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REVIEW

Emerging Insights on the Interaction Between Anticancer and Immunosuppressant Drugs and Intestinal Microbiota in Pediatric Patients

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Diseases affecting the immune system, such as inflammatory bowel disease (IBD), juvenile idiopathic arthritis (JIA), and acute lymphoblastic leukemia (ALL), are pathological conditions affecting the pediatric population and are often associated with alterations in the intestinal microbiota, such as a decrease in bacterial diversity. Growing evidence suggests that gut microbiota can interfere with chemotherapeutic and immunosuppressant drugs, used in the treatment of these diseases, reducing or facilitating drug efficacy. In particular, the effect of intestinal microflora through translocation, immunomodulation, metabolism, enzymatic degradation, and reduction of bacterial diversity seems to be one of the reasons of interindividual variability in the therapeutic response. Although the extent of the role of intestinal microflora in chemotherapy and immunosuppression remains still unresolved, current evidence on bacterial compositional shifts will be taken in consideration together with clinical response to drugs for a better and personalized therapy. This review is focused on the effect of the intestinal microbiota on the efficacy of pharmacological therapy of agents used to treat IBD, JIA, and ALL.

Over 35,000 bacterial species are present in the human gut microbiota, belonging mainly to the phyla of Firmicutes and Bacteroidetes, followed by Proteobacteria and Actinobacteria, and to a lesser extent to Fusobacteria, Verrucomicrobia, Cyanobacteria, and Spirochetes[1,2] (**Table 1**). They are distributed along the alimentary tract with an increasing gradient of density, depending on pH values, and with a different composition, depending on nutrients availability and oxygen tension. In the small intestine, besides species belonging to Bacteroides (Bacteroidetes) and Clostridiales (Firmicutes), which strictly adhere to the mucous epithelium forming the resident microflora, Proteobacteria and Lactobacillales (Firmicutes) are found in the lumen (transient microflora) due to the presence of monosaccharides and disaccharides. In the colon, where bacterial population reaches the highest concentration (about 10^{12}–10^{13} CFU/mL), Proteobacteria greatly decrease, replaced by anaerobic species able to ferment carbohydrates with production of short chain fatty acids (SCFAs), such as acetate, propionate, and butyrate. There are evidences that gut microbiota plays a fundamental role in the healthy immune status maintenance. In particular, intestinal microflora and immune system are constantly shaping each other in a mutual aim to flourish and to keep the healthy individual in balance.[3–5] The healthy state of the immune system in adulthood is related to the presence of a diversified microflora, which develops in early childhood thanks to a correct colonization sequence by different microorganisms.

The initial colonization of the infant gut takes place during delivery and undergoes a lot of modifications during the first 3 years of life, depending on the diet. It is commonly considered a healthy microbiota, the one developed by full term, vaginally delivered, and breast-fed infants. These babies are initially colonized by vagina-associated species, mostly *Lactobacillus* (Firmicutes), *Prevotella* (Bacteroidetes), and, to a lesser extent, *Bifidobacterium* (Actinobacteria)[6–8]; then, breastfeeding stimulates the proliferation of few species of *Bifidobacterium* (*B. breve*, *B. bifidum*, and *B. longum*), which become the most prevalent, thanks to their ability to use, as the sole carbon source, some human milk oligosaccharides abundant in maternal milk and not degraded by the infant's enzymes.[9] The microflora composition remains highly stable during the feeding period and only after the beginning of weaning a diversification is observed: 5 days of breast milk cessation are sufficient to reduce the level of *Bifidobacterium* and *Lactobacillus* and increase that of *Bacteroides*, *Blautia*, and *Ruminococcus*[10]; protein and fiber intake have been correlated with an increase of *Lachnospiraceae* and *Prevotellaceae*, respectively[11]; species like *Faecalibacterium prausnitzii* and *Akkermansia muciniphila*, almost absent in the infant gut, increase significantly during the next 12 months, and since 3-years-old, the microbiota achieves the complex and stable composition typical of the adult, consisting for > 80% of species belonging to Firmicutes and Bacteroidetes.[12]

