss [98], strengthening the notion that RYGB alters various aspects of gut microbiota in relation to obesity and metabolic health.

16.5 Bile Acids as Regulators of Energy Metabolism and Body Weight

Bile acids are produced in the liver, stored in the gall bladder, and secreted into the duodenum upon meal consumption. Bile acid levels nearly triple following meal consumption as they are important in not only the facilitation of micelle formation, which promotes the processing/digestion of dietary fat and fat-soluble vitamins, but also energy metabolism. Although fasting bile acids do not differ between lean and obese individuals, obesity appears to blunt the rise in some circulating postprandial bile acids, while other circulating glycemic conjugated forms preferentially decrease [103, 104]. The rise in postprandial bile acids is particularly relevant to energy metabolism and weight regulation as their hormonal effects include stimulating FGF19, GLP-1, and brown adipose activity. Interestingly, fasting total serum bile acids and individual levels of taurodeoxycholic, glycocholic, glycochenodeoxycholic, and glycodeoxycholic acids are elevated after RYGB surgery compared to preoperatively and when compared with weight-matched nonsurgical controls [103, 105]. The exact mechanism responsible for elevated bile acids following bariatric surgery is unclear, but animal work suggests that increased nutrient delivery to the ileum leads to increased satiety hormone levels and weight loss [106]. This suggests that nutrient flow to the distal small intestine is a potentially important mechanism linking altered bile acid levels seen following RYGB with changes in gut hormone secretion. However, despite immediate elevations in bile acids following RYGB, the rise in bile acids appears even greater several months post-operation. As such, it is likely that intestinal adaptation, including genes that synthesize and regulate transporters important for bile acid uptake, plays a key role in explaining elevated postprandial bile acid levels [103]. RYGB also alters intestinal gut microbiota, which are key regulators of bile acid conjugation and secondary bile acid formation [77, 93]. Interestingly, germfree mice also have low bile acid levels and diversity compared with wild-type controls, highlighting that gut microbiota may contribute to bile acid diversity and impact not only GLP-1 secretion but also energy expenditure. Concomitantly, it is important to note that conjugated bile acids entering the duodenum from the enterohepatic cycle circulate to the ileum where they are deconjugated, and the portion of these later bile acids might directly affect the composition of the microbiota [73].

Bile acids have been implicated in the improvement in weight and glucose metabolism following bariatric surgery. Pournaras et al. [106] demonstrated that fasting total serum bile acids are elevated within days following RYGB, but not

LAGB, suggesting that bile acids may contribute to weight-independent improvements in glucose homeostasis. Indeed, fasting total bile acids are inversely correlated with postprandial glucose and positively correlated with peak GLP-1 levels [107]. In line with this observation, bile acids are known to act on TGR5 receptors located on enteroendocrine cells and promote the secretion of GLP-1 release [108], which may contribute to satiety and beta-cell insulin secretion. In addition, farnesoid X receptor (FXR) in pancreatic beta-cells may directly respond to the rise in bile acids, thereby increasing insulin release [109]. In parallel, through an FXR-mediated pathway in the intestine, bile acids stimulate the secretion of FGF19, a protein that contributes to improved peripheral glucose disposal and lipid homeostasis [110, 111]. Thus, the physiologic effects of bile acids likely extend beyond that of gut-pancreas "cross-talk," since TGR5 receptors are also located on the skeletal muscle. TGR5 receptors are also present in brown adipose tissue, and the binding of bile acids to TGR5 in the skeletal muscle and brown adipose tissue may contribute to enhanced action of thyroid hormones to foster weight loss by increasing energy expenditure. Watanabe et al. [112] have reported that bile acids increase energy expenditure in both skeletal muscle and brown adipose tissue, and Ockenga et al. [113] found that postprandial levels of circulating bile acids are strongly associated with postprandial energy expenditure in lean individuals. Collectively, these findings suggest that restoration of elevated bile acids in obese individuals after bariatric surgery may facilitate weight loss.

16.6 Risks of Bariatric Surgery and Nutrient Deficiencies

A misconception about bariatric surgery is that it is highly related with risk of postsurgical complications and mortality. However, the prospective Longitudinal Assessment of Bariatric Surgery study [113] reported that the 30-day death rate of adults undergoing bariatric surgery (RYGB or LAGB) was 0.3 % [114]. Thus, there is little evidence for higher risk of mortality than standard operation (e.g., cardiovascular), and if anything, data suggest that bariatric surgery increases life expectancy [5, 115] due to reductions in obesity-induced cardiovascular risk factors such as diabetes. Although in rare cases, symptomatic hypoglycemia has been documented after RYGB and is associated with postprandial hyperinsulinemia due to elevated GLP-1 levels [116], the incidence of neuroglycopenia and seizures is rare. The conventional treatment of hypoglycemia in these patients involves carbohydrate restriction to minimize hypoglycemic-related episodes [117]. Despite little evidence for higher death rates after bariatric surgery, it is important to consider these risks against the long-term cardiovascular risk of continued obesity.

LAGB is viewed as the safest of the current bariatric procedures. It does not involve bowel anastomosis, and the risk of major hemorrhage, gastric perforation, and pulmonary embolism is less than 1 %. Late complications requiring reoperation include band slippage or prolapse (5–10 %) and band erosion (1–3 %). The entire

intestinal tract is left intact, so subsequent nutritional deficiencies are rare [118]. RYGB, on the other hand, carries an overall risk of major complication of 10–15 %. Anastomotic leak (1–5 %), pulmonary embolism (<1 %), and hemorrhage (1–4 %) can be life-threatening but are rare. Late complications such as ulcer or stricture formation at the gastrojejunostomy site occur in 5–10 % of cases and are managed nonoperatively [46].

Nutritional deficiencies occur in 30–70 % of patients. Patients at high risk of developing severe nutritional deficiencies include those who have lost more than 10 % of their body weight by 1 month and those with: anastomotic stenosis, surgical revision requirements, and persistent vomiting [119]. Protein calorie malnutrition is also a concern and can be recognized by signs such as edema, hypoalbuminemia, anemia, and hair loss. To minimize these effects, it is generally recommended that patients consume between 60 and 80 g/day of protein and approximately 800 kcal/day. Vitamin deficiencies can lead to peripheral neuropathy (B_{12}), Wernicke encephalopathy (B_1), and metabolic bone disease (Vitamin D). Subsequently, in addition to multivitamin supplementation, monitoring nutrient and vitamin levels after bariatric surgery is recommended at least every 6 months [119–121].

16.7 Implications for Gut Physiology Following Bariatric Surgery in Metabolic Disease

Bariatric surgery has proven valuable in identifying the gut as a critical organ regulating energy balance and glucose homeostasis (Fig. 16.5). The resulting weight loss from bariatric surgery is persistent in most patients, and the durability in weight loss is considered an underlying mechanism responsible for the majority of long-term glycemic control benefit and cardiovascular risk reduction. However, it is clear that alterations in gut physiology have distinct effects on not only appetite but also on insulin resistance, beta-cell function, hypertension, and blood lipids. Specifically, the prevailing view at this point in time is that alterations in enteroendocrine cells following bariatric surgery induce elevations in gut hormones (e.g., GLP-1, GIP, PYY, etc.) that promote satiety and improve insulin action, whereas the changes in gut microbiome and bile acids are new physiologic mechanisms contributing to the overall improvement in body fat and cardiovascular benefits following bariatric surgery. Taken together, the interaction between the gut and metabolic improvements seen following bariatric surgery warrants consideration of obesity-induced type 2 diabetes and cardiometabolic risk as "intestinal-related diseases." Thus, there is an opportunity going forward to stratify patients prior to bariatric surgery from a metabolic and gut microbial point of view, and this may impact the success of surgery on obesity-related disease. Further understanding of mechanisms related to gut metabolism following bariatric surgery, with or without lifestyle modification, will likely promote new medical strategies that will improve treatment and resolution of obesity, type 2 diabetes, and cardiovascular disease.

References

1. Obesity and overweight. Fact sheet no 311. Geneva: World Health Organization; 2013.
2. Flegal KM, Carroll MD, Kit BK, Ogden CL. Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999–2010. JAMA. 2012;307(5):491–7.
3. Ogden C, Carroll M, Curtin L, McDowell M, Tabak C, Flegal K. Prevalence of overweight and obesity in the United States, 1999–2004. JAMA. 2006;295(13):1549–55.
4. Noria SF, Grantcharov T. Biological effects of bariatric surgery on obesity-related comorbidities. Can J Surg. 2013;56(1):47–57.
5. Adams KF, Schatzkin A, Harris TB, Kipnis V, Mouw T, Ballard Barbash R, Hollenbeck A, Leitzmann MF. Overweight, obesity, and mortality in a large prospective cohort of persons 50 to 71 years old. N Engl J Med. 2006;355(8):763–78.
6. NIH conference. Gastrointestinal surgery for severe obesity. Consensus development conference panel. Ann Intern Med. 1991;115(12):956–61.
7. Steinbrook R. Surgery for severe obesity. N Engl J Med. 2004;350(11):1075–9.
8. Colquitt JL, Picot J, Loveman E, Clegg AJ. Cochrane Database Syst Rev. 2009;2:CD003641. doi:10.1002/14651858.CD003641.pub3. Review.
9. Parikh MS, Fielding GA, Ren CJ. U.S. experience with 749 laparoscopic adjustable gastric bands: intermediate outcomes. Surg Endosc. 2005;19(12):1631–5.
10. Gagner M, Deitel M, Kalberer TL, Erickson AL, Crosby RD. The second international consensus summit for sleeve gastrectomy. Surg Obes Relat. 2009;5(4):476–85.
11. Hutter MM, Schirmer BD, Jones DB, Ko CY, Cohen ME, Merkow RP, Nguyen NT. First report from the American college of surgeons bariatric surgery center network: laparoscopic sleeve gastrectomy has morbidity and effectiveness positioned between the band and the bypass. Ann Surg. 2011;254(3):410–20.
12. Kumar NT, Christopher CT. Endoscopic solutions for weight loss. Curr Opin Gastroenterol. 2011;27(5):407–11.
13. Rubino F. Bariatric surgery: effects on glucose homeostasis. Curr Opin Clin Nutr Metab Care. 2006;9(4):497–507.
14. Buchwald H, Estok R, Fahrbach K, Banel D, Jensen M, Pories W, Bantle J, Sledge I. Weight and type 2 diabetes after bariatric surgery: systematic review and meta-analysis. Am J Med. 2009;122(3):248–56. e5.
15. Stimac DM, Sanja. Endoscopic approaches to obesity. Dig Dis. 2012;30(2):187–95.
16. Blomain ES, Dirhan DA, Valentino MA, Kim GW, Waldman SA. ISRN Obes. 2013;2013:210524. doi:10.1155/2013/210524. eCollection 2013. Review.
17. Sjostrom L, Lindroos AK, Peltonen M, et al. Lifestyle, diabetes, and cardiovascular risk factors 10 years after bariatric surgery. N Engl J Med. 2004;351(26):2683–93.
18. Sjostrom L, Peltonen M, Jacobson P, et al. Bariatric surgery and long-term cardiovascular events. JAMA. 2012;307(1):56–65.
19. Buchwald H, Avidor Y, Braunwald E, Jensen MD, Pories W, Fahrbach K, Schoelles K. Bariatric surgery: a systematic review and meta-analysis. JAMA. 2004;292(14):1724–37.
20. Chapman AE, Kiroff G, Game P, Foster B, O'Brien P, Ham J, Maddern G. Laparoscopic adjustable gastric banding in the treatment of obesity: a systematic literature review. Surgery. 2004;135(3):326–51.
21. Brethauer SA, Chand B, Schauer PR. Risks and benefits of bariatric surgery: current evidence. Cleve Clin J Med. 2006;73(11):993–1007.
22. Despres JP, Lemieux I, Prud'homme D. Treatment of obesity: need to focus on high risk abdominally obese patients. BMJ. 2001;322(7288):716–20.
23. DeFronzo RA, Abdul Ghani MA. Preservation of ß-cell function: the key to diabetes prevention. J Clin Endocrinol Metab. 2011;96(8):2354–66.
24. Samuel VT, Shulman GI. Mechanisms for insulin resistance: common threads and missing links. Cell. 2012;148(5):852–71.