A correct colonization sequence is considered very important for the development of this healthy microbiota: the initial proliferation of a low number of aerotolerant species is needed for oxygen consumption, which prepares an advantageous environment for the colonization by a high number of

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Table 1 Classification of bacteria and interaction with disease and drug therapy

Class	Order	Family	Genus	Species	Disease or experimental design	Drugs	Interactions	Observations	Ref.
Firmicutes phylum									
					Mouse model	Thiopurines	Microbiota as IBD therapy response biomarker	↑ after therapy	56
					Mouse model	Fluorouracil	Reduced diversity	↓ after therapy	23
Clostridia	Clostridiales	*Clostridiaceae*			ALL		Microbiota as ALL chemotherapy response biomarker	↑ after therapy	68
					JIA		Microbiota and JIA susceptibility	↓ in JIA patients[a]	75
			Clostridium		*In vitro*	Sulfasalazine	Microbiota species involved in IBD therapeutics metabolism	Drug activation	32
					Mouse model	Cyclophosphamide	Reduced diversity	↓ after therapy	22
			Clostridium	*Scindens*	*In vitro*	Dexamethasone	Microbiota species involved in ALL chemotherapeutics metabolism	Dexamethasone side chain cleavage	32
			Clostridium	*Bolteae*	*In vitro*	Dasatinib	Microbiota species involved in ALL chemotherapeutics metabolism	Drug metabolism	32
			Clostridium	*Cluster IV*	JIA		Microbiota as JIA therapy response biomarker	↑ in responder patients	81
			Clostridum	*Cluster XIVb*	JIA		Microbiota and JIA susceptibility	↑ in JIA patients[a]	81
			Clostridum	*Cluster XVIII*	JIA		Microbiota as JIA therapy response biomarker	↑ in responder patients	81
		Acidaminococcaceae	*Phascolarctobacterium*		IBD	Infliximab	Microbiota as IBD therapy response biomarker	↓ in responder patients	59
						Adalimumab	Microbiota as IBD therapy response biomarker	↓ in responder patients	59
		Lachnospiraceae			Mouse model	Methotrexate	Microbiota as ALL chemotherapy response biomarker	↑ after therapy	39
			Lachnospira		IBD	Infliximab	Microbiota as IBD therapy response biomarker	↑ in responder patients	59

(Continues)

Table 1 (Continued)

Class	Order	Family	Genus	Species	Disease or experimental design	Drugs	Interactions	Observations	Ref.
						Adalimumab	Microbiota as IBD therapy response biomarker	↑ in responder patients	59
					Mouse model	Cyclophosphamide	Reduced diversity	↓ after therapy	22
			Coprococcus		ALL		Microbiota and ALL susceptibility	↓ in ALL patients[a]	66
					Mouse model	Cyclophosphamide	Reduced diversity	↓ after therapy	22
			Anorestipes		ALL		Microbiota and ALL susceptibility	↓ in ALL patients[a]	66
			Blautia		IBD	Ustekinumab	Microbiota as IBD therapy response biomarker	↑ in responder patients	61
					ALL		Microbiota and ALL susceptibility	↓ in ALL patients[a]	67
			Roseburia		ALL		Microbiota and ALL susceptibility	↓ in ALL patients[a]	66
					IBD	Ustekinumab	Microbiota as IBD therapy response biomarker	↑ in responder patients	61
					Mouse model	Cyclophosphamide	Reduced diversity	↓ after therapy	22
			Roseburia	*Faecis*	IBD	Infliximab	Microbiota as IBD therapy response biomarker	↑ in responder patients	59
						Adalimumab	Microbiota as IBD therapy response biomarker	↑ in responder patients	59
						Ustekinumab	Microbiota as IBD therapy response biomarker	↑ in responder patients	61
			Roseburia	*Inulinivorans*	IBD	Vedolizumab	Microbiota as IBD therapy response biomarker	↑ in responder patients	60
		Mogibacteriaceae			JIA	Standard therapies (biological agents, methotrexate, NSAIDs, steroids)	Microbiota as JIA therapy response biomarker	↓ in responder patients	92
		Peptostreptococcaceae			JIA		Microbiota and JIA susceptibility	↓ in JIA patients[a]	81
		Ruminococcaceae			ALL		Microbiota as ALL chemotherapy response biomarker	↓ after therapy	68