25. Pories WJ, Caro JF, Flickinger EG, Meelheim HD, Swanson MS. The control of diabetes mellitus (NIDDM) in the morbidly obese with the greenville gastric bypass. Ann Surg. 1987;206(3):316–23.
26. Pories WJ, Swanson MS, MacDonald KG, Long SB, Morris PG, Brown BM, Barakat HA, deRamon RA, Israel G, Dolezal JM. Who would have thought it? An operation proves to be the most effective therapy for adult-onset diabetes mellitus. Ann Surg. 1995;222(3):339–50.
27. Scopinaro N, Papadia F, Marinari G, Camerini G, Adami G. Long-term control of type 2 diabetes mellitus and the other major components of the metabolic syndrome after biliopancreatic diversion in patients with BMI < 35 kg/m2. Obes Surg. 2007;17(2):185–92.
28. Scopinaro N, Marinari GM, Camerini GB, Papadia FS, Adami GF. Specific effects of biliopancreatic diversion on the major components of metabolic syndrome: a long-term follow-up study. Diabetes Care. 2005;28(10):2406–11.
29. Brethauer SA, Aminian A, Romero-Talamás H, Batayyah E, Mackey J, Kennedy L, Kashyap SR, Kirwan JP, Rogula T, Kroh M, Chand B, Schauer PR. Can diabetes be surgically cured? Long-term metabolic effects of bariatric surgery in obese patients with type 2 diabetes mellitus. Ann Surg. 2013;258(4):628–36.
30. Torquati A, Lutfi R, Abumrad N, Richards WO. Is roux-en-Y gastric bypass surgery the most effective treatment for type 2 diabetes mellitus in morbidly obese patients? J Gastrointest Surg. 2005;9(8):1112–6.
31. Pories WJ, MacDonald KG, Flickinger EG, Dohm GL, Sinha MK, Barakat HA, May HJ, Khazanie P, Swanson MS, Morgan E. Is type II diabetes mellitus (NIDDM) a surgical disease? Ann Surg. 1992;215(6):633–42.
32. Sugerman HJ, Wolfe LG, Sica DA, Clore JN. Diabetes and hypertension in severe obesity and effects of gastric bypass-induced weight loss. Ann Surg. 2003;237(6):751–6.
33. Dixon JB, O'Brien PE, Playfair J, Chapman L, Schachter LM, Skinner S, Proietto J, Bailey M, Anderson M. Adjustable gastric banding and conventional therapy for type 2 diabetes: a randomized controlled trial. JAMA. 2008;299(3):316–23.
34. Mingrone G, Panunzi S, De Gaetano A, Guidone C, Iaconelli A, Leccesi L, Nanni G, Pomp A, Castagneto M, Ghirlanda G, Rubino F. Bariatric surgery versus conventional medical therapy for type 2 diabetes. N Engl J Med. 2012;366(17):1577–85.
35. Ikramuddin S, Korner J, Lee WJ, Connett JE, Inabnet WB, Billington CJ, Thomas AJ, Leslie DB, Chong K, Jeffery RW, Ahmed L, Vella A, Chuang LM, Bessler M, Sarr MG, Swain JM, Laqua P, Jensen MD, Bantle JP. Roux-en-Y gastric bypass vs. intensive medical management for the control of type 2 diabetes, hypertension, and hyperlipidemia: the diabetes surgery study randomized clinical trial. JAMA. 2013;309(21):2240–9.
36. Schauer PR, Kashyap SR, Wolski K, Brethauer SA, Kirwan JP, Pothier CE, Thomas S, Abood B, Nissen SE, Bhatt DL. Bariatric surgery versus intensive medical therapy in obese patients with diabetes. N Engl J Med. 2012;366(17):1567–76.
37. Bouldin MJ, Ross LA, Sumrall CD, Loustalot FV, Low AK, Land KK. The effect of obesity surgery on obesity comorbidity. Am J Med Sci. 2006;331(4):183–93.
38. Neter JE, Stam BE, Kok FJ, Grobbee DE, Geleijnse JM. Influence of weight reduction on blood pressure: a meta-analysis of randomized controlled trials. Hypertension. 2003;42(5):878–84.
39. Dornfeld LP, Maxwell MH, Waks AU, Schroth P, Tuck ML. Obesity and hypertension: long-term effects of weight reduction on blood pressure. Int J Obes. 1985;9(6):381–9.
40. Hypertension Prevention Trial Research Group. The hypertension prevention trial: three-year effects of dietary changes on blood pressure. Hypertension prevention trial research group. Arch Intern Med. 1990;150(1):153–62.
41. Sjöström CD, Peltonen M, Sjöström L. Blood pressure and pulse pressure during long-term weight loss in the obese: the Swedish obese subjects (SOS) intervention study. Obes Res. 2001;9(3):188–95.
42. Benetos A, Rudnichi A, Safar M, Guize L. Pulse pressure and cardiovascular mortality in normotensive and hypertensive subjects. Hypertension. 1998;32(3):560–4.

43. Rubino F, R'bibo SL, del Genio F, Mazumdar M, McGraw T. Metabolic surgery: the role of the gastrointestinal tract in diabetes mellitus. Nat Rev Endocrinol. 2010;6(2):102–9.
44. Marceau P, Hould FS, Simard S, Lebel S, Bourque RA, Potvin M, Biron S. Biliopancreatic diversion with duodenal switch. World J Surg. 1998;22(9):947–54.
45. Brolin R, LaMarca L, Kenler H, Cody R. Malabsorptive gastric bypass in patients with superobesity. J Gastrointest Surg. 2002;6(2):195–203.
46. Kashyap SR, Gatmaitan P, Brethauer SA, Schauer PR. Bariatric surgery for type 2 diabetes: weighing the impact for obese patients. Cleve Clin J Med. 2010;77(7):468–76.
47. Cummings DE. Endocrine mechanisms mediating remission of diabetes after gastric bypass surgery. Int J Obes. 2009;33 Suppl 1:S33–40.
48. Pournaras DJ, le Roux CW. Obesity, gut hormones, and bariatric surgery. World J Surg. 2009;33(10):1983–8.
49. Mithieux G. A novel function of intestinal gluconeogenesis: central signaling in glucose and energy homeostasis. Nutrition. 2009;25(9):881–4.
50. Rubino F. The early effect of the roux-en-Y gastric bypass on hormones involved in body weight regulation and glucose metabolism. Ann Surg. 2004;240(2):236–42.
51. Salinari S, Debard C, Bertuzzi A, Durand C, Zimmet P, Vidal H, et al. PLoS One. 2013;8(2):e56258. doi:10.1371/journal.pone.0056258. Epub 2013 Feb 20.
52. Ionut V, Bergman RN. Mechanisms responsible for excess weight loss after bariatric surgery. J Diabetes Sci Technol. 2011;5(5):1263–82.
53. Saeidi N, Meoli L, Nestoridi E, Gupta NK, Kvas S, Kucharczyk J, Bonab AA, Fischman AJ, Yarmush ML, Stylopoulos N. Reprogramming of intestinal glucose metabolism and glycemic control in rats after gastric bypass. Science. 2013;341(6144):406–10.
54. Vollmer K, Holst JJ, Baller B, Ellrichmann M, Nauck MA, Schmidt WE, Meier JJ. Predictors of incretin concentrations in subjects with normal, impaired, and diabetic glucose tolerance. Diabetes. 2008;57(3):678–87.
55. Holst JJ, Vilsbll T, Deacon CF. The incretin system and its role in type 2 diabetes mellitus. Mol Cell Endocrinol. 2009;297(1–2):127–36.
56. Laferrère B, Teixeira J, McGintry J, Tran H, Egger JR, Colarusso A, Kovack B, Bawa B, Koshy N, Lee H, Yapp K, Olivan B. Effect of weight loss by gastric bypass surgery versus hypocaloric diet on glucose and incretin levels in patients with type 2 diabetes. J Clin Endocrinol Metab. 2008;93(7):241979–85.
57. Samat A, Malin SK, Huang H, Schauer PR, Kirwan JP, Kashyap SR. Ghrelin suppression is associated with weight loss and insulin action following gastric bypass surgery at 12 months in obese adults with type 2 diabetes. Diabetes Obes Metab. 2013;15(10):963–6.
58. Kashayp SR, Bhatt DL, Wolski K, Wantanabe RM, Abdul-Ghani M, Abood B, Pothier CE, Brethauer SA, Nissen S, Gupta M, Kirwan JP, Schauer PR. Metabolic effects of bariatric surgery in patients with moderate obesity and type 2 diabetes: analysis of a randomized control trial comparing surgery vs. intensive medical treatment. Diabetes Care. 2013;36(8):2175–82.
59. Malin SK, Samat A, Wolski K, Abood B, Pothier CE, Bhatt DL, Nissen S, Brethauer SA, Schauer PR, Kirwan JP, Kashyap SR. Improved acylated ghrelin suppression at 2 years in obese patients with type 2 diabetes: effects of bariatric surgery vs standard medical therapy. Int J Obes. 2013;36(8):2175–82.
60. Kashyap SR, Daud S, Kelly KR, Gastaldelli A, Win H, Brethauer S, Kirwan JP, Schauer PR. Acute effects of gastric bypass versus gastric restrictive surgery on beta-cell function and insulinotropic hormones in severely obese patients with type 2 diabetes. Int J Obes. 2010;34(3):462–71.
61. Quercia I, Dutia R, Kotler DP, Belsley S, Laferrerre B. Gastrointestinal changes after bariatric surgery. Diabetes Metab. 2013;S1262–3636
62. Korner J, Bessler M, Inabnet W, Taveras C, Holst JJ. Exaggerated glucagon-like peptide-1 and blunted glucose-dependent insulinotropic peptide secretion are associated with roux-en-Y gastric bypass but not adjustable gastric banding. Surg Obes Relat Dis. 2007;3(6):597–601.