(Continues)

Table 1 (Continued)

Class	Order	Family	Genus	Species	Disease or experimental design	Drugs	Interactions	Observations	Ref.
					JIA		Microbiota and JIA susceptibility	↑ in JIA patients[a]	81
			Ruminococcus		Mouse model	Methotrexate	Microbiota as ALL chemotherapy response biomarker	↓ after therapy	71
					IBD	Ustekinumab	Microbiota as IBD therapy response biomarker	↑ in responder patients	61
					Melanoma	Anti PD-1	Reduced diversity	↑ in responder patients	36,66
					ALL	polychemotherapy	Microbiota and ALL susceptibility	↓ in ALL patients[a]	
			Ruminococcus	*Gnavus*	IBD		Microbiota and IBD susceptibility	↑ in IBD patients[a]	48–50
					In vitro	Sulfasalazine	Microbiota species involved in IBD therapeutics metabolism	Drug activation	32
			Faecalibacterium		ALL	Polychemotherapy	Microbiota and ALL susceptibility	↑ in ALL patients[a]	66
							Microbiota as ALL chemotherapy response biomarker	↓ after therapy	68
			Faecalibacterium	*Prausnitzii*	IBD	Infliximab	Microbiota as IBD therapy response biomarker	↑ in responder patients	57
						Adalimumab	Microbiota as IBD therapy response biomarker	↑ in responder patients	57
						Ustekinumab	Microbiota as IBD therapy response biomarker	↑ in responder patients	61
					JIA		Microbiota and JIA susceptibility	↓ in JIA patients[a]	79,89,90
		Eubacteriaceae	*Eubacterium*		*In vitro*	Sulfasalazine	Microbiota species involved in IBD therapeutics metabolism	Drug activation	32
			Eubacterium	*Rectale*			Introduction	Immunomodulation	15
			Eubacterium	*Hallii*			Introduction	Immunomodulation	15

(Continues)

Table 1 (Continued)

Class	Order	Family	Genus	Species	Disease or experimental design	Drugs	Interactions	Observations	Ref.
Bacilli	Lactobacillales	*Enterococcaceae*			ALL		Microbiota and ALL susceptibility	↑ in ALL patients[a]	67
							Microbiota and ALL susceptibility	↑ after therapy	68
							Microbiota and ALL susceptibility	↑ chemotherapy-induced HEM and GI toxicity	68
							Microbiota and ALL susceptibility	↑ after therapy	68
					Mouse model	Cyclophosphamide	Reduced diversity		20
			Enterococcus		ALL	Polychemotherapy	Microbiota as ALL chemotherapy response biomarker	↑ after salvage therapy	69
					JIA		Microbiota and JIA susceptibility	↑ in JIA patients[a]	85
			Enterococcus	*Faecium*	Melanoma	Anti-PD-1	Reduced diversity	↑ in responder patients	37
			Enterococcus	*Faecalis*	*In vitro*	Thiopurines	Microbiota as IBD therapy response biomarker	Drug activation	56
			Enterococcus	*Hirae*	Mouse model	Cyclophosphamide	Translocation and immunomodulation	Translocation	22
		Streptococcaceae			ALL	Polychemotherapy	Microbiota as ALL chemotherapy response biomarker	↑ after therapy ↑ chemotherapy-induced GI toxicity	68
			Streptococcus		Mouse model	Methotrexate	Microbiota as ALL chemotherapy response biomarker	↓ after therapy	71
		Lactobacillaceae			ALL	Polychemotherapy	Microbiota as ALL chemotherapy response biomarker	↑ after chemotherapy	68
			Lactobacillus	*Johnsonii*	Mouse model	Cyclophosphamide	Translocation and immunomodulation	Translocation	22
	Erysipelotrichiales	*Erysipelotrichiaceae*			ALL		Microbiota and ALL susceptibility	↓ in ALL patients[a]	67
					JIA		Microbiota and JIA susceptibility	↑ in JIA patients[a]	90

(Continues)

Table 1 (Continued)

Class	Order	Family	Genus	Species	Disease or experimental design	Drugs	Interactions	Observations	Ref.
			Allobaculum		JIA		Microbiota and JIA susceptibility	↑ in JIA patients[a]	90
Negativicutes	Selenomonadales	*Veillonellaceae*			IBD		Microbiota and IBD susceptibility	↑ in IBD patients[a]	48–50
					JIA		Microbiota and JIA susceptibility	↑ in JIA patients[a]	81
Bacteroidetes phylum									
Bacteroidia	Bacteroidales				Mouse model	Methotrexate	Microbiota as ALL chemotherapy response biomarker	↓ after therapy	39
		Porphyromonadaceae	*Parabacteroides*		IBD	5-aminosalicylic acid	Microbiota as IBD therapy response biomarker	↓ after therapy	54
			Parabacteroides	*Johnsonii*	*In vitro*	Imatinib	Microbiota as ALL chemotherapy response biomarker	Drug metabolism	32
			Odoribacter		JIA		Microbiota as JIA therapy response biomarker	↑ in responder patients	81
		Prevotellaceae			JIA		Microbiota and JIA susceptibility	↓ in JIA patients[a]	85
			Prevotella		ALL		Microbiota and ALL susceptibility	↑ in ALL patients[a]	66
			Prevotella		JIA		Microbiota and JIA susceptibility	↓ in JIA patients[a]	85
			Prevotella		*In vitro*	Sulfasalazine	Microbiota species involved in IBD therapeutics metabolism	Drug activation	32
					IBD	5-Aminosalicylic acid	Microbiota as IBD therapy response biomarker	↓ after treatment	54
		Bacteriodaceae			JIA		Microbiota and JIA susceptibility	↑ in JIA patients[a]	85
			Bacteroides		Mouse model	Methotrexate	Microbiota as ALL chemotherapy response biomarker	↑ after therapy	71
					IBD	5-Aminosalicylic acid	Microbiota as IBD therapy response biomarker	↓ after therapy	54
					ALL		Microbiota and ALL susceptibility	↓ in ALL patients[a]	66

(Continues)

Table 1 (Continued)

Class	Order	Family	Genus	Species	Disease or experimental design	Drugs	Interactions	Observations	Ref.
					JIA		Microbiota and JIA susceptibility	↑ in JIA patients[a]	85
			Bacteroides	*Thetaiotaomicron*	*In vitro*	Thiopurines	Microbiota as IBD therapy response biomarker	Drug activation	56
			Bacteroides	*Dorei*	*In vitro*	Corticosteroids	Microbiota species involved in ALL chemotherapeutics metabolism	Drug metabolism	32
			Bacteroides	*Eggerthii*	*In vitro*	Imatinib	Microbiota species involved in ALL chemotherapeutics metabolism	Drug metabolism	32
			Bacteroides	*Vulgatus*	*In vitro*	Imatinib	Microbiota species involved in ALL chemotherapeutics metabolism	Drug metabolism	32
			Bacteroides	*Stercoris*	*In vitro*	Imatinib	Microbiota species involved in ALL chemotherapeutics metabolism	Drug metabolism	32
			Bacteroides	*fragilis*	Mouse model	Ipilimumab	Translocation and immunomodulation	↑ response	24
					In vitro	Dasatinib	Microbiota species involved in ALL chemotherapeutics metabolism	Drug metabolism	32
					JIA		Microbiota and JIA susceptibility	↑ in JIA pediatric patients[a]	89
		Rikenellaceae	*Alistipes*		*In vitro*	Sulfasalazine	Microbiota species involved in IBD therapeutics metabolism	Drug activation	32
			Alistipes	*Indistinctus*	*In vitro*	Cyclophosphamide	Microbiota species involved in ALL chemotherapeutics metabolism	Drug metabolism	32
Proteobacteria phylum									
					ALL	Polychemotherapy	Microbiota as ALL chemotherapy response biomarker	↑ chemotherapy-induced HEM toxicity	68