63. Hanusch Enserer U, Ghatei MA, Cauza E, Bloom SR, Prager R, Roden M. Relation of fasting plasma peptide YY to glucose metabolism and cardiovascular risk factors after restrictive bariatric surgery. Wien Klin Wochenschr. 2007;119(9–10):291–6.
64. le Roux CW, Aylwin SJ, Batterham RL, Borg CM, Coyle F, Prasad V, Shurey S, Ghatei MA, Patel AG, Bloom SR. Gut hormone profiles following bariatric surgery favor an anorectic state, facilitate weight loss, and improve metabolic parameters. Ann Surg. 2006;243(1):108–14.
65. Laferrère B, Swerdlow N, Bawa B, Arias S, Bose M, Olivan B, Teixeira J, McGinty J, Rother KI. Rise of oxyntomodulin in response to oral glucose after gastric bypass surgery in patients with type 2 diabetes. J Clin Endocrinol Metab. 2010;95(8):4072–6.
66. Tong J, Prigeon RL, Davis HW, Bidlingmaier M, Kahn SE, Cummings D, Tschop M, D'Alessio D. Ghrelin suppresses glucose-stimulated insulin secretion and deteriorates glucose tolerance in healthy humans. Diabetes. 2010;59(9):2145–51.
67. Cummings DE, Weigle DS, Frayo RS, Breen P, Ma M, Dellinger EP, Purnell J. Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. N Engl J Med. 2002;346(21):1623–30.
68. Falken Y, Hellstrom PM, Holst JJ, Naslund E. Changes in glucose homeostasis after roux-en-Y gastric bypass surgery for obesity at day three, two months, and one year after surgery: role of gut peptides. J Clin Endocrinol Metab. 2011;96(7):2227–35.
69. Breen DM, Rasmussen BA, Cote CD, Jackson VM, Lam TK. Nutrient-sensing mechanisms in the gut as therapeutic targets for diabetes. Diabetes. 2013;62(9):3005–13.
70. Hildebrandt MA, Hoffmann C, Sherrill-Mix SA, Keilbaugh SA, Hamady M, Chen YY, Knight R, Ahima RS, Bushman F, Wu GD. High-fat diet determines the composition of the murine gut microbiome independently of obesity. Gastroenterology. 2009;137(5):1716–24.
71. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature. 2006;444(7122):1022–3.
72. Ding S, Chi MM, Scull BP, Rigby R, Schwerbrock NM, Magness S, et al. PLoS One. 2010;5(8):e12191. doi:10.1371/journal.pone.0012191.
73. Aron-Wisnewsky J, Dore J, Clement K. The importance of the gut microbiota after bariatric surgery. Nat Rev Gastroenterol Hepatol. 2012;9(10):590–8.
74. Swartz TD, Duca FA, de Wouters T, Sakar Y, Covasa M. Up-regulation of intestinal type 1 taste receptor 3 and sodium glucose luminal transporter-1 expression and increased sucrose intake in mice lacking gut microbiota. Br J Nutr. 2012;107(5):621–30.
75. Schwiertz A, Taras D, Schafer K, Beijer S, Bos NA, Donus C, Hardt PD. Microbiota and SCFA in lean and overweight healthy subjects. Obesity. 2010;18(1):190–5.
76. Zhang H, DiBaise JK, Zuccolo A, Kudrna D, Braidotti M, Yu Y, Parameswaran P, Crowell MD, Wing R, Rittmann BE, Krajmalnik-Brown R. Human gut microbiota in obesity and after gastric bypass. Proc Natl Acad Sci U S A. 2009;106(7):2365–70.
77. Furet JP, Kong LC, Tap J, Poitou C, Basdevant A, Bouillot JL, Mariat D, Corthier G, Dore J, Henegar C, Rizkalla S, Clement K. Differential adaptation of human gut microbiota to bariatric surgery-induced weight loss: links with metabolic and low-grade inflammation markers. Diabetes. 2010;59(12):3049–57.
78. Kong LC, Tap J, Aron-Wisnewsky J, Pelloux V, Basdevant A, Bouillot JL, Zucker JD, Dore J, Clement K. Gut microbiota after gastric bypass in human obesity: increased richness and associations of bacterial genera with adipose tissue genes. Am J Clin Nutr. 2013;98(1):16–24.
79. Vrieze A, Van Nood E, Holleman F, Salojarvi J, Kootte RS, Bartelsman JF, Dallinga-Thie GM, Ackermans MT, Serlie MJ, Oozeer R, Derrien M, Druesne A, Van Hylckama Vlieg JE, Bloks VW, Groen AK, Heilig HG, Zoetendal EG, Stroes ES, de Vos WM, Hoekstra JB, Nieuwdorp M. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. Gastroenterology. 2012;143(4):913–6.e7.
80. Nie B, Park HM, Kazantzis M, Lin M, Henkin A, Ng S, Song S, Chen Y, Tran H, Lai R, Her C, Maher JJ, Forman BM, Stahl A. Specific bile acids inhibit hepatic fatty acid uptake in mice. Hepatology. 2012;56(4):1300–10.

81. Walker AW, Duncan SH, McWilliam Leitch EC, Child MW, Flint H. pH and peptide supply can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities from the human colon. Appl Environ Microbiol. 2005;71(7):3692–700.
82. Laferrère B. Gut feelings about diabetes. Endocrinol Nutr. 2012;59(4):254–60.
83. Harris K, Kassis A, Major G, Chou CJ. J Obes. 2012;2012:879151. doi:10.1155/2012/879151. Epub 2012 Jan 24. Erratum in: J Obes. 2012;2012:782920.
84. Tilg H, Kaser A. Gut microbiome, obesity, and metabolic dysfunction. J Clin Invest. 2011;121(6):2126–32.
85. Cani PD, Knauf C, Iglesias MA, Drucker DJ, Delzenne NM, Burcelin R. Improvement of glucose tolerance and hepatic insulin sensitivity by oligofructose requires a functional glucagon-like peptide 1 receptor. Diabetes. 2006;55(5):1484–90.
86. Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, Gibson GR, Delzenne NM. Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia. 2007;50(11):2374–83.
87. Troseid M, Nestvold TK, Rudi K, Thoresen H, Nielsen E, Lappegard KT. Plasma lipopolysaccharide is closely associated with glycemic control and abdominal obesity: evidence from bariatric surgery. Diabetes Care. 2013;36(11):3627–32.
88. Creely SJ, McTernan PG, Kusminski CM, Fisher fM, Da Silva NF, Khanolkar M, Evans M, Harte AL, Kumar S. Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. Am J Physiol Endocrinol Metab. 2007;292(3):E740–7.
89. Lassenius MI, Pletillainen KH, Kaartinen K, Pussinen PJ, Syrjanen J, Forsblom C, Porsti I, Rissanen A, Kaprio J, Mustonen J, Groop PH, Lehto. Bacterial endotoxin activity in human serum is associated with dyslipidemia, insulin resistance, obesity, and chronic inflammation. Diabetes Care. 2011;34(8):1809–15.
90. Shen J, Obin MS, Zhao L. The gut microbiota, obesity and insulin resistance. Mol Aspects Med. 2013;34(1):39–58.
91. Arora T, Sharma R, Frost G. Propionate. Anti-obesity and satiety enhancing factor? Appetite. 2011;56(2):511–5.
92. Soliman MM, Ahmed MM, Salah-Eldin AE, Abdel-Aal A. Butyrate regulates leptin expression through different signaling pathways in adipocytes. J Vet Sci. 2011;12(4):319–23.
93. Liou AP, Paziuk M, Luevano Jr JM, Machineni S, Turnbaugh PJ, Kaplan LM. Sci Transl Med. 2013;5(178):178–41. doi:10.1126/scitranslmed.3005687.
94. Backhed F, Manchester JK, Semenkovich CF, Gordon J. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci U S A. 2007;104(3):979–84.
95. Peng Y, Rideout DA, Rakita SS, Gower Jr WR, You M, Murr MM. Does LKB1 mediate activation of hepatic AMP-protein kinase (AMPK) and sirtuin1 (SIRT1) after roux-en-Y gastric bypass in obese rats? J Gastrointest Surg. 2010;14(2):221–8.
96. Bjursell M, Admyre T, Goransson M, Marley AE, Smith DM, Oscarsson J, Bohlooly-Y M. Improved glucose control and reduced body fat mass in free fatty acid receptor 2-deficient mice fed a high-fat diet. Am J Physiol Endocrinol Metab. 2011;300(1):E211–20.
97. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, Feldstein AE, Britt EB, Fu X, Chung YM, Wu Y, Schauer PR, Smith JD, Allayee H, Tang WH, DiDonato JA, Lusis AJ, Hazen SL. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature. 2011;472(7341):57–63.
98. Seyfried F, Li JV, Miras AD, Cluny NL, Lannoo M, Fenske WK, Sharkey KA, Nicholson JK, le Roux CW, Holmes E. Urinary phenotyping indicates weight loss-independent metabolic effects of roux-en-Y gastric bypass in mice. J Proteome Res. 2013;12(3):1245–53.
99. Tang WH, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, Wu Y, Hazen SL. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med. 2013;368(17):1575–84.
100. Nicholson JK, Holmes E, Wilson ID. Gut microorganisms, mammalian metabolism and personalized health care. Nat Rev Microbiol. 2005;3(5):431–8.