(Continues)

Table 1 (Continued)

Class	Order	Family	Genus	Species	Disease or experimental design	Drugs	Interactions	Observations	Ref.
Betaproteobacteria	Burkholderiales				IBD	Vedolizumab	Microbiota as IBD therapy response biomarker	↑ in responder patients	60
					Rheumatoid arthritis	Etanercept	Microbiota as JIA therapy response biomarker	↑ in responder patients	94
						Adalimumab	Microbiota as JIA therapy response biomarker	↑ in responder patients	94
						Infliximab	Microbiota as JIA therapy response biomarker	↑ in responder patients	94
		Sutterellaceae	*Sutterella*		IBD		Microbiota and IBD susceptibility	↓ in IBD patients[a]	48–50
					JIA		Microbiota as JIA therapy response biomarker	↓ in responder patients[a]	81
		Sutterellaceae	*Parasutterella*		JIA		Microbiota as JIA therapy response biomarker	↑ in responder patients	81
Gammaproteobacteria	Enterobacteriales	*Enterobacteriaceae*			JIA		Microbiota and JIA susceptibility	↑ in JIA patients[a]	85
					Mouse model	Cyclophosphamide	Reduced diversity	↑ after therapy	20
			Salmonella	*Typhimurium*	*in vitro*	Sulfasalazine	Microbiota species involved in IBD therapeutics metabolism	↓ drug activation	32
			Raoultella	*Planticola*	Mouse model	Doxorubicin	Enzymatic drug modification and metabolism	Drug inactivation	26
			Escherichia	*Coli*	IBD	5-Aminosalicylic acid	Microbiota as IBD therapy response biomarker	↓ after therapy	54
					In vitro	Sulfasalazine	Microbiota species involved in IBD therapeutics metabolism	↓ drug activation	32
					Mouse model	Doxorubicin	Enzymatic drug modification and metabolism	Drug inactivation	26

(Continues)

Table 1 (Continued)

Class	Order	Family	Genus	Species	Disease or experimental design	Drugs	Interactions	Observations	Ref.
					In vitro	Thiopurine	Microbiota as IBD therapy response biomarker	Drug activation	56
					IBD	Ustekinumab	Microbiota as IBD therapy response biomarker	↓ in responder patients	61
					In vitro	Gemcitabine	Enzymatic drug modification and metabolism	Drug inactivation	31
			Shigella		IBD	5-Aminosalicylic acid	Microbiota as IBD therapy response biomarker	↓ after therapy	54
						Ustekinumab	Microbiota as IBD therapy response biomarker	↓ in responder patients	61
			Klebsiella		JIA IBD		Microbiota and JIA susceptibility	↑ in JIA patients[a] ↑ severe clinical course	59,85
			Klebsiella	*Pneumoniae*	Mouse model	Doxorubicin	Enzymatic drug modification and metabolism	Drug inactivation	26
	Pasteurellales	*Pasteurellaceae*			IBD		Microbiota and IBD susceptibility	↑ in IBD patients[a]	48–50
	Pseudomonadales	*Pseudomonadaceae*			Mouse model	Cyclophosphamide	Reduced diversity	↑ after therapy	20
Actinobacteria phylum					Mouse model	Fluorouracil	Reduced diversity	↑ after therapy	23
					ALL	Polychemotherapy	Microbiota as ALL chemotherapy response biomarker	↓ after therapy	68
Actinobacteria	Bifidobacteriales	*Bifidobacteriaceae*	*Bifidobacterium*		IBD	Infliximab	Microbiota as IBD therapy response biomarker	↑ in responder patients	59
						Adalimumab	Microbiota as IBD therapy response biomarker	↑ in responder patients	59
			Bifidobacterium	*Bifidum*			Introduction	↑ in breast-fed infants	9
			Bifidobacterium	*Breve*			Introduction	↑ in breast-fed infants	9
			Bifidobacterium	*Longum*	Melanoma	Anti-PD-1	Reduced diversity	↑ in responder patients	37