101. Calvani R, Miccheli A, Capuani G, Tomassini Miccheli A, Puccetti C, Delfini M, Iaconelli A, Nanni G, Mingrone G. Gut microbiome-derived metabolites characterize a peculiar obese urinary metabotype. Int J Obes. 2010;34(6):1095–8.
102. Holmes E, Loo RL, Stamler J, Bictash M, Yap IK, Chan Q, Ebbels T, De Iorio M, Brown IJ, Veselkov KA, Daviglus ML, Kesteloot H, Ueshima H, Zhao L, Nicholson JK, Elliott P. Human metabolic phenotype diversity and its association with diet and blood pressure. Nature. 2008;453(7193):396–400.
103. Ahmad NN, Pfalzer A, Kaplan LM. Roux-en-Y gastric bypass normalizes the blunted postprandial bile acid excursion associated with obesity. Int J Obes. 2013;37(12):1553–9.
104. Glicksman C, Pournaras DJ, Wright M, Roberts R, Mahon D, Welbourn R, Sherwood R, Alaghband-Zadeh J, le Roux CW. Postprandial plasma bile acid responses in normal weight and obese subjects. Ann Clin Biochem. 2010;47(5):482–4.
105. Patti ME, Houten SM, Bianco AC, Bernier R, Larsen PR, Holst JJ, Badman MK, Maratos-Flier E, Mun EC, Pihlajamaki J, Auwerx J, Goldfine AB. Serum bile acids are higher in humans with prior gastric bypass: potential contribution to improved glucose and lipid metabolism. Obesity. 2009;17(9):1671–7.
106. Pournaras DJ, Glicksman C, Vincent RP, Kuganolipava S, Alaghband-Zadeh J, Mahon D, Bekker JH, Ghatei MA, Bloom SR, Walters JR, Welbourn R, le Roux CW. The role of bile after roux-en-Y gastric bypass in promoting weight loss and improving glycaemic control. Endocrinology. 2012;153(8):3613–9.
107. Katsuma S, Hirasawa A, Tsujimoto G. Bile acids promote glucagon-like peptide-1 secretion through TGR5 in a murine enteroendocrine cell line STC-1. Biochem Biophys Res Commun. 2005;329(1):386–90.
108. Thomas C, Gioiello A, Noriega L, Strehle A, Oury J, Rizzo G, Macchiarulo A, Yamamoto H, Mataki C, Pruzanski M, Pellicciari R, Auwerx J, Schoonjans K. TGR5-mediated bile acid sensing controls glucose homeostasis. Cell Metab. 2009;10(3):167–77.
109. Ryan KK, Tremaroli V, Clemmensen C, Kovatcheva-Datchary P, Myronovych A, Karns R, et al. Nature. 2014;509(7499):183–8. doi:10.1038/nature13135. Epub 2014.
110. Düfer M, Hörth K, Wagner R, Schittenhelm B, Prowald S, Wagner TF, Oberwinkler J, Lukowski R, Gonzalez FJ, Krippeit-Drews P, Drews G. Bile acids acutely stimulate insulin secretion of mouse beta-cells via farnesoid X receptor activation and K(ATP) channel inhibition. Diabetes. 2012;61(6):1479–89.
111. Lundasen T, Galman C, Angelin B, Rudling M. Circulating intestinal fibroblast growth factor 19 has a pronounced diurnal variation and modulates hepatic bile acid synthesis in man. J Intern Med. 2006;260(6):530–6.
112. Watanabe M, Houten SM, Mataki C, Christoffolete MA, Kim BW, Sato H, Messaddeq N, Harney JW, Ezaki O, Kodama T, Schoonjans K, Bianco AC, Auwerx J. Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. Nature. 2006;439(7075):484–9.
113. Ockenga J, Valentini L, Schuetz T, Wohlgemuth F, Glaeser S, Omar A, Kasim E, duPlessis D, Featherstone K, Davis JR, Tietge UJ, Kroencke T, Biebermann H, Köhrle J, Brabant G. Plasma bile acids are associated with energy expenditure and thyroid function in humans. J Clin Endocrinol Metab. 2012;97(2):535–42.
114. Flum DR, Belle SH, King WC, Wahed AS, Berk P, Chapman W, Pories W, Courcoulas A, McCloskey C, Mitchell J, Patterson E, Pomp A, Staten MA, Yanovski SZ, Thirlby R, Wolfe B. Perioperative safety in the longitudinal assessment of bariatric surgery. N Engl J Med. 2009;361(5):445–54.
115. Sjöström L, Narbro K, Sjöström CD, Karason K, Larsson B, Wedel H, Lystig T, Sullivan M, Bouchard C, Carlsson B, Bengtsson C, Dahlgren S, Gummesson A, Jacobson P, Karlsson J, Lindroos AK, Lönroth H, Näslund I, Olbers T, Stenlöf K, Torgerson J, Agren G, Carlsson LM. Effects of bariatric surgery on mortality in Swedish obese subjects. N Engl J Med. 2007;357(8):741–52.
116. Service GJ, Thompson GB, Service FJ, Andrews JC, Collazo-Clavell ML, Lloyd RV. Hyperinsulinemic hypoglycemia with nesidioblastosis after gastric-bypass surgery. N Engl J Med. 2005;353(3):249–54.

117. Goldfine AB, Mun EC, Devine E, Bernier R, Baz Hecht M, Jones DB, Schneider BE, Holst JJ, Patti ME. Patients with neuroglycopenia after gastric bypass surgery have exaggerated incretin and insulin secretory responses to a mixed meal. J Clin Endocrinol Metab. 2007;92(12):4678–85.
118. Tucker ON, Szomstein S, Rosenthal RJ. Nutritional consequences of weight-loss surgery. Med Clin N Am. 2007;91(3):499–514. xii.
119. Davies DJ, Baxter JM. Nutritional deficiencies after bariatric surgery. Obes Surg. 2007;17(9):1150–8.
120. Ritz P, Becouarn G, Douay O, Salle A, Topart P, Rohmer V. Gastric bypass is not associated with protein malnutrition in morbidly obese patients. Obes Surg. 2009;19(7):840–4.
121. Angstadt JD, Bodziner RA. Peripheral polyneuropathy from thiamine deficiency following laparoscopic roux-en-Y gastric bypass. Obes Surg. 2005;15(6):890–2.

ERRATUM

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Chapter 4

The aim of this erratum is to acknowledge the original sources used in this book. Permission has been obtained for the reused text, however in some cases the authors omitted to credit the source correctly.

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Where reference 29 is cited please note the following credit text reprinted with permission of Future Medicine Ltd from Sun J, Beger RD, Schnackenberg LK. Metabolomics as a tool for personalizing medicine: 2012 update. Personalized Medicine. 2013;10(2):149–61**.** With permission from Future Medicine Ltd.

Please also note that the following citations were omitted:

"Many phenotypic and genotypic states, such as a toxic response to a drug, are predicted by differences in the concentrations of functionally relevant metabolites in biological fluids and tissues" [14].
The previous reference was: [none].

The online version of the original book can be found at
http://dx.doi.org/10.1007/978-1-4471-6539-2

“Therefore, it is critical to be able to assess an individual’s metabolic phenotype, which will provide useful information for determining the correct drug and dose treatment and predicting the individual response following a therapeutic intervention.”
“The metabolic phenotype (metabotype) is a result of the overall influences of the patient’s physiological status, gut microbiome status, and chemical, genetic, and other environmental factors. Changes in the metabotype reflected in the biofluid or tissue evaluated occur downstream of alterations in gene and protein expression. As such, the metabotype, which comprises the genotype and phenotype, represents the ultimate biological endpoint and can provide useful information about an individual’s current physiological status that can be used for predicting the outcome prior to a therapeutic intervention” [29].
The previous reference was: [none].

“metabonomics provides the capability to analyze large arrays of metabolites for extracting biochemical information that reflects true functional endpoints of overt biological events, whereas other functional genomics technologies such as transcriptomics and proteomics merely indicate the potential cause for phenotypic response”. “Metabonomics bridges this information gap by depicting, in particular, such functional information because metabolite differences in biological fluids and tissues provide the closest link to the various phenotypic responses. Such changes in the biochemical phenotype are of direct interest to pharmaceutical, biotech, and health industries once appropriate technology allows the cost-efficient mining and integration of this information” [14].
The previous reference was: [none].

“necessarily predict drug effects, toxicological response, or disease states at the phenotype level unless functional validation is added” [14].
The previous reference was: [none].

“Understanding these connections, in turn, may eventually lead to more targeted nutrition or therapies and more refined disease risk stratification. These could result in a critical step towards personalized health care and nutrition based on a combination of genotyping and metabolic characterization” [14].
The previous reference was: [none].

“will provide a more personalized approach to patient treatment with a more positive outcome by diagnosing not only the disease but also the disease phenotype” [29].
The previous reference was: [none].

“The metabolic profile represents the phenotype of the organism and reflects the overall biological influences, including interactions between multiple genomes (e.g., genomes from animals or humans and their gut microbiome)”. “Pharmacometabonomics uses the pre-dose metabolite profiling in the biofluids or fecal extracts to predict the responses of an individual to a drug/nutritional intervention and to identify surrogate markers for subsequent drug administration.

Furthermore, pharmacometabonomics is capable of providing useful drug pharmacokinetic and drug metabolite information for an individual, which can provide a mechanistic understanding of varied responses between individuals to the efficacy, side effects, and toxicity of a drug" [29].
The previous reference was: [none].

"In view of the chemical and physical diversity of small biological molecules, the challenge remains in developing protocols to gather the whole "metabolome"" [14].
The previous reference was: [none].

"Metabonomics studies demonstrate its potential impact on the drug discovery process by enabling the incorporation of safety endpoints much earlier in the drug discovery process, reducing the likelihood (and cost) of later stage attrition" [14].
The previous reference was: [none].

"The metabolic profile of the pre-dose urine samples can predict both individual susceptibility to acetaminophen-induced toxicity and liver injury and also can predict the relative excretion levels of acetaminophen metabolites in the forms of glucuronide and sulfate conjugates" [29].
The previous reference was: [none].

"NMR-based metabonomics approaches were employed to profile pre- and postdose urinary metabolites and discovered that human subjects with high pre-dose levels of *p*-cresol (one of the metabolites related to an individual's gut microbiome) had lower concentrations of acetaminophen metabolites" [29]. From postdose urine samples, it was possible to determine the proportions of the various drug metabolites excreted by each subject, which was known to show considerable intersubject variation. The findings indicate that each individual, colonized by a unique assortment of trillions of microbes, responds to a drug differently, either beneficially or adversely. It provides the information of how a particular drug is metabolized and excreted by each individual. Such information may have a major influence on the drug safety and efficacy. "This study demonstrates that evaluation of a metabolic phenotype by metabolic profiling could play an important role in drug metabolism and toxicity, as well as in personalized health care" [29].
The previous reference was: [none].

"In both preclinical screening and mechanistic exploration, metabolic profiling can offer rapid, noninvasive toxicological information that is robust and reproducible, with little or no added technical resources to existing studies in drug metabolism and toxicity" [14].
The previous reference was: [none].

"The metabolome, or the complete metabolite composition of a system such as a cell or organism, is the end product not only of the genetic blueprint of an organism but also all influential factors to which the organism is exposed, such as nutrition, environmental factors, or treatments" [55].
The previous reference was: [none].

"Metabonomic strategies together with advanced chemometric and bioinformatic tools [44, 51, 52] can help track the interaction between nutrients and human metabolism, as well as the involvement of the genome and the gut microbiome, in overall human health, and can be considered critical measures of function or phenotype" [53] [55].
The previous reference was: [53].

"component of nutritional phenotypes and will enable individualized dietary recommendations. The relation between diet and metabonomic profiles as well as between those profiles and health and disease needs to be established" [14].
The previous reference was: [none].

"Many progresses are made through a systematic inventory of all relevant parameters by using different "-omics" technologies and application of new bioinformatics tools together with extensive data warehousing to unravel disease mechanisms, define biomarkers, or apply personalized medication" (Fig. 4.4) [55].
The previous reference was: [none].

"Likely, in cases of impairment of human homeostasis, the patients would thus develop a coordinated approach to reestablish a metabolic trajectory for the individual consistent with their metabolic phenotype" [55].
The previous reference was: [none].

"The results of this study highlight the diversity of physiological variations of human metabolism and emphasize the effect of nutritional phytochemicals in modulating human metabolism and maintaining homeostasis of human gut eco-system" [55].
The previous reference was: [none].

"Xie et al. [59] performed a study on 20 volunteers to investigate the human metabolic response to drinking Pu-erh tea over a 6-week period, using a UPLCQTOFMS-based metabonomics approach. The final metabolic profile was greatly altered by Pu-erh tea consumption. The trajectory of the PCA scores plot based on urine data revealed a clear separation tendency of samples obtained before (days 1 and 7), during (days 16, 21, and 28), and after tea ingestion (washout period; days 30, 36, 42). Interestingly, the metabolic patterns of samples obtained 2 weeks after tea intake are still distinct from the pre-dose pattern, probably due to the possibility that Pu-erh tea may change the structure of the resident gut microbiota" [55].
The previous reference was: [none].

This was followed by a more in-depth study of Pu-erh tea in human subjects [60]. "Urine samples were collected at 0, 1, 3, 6, 9, 12, and 24 h within the first 24 h of tea intake and once a day during a 2-week daily Pu-erh tea ingestion phase and a 2-week "washout" phase. The dynamic concentration profile of bioavailable plant molecules (due to in vivo absorption and the hepatic and gut bacterial metabolism) and the human metabolic response profile were identified and correlated with each

other, highlighting the great potential of metabonomic strategy to unravel the complex interactions between multicomponent nutraceuticals and human metabolic system in nutritional studies" [55].
The previous reference was: [none].

"The goal of nutrition has extended beyond just ameliorating or curing diseases and now aims to achieve an overall objective in preventing diseases and improving health. Therefore, the pivotal scientific objective has become understanding the relationship between diet (both macroand micronutrients) and health/diseases. The comprehensive analysis of the metabolome via metabonomics will serve as the bioinformational base for modern nutritional science. Biomarkers and/or patterns of expression will undoubtedly have the potential to be used for human health assessment (Fig. 4.5). Together this indicates that the future goal of nutritional research will be to predict the likelihood of future diseases within the context of an individual's overall heath and identify causal risk factors, leading to recommendations for appropriate intervention, such as to change dietary habits or to avoid homeostasis loss and maintain healthy status" [55].
The previous reference was: [none].

"Whereas the human genome is the set of all genes in a human being, the human metabolome is the set of all metabolites in a human being. Metabonomics bridges the gap between the genotype and the phenotype and is an important basis of personalized medicine. Metabonomics has been used to identify biomarkers for disease and the effects of drugs" [14]. Various metabonomic technologies including NMR and MS have been intensively applied to metabonomics study. "Pharmacometabonomic approach to personalizing drug treatment uses a combination of pre-dose metabolite profiling and chemometrics to model and predict the responses of individual subjects. Metabonomics also has a role to play in assessing drug toxicity and in guiding nutrition" [14].
The previous reference was: [none].