(Continues)

Table 1 (Continued)

Class	Order	Family	Genus	Species	Disease or experimental design	Drugs	Interactions	Observations	Ref.
			Bifidobacterium	*Ruminatum*	*In vitro*	Dasatinib	Microbiota species involved in ALL chemotherapeutics metabolism	Drug metabolism	32
	Coriobacteriales	*Coriobacteriaceae*	*Collinsella*		IBD	Infliximab	Microbiota as IBD therapy response biomarker	↑ in responder patients	59
						Adalimumab	Microbiota as IBD therapy response biomarker	↑ in responder patients	59
					JIA	NSAIDs and sulfasalazine therapy	Microbiota as JIA therapy response biomarker	↑ in responder patients	91
			Collinsella	*Aerofaciens*	Melanoma	Anti-PD-1	Reduced diversity	↑ in responder patients	37
			Eggerthella		IBD	Infliximab	Microbiota as IBD therapy response biomarker	↑ in responder patients	59
						Adalimumab	Microbiota as IBD therapy response biomarker	↑ in responder patients	59
Tenericutes phylum									
Mollicutes	Mycoplasmatales	*Mycoplasmataceae*	*Mycoplasma*		*In vitro*	5-Fluoro-deoxyuridine	Enzymatic drug modification and metabolism	Drug inactivation	30
Fusobacteria phylum									
Fusobacteria	Fusobacteriales	*Fusobacterium*			IBD		Microbiota and IBD susceptibility	↑ in IBD patients[a]	48–50
Verrucobacteria phylum									
					ALL	Polychemotherapy	Microbiota as ALL chemotherapy response biomarker	↓ after therapy	68
Verrucomicrobiae	Verrucomicrobiales	*Verrucomicrobiaceae*			Mouse model	Fluorouracil	Reduced diversity	↑ after therapy	23
			Akkermansia	*Muciniphila*	Melanoma	Anti-PD-1	Reduced diversity	↑ in responder patients	38
					JIA		Microbiota and JIA susceptibility	↑ in JIA patients[a]	79
Cyanobacteria phylum									
Nostocophycideae	Nostocales				Rheumatoid arthritis	Etanercept	Microbiota as JIA therapy response biomarker	↑ after therapy	95

ALL, acute lymphoblastic leukemia; GI, gastrointestinal; HEM, hematological; IBD, inflammatory bowel disease; JIA, juvenile idiopathic arthritis; NSAIDs, nonsteroidal anti-inflammatory drugs; PD-1, programmed cell death 1.
[a]Compared with healthy subjects.

anaerobes, including species able to produce SCFA. Events like pre-term birth, caesarean section delivery, and feeding by formula-milk, are related to formation of a different, disadvantageous, microflora[13]: pre-term babies are initially colonized mainly by opportunistic pathogens, like *Enterococcus* and species belonging to *Enterobacteriaceae*[7]; caesarean section results in colonization by environmental microorganisms (present on the maternal skin or carried by the hospital staff), with a low amount of Actinobacteria and a high amount of Firmicutes, mainly *Clostridia*[6,7]; in the microbiota of formula-fed babies, high amounts of different potential pathogens, like *Staphylococcus*, *Enterobacteriaceae*, *Enterococcus*, and *Clostridium* have been found.[14] In all cases, the colonization by *Bifidobacteria* and *Lactobacilli* is delayed if not seriously impaired, dramatically affecting the subsequent colonization by microorganisms that play a fundamental role in the maturation and maintenance of the immune system. For example, *Eubacterium rectale*, *Eubacterium hallii*, *Faecalibacterium prausnitzii*, and *Roseburia faecis*, all belonging to Firmicutes phylum, contribute to the intestinal immunoregulation, producing butyrate through bacterial fermentation of indigestible dietary components introduced by the host in the colon environment.[15] Butyrate positively influences cell proliferation and differentiation, contributing to the stability of the gut epithelial barrier. Moreover, it has anti-inflammatory and immunomodulatory properties inhibiting the nuclear factor kappa β of macrophages, interfering with TNFα, interleukin (IL)-6, and myeloperoxidase activity, and inducing regulatory T cell (Treg) differentiation.[16,17]