"An approach referred to as integrative personal "-omics" profile evaluated genomic, transcriptomic, proteomic, metabonomic, and antibody profiles from a single individual over a 14-month period. The study revealed changes in the "-omics" profiles between healthy and viral states and between nondiabetic and diabetic states throughout the study period. Furthermore, it was noted that disease risk could be assessed from the individual and maternal genome sequences. This study demonstrated that the integration of genomics data with other dynamic "-omics" datasets can be used to predict various medical risks and the health status of an individual. Such datasets for many individuals may provide a database that can be used for enhancing diagnostics, monitoring, and treatment in the future with metabonomics playing a critical role" [29].
The previous reference was: [none].

Chapter 9

The aim of this erratum is to acknowledge the original sources used in this book. The authors omitted a reference from the list and apologize for this oversight.

The following reference is missing from the list:
95. S Collino, FP Martin, LG Karagounis, et al. Musculoskeletal system in the old age and the demand for healthy ageing biomarkers. Mech.Ageing Dev. 2013; 134: 541-7.

All excerpts from this reference are with kind permission from Elsevier.

The corrected citations:
"Aging can commonly be characterized as a progressive, generalized impairment of biological functions resulting in an increased vulnerability to environmental challenge and a higher risk of disease and death" [1, 95].
The previous reference was: [1].

"Understanding the physiology of aging is of tremendous importance to allow populations to grow old disease-free and with a good quality of life. In this respect, it is important to understand the natural aging process and to elucidate where lifestyle and/or dietary interventions can have an impact" [95].
The previous reference was: [none].

"Imaging techniques and flux analysis using stable isotopes are parallel technologies to obtain metabolite information. Multivariate statistical and bioinformatics techniques are ultimately used for data mining the complex metabolic profiles which encapsulate information on genetics, environmental factors, gut microbiota activity, and lifestyle and food habits. This combined strategy sustains the complex process of identifying emerging biomarkers indicative of the individual response to specific physiological factors and/or nutritional/physical interventions" [95].
The previous reference was: [none].

"In addition, elderly may be also prone to be resistant to anabolic stimuli which is likely a key factor in the loss of skeletal muscle mass with aging [95]".
The previous reference was: [none].

"As centenarians well represent the model of successful and healthy aging [14], there are many important implications in revealing the underlying molecular mechanisms behind such acquired longevity" [95].
The previous reference was: [none].

"Untargeted metabonomics profiling of urine revealed that the longevity process is marked by changes in gut microbial metabolites, as displayed by increase urinary excretions of phenylacetylglutamine, p-cresol sulfate, and 2-hydroxybenzoate. Moreover, centenarian offsprings, who are reported to have delay in age-related diseases, have a distinct serum metabolic phenotype from siblings of non-long-living parents, with changes in amino acids (serine, phenylalanine) and lysophosphatidylcholines" [95].

The previous reference was: [none].

“Additionally, an investigation on specific lipids associated with familial longevity in females was explored by Gonzalez-Covarrubias et al. in the plasma lipidome by measuring 128 lipid species in 1,526 offspring of nonagenarians (59 years±6.6) and 675 (59 years±7.4) controls from the Leiden Longevity Study” [16,95].
The previous reference was: [16].

“Here in women 19 lipid species associated with familial longevity with ether phosphocholine and sphingomyelin species are identified as candidate longevity markers. While this population reflects a different cohort with plausible differences in lifestyle and dietary habits, common to the previous study, the authors postulated that lipid signatures in plasma lipidome of female individuals could suggest a better antioxidant capacity and lower lipid peroxidation capabilities with probable effects on the longevity process” [95]
The previous reference was: [none].

“The development of systems biology approaches and the new generation of biomarker patterns will provide the opportunity to associate complex metabolic regulations with key aging biological processes” [95].
The previous reference was: [none].

“The gastrointestinal tract (GIT) is one of the most essential interfaces of mammalian organism interacting with nutrients, exogenous compounds, and gut microbiota, and its condition is influenced by the complex interplay between these environmental factors and host genetic elements. Along the GIT, the gut microbiota is a key determinant of the gut functional ecology and metabolic homeostasis, through fine interactions with regulatory processes involved in the absorption, digestion, metabolism, and excretion of dietary nutrients as well as barrier integrity, motility, and mucosal immunity” [23, 24, 95].
The previous reference was: [23,24].

“Increasing scientific evidence has been reported on the fundamental role of gut microbiota in both positive and negative triggers of specific metabolic states of individuals and populations” [61, 65, 95].
The previous reference was: [61,65].

“Systems biology approaches, including metabonomics, have emerged over the last two decades as a novel way forward to provide insights into the role of mammalian gut microbial metabolic interactions in individual susceptibility to health and disease outcomes” [95].
The previous reference was: [none].

“A series of investigations in human [74] and animal models [75–77] have provided a set of reference metabolic profiles of gut intestinal biopsies that can be used to assess not only compartment structure and function but also the gut microbial impact at the tissue level” [78, 95].
The previous reference was: [78].

"Such applications will help in identifying main metabolic processes conserved across species on which gut microbiota modulates to shape the microenvironment. For instance, the investigations illustrated how microbial-dependent variations along the upper intestine, an element often underestimated due to low bacterial populations, may affect utilization efficiency of dietary proteins and amino acids and their subsequent availability to extra-intestinal tissues. Moreover, some reference data were generated to investigate changes in gut functionality, such as gut permeability, using metabolic profiling of biofluids" [79, 80, 95].
The previous reference was: [78, 80].

"Both manifestations of IBD, ulcerative colitis (UC) and Crohn's disease (CD), are mediated by common and distinct mechanisms influenced by multiple environmental factors and specific genetic predispositions, including gut microbiota. Advancing knowledge regarding the mechanisms of IBD has led to the development of different therapeutic solutions based on surgery [82], cannabinoids [83], immunosuppression [84], and alternatively probiotic supplementation [85]. Although prognostic and monitoring tools are currently lacking, metabolic profiling in combination with state-of-the-art clinical and medical readouts is foreseen to be a valuable tool to differentiate and follow-up IBD evolution and response to disease-modifying interventions" [95]
The previous reference was: [none].

"Winterkamp et al. reported previously how N-methylhistamine, a key metabolite in mast cell metabolism involved in the pathogenesis of IBD, could be used as an indicator of disease activity in patients [86]. In this study, the urinary excretion of N-methylhistamine was associated with elevated histamine production and metabolism in CD and UC and could be used as a reliable diagnostic tool to monitor clinical and endoscopic disease activity in IBD. Additional proofs of concept on the feasibility to identify some metabolic indicators of early onsets of chronic inflammatory development offer also novel promising directions for patient monitoring and early patient stratification [87]. Additional applications of noninvasive profiling of stool from patients provided novel insights into the remodeling of the gut microbial communities and activities, concomitant to malabsorption and element of protein-losing enteropathy" [88, 89, 95].
The previous reference was: [88, 89].

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