Furthermore, commensal bacteria are responsible for triggering several pathways involved in the regulation of the immune system.[3] A general consensus exists that Treg cells are key mediators of immune responses, maintaining both peripheral and mucosal homeostasis.[3,5] Although the primary site of differentiation is the thymus, the induction of Treg cells can occur also in the gastrointestinal tract after the production of inducing factors by dendritic cells presenting antigens derived from commensal bacteria.[3] The most significant demonstration that intestinal microflora could promote the differentiation of CD4-positivity in Treg cells and the consequent modulation of immune response was provided by Mazmanian *et al.*[18] This study showed that the antigen polysaccharide A of the symbiont *Bacteroides fragilis*, administered to animals with induced colitis, suppresses the production of pro-inflammatory IL-17, stimulating the induction of Treg cells.

Other pathways involving toll-like receptors and NOD-like receptors have been found to be responsible for the normal development of the intestinal mucosal immune system.[5] These receptors are able to recognize microbe-associated molecular patterns, such as peptidoglycan components, and to trigger, respectively, either the innate intestinal immune response or the assembly of inflammasomes.[5]

From another point of view, the immune system is able both to maintain a symbiotic relationship with commensal bacteria and to contain microorganisms with pathogenic potential.[3] The host reaches a condition of homeostasis minimizing the contact between pathogenic microorganisms and the epithelial cell surface through a primary barrier consisting of mucus, antimicrobial peptides, and secretion of immunoglobulin A.[3] These structural and immunological components are recognized as the "mucosal firewall."

The ability of the microbiota to directly affect either positively or negatively the pharmacological properties of drugs, especially if orally administered, by enzymatic modification, has been recognized about 50 years back. However, in the last 10 years, additional systems through which the microbiota can modulate the efficacy and/or the toxicity of pharmacological treatments have been described and recently outlined in five basic key mechanisms structured as the "TIMER framework"[19]: translocation, immunomodulation, metabolism, enzymatic modification, and reduced diversity of the microflora components.

Translocation and immunomodulation

The risk of bacterial translocation through the mucosal barrier is strictly connected with mucosal damage and intestinal permeability.[20] Doxorubicin-induced cytotoxicity within the gastrointestinal tract manifests as an increase in apoptosis in the stem cell zone of the jejunum and as mucosal damage, including a decrease in crypt number and villus height. Recently, Rigby *et al.*[21] demonstrated that mucosal damage is dependent on enteric bacteria. The authors observed that doxorubicin chemotherapy caused similar levels of apoptosis in both conventionally raised and germ-free (GF) mice's small intestine but alterations in crypt depth and number were observed only in conventionally raised mice, suggesting that the presence of enteric bacteria could cause an increase of epithelial barrier permeability due to their translocation.[21]

Gut epithelial barrier alterations are characterized by shortening of small intestinal villi, interstitial edema, epithelial discontinuities, and focal invasion of mononuclear cells in lamina propria after therapy with both doxorubicin and cyclophosphamide.[22] Several studies on mouse models validated the role of the chemotherapeutic agent cyclophosphamide in increasing intestinal permeability and in the resulting bacterial translocation.[20,22] Adherens and tight junctions, localized at the apical-lateral and lateral membrane, contribute considerably to the regulation of paracellular permeability. According to this, Yang and colleagues conducted *in vivo* intestinal permeability assays in male Balb/c mice and investigated the expression of tight and adherens junctions' proteins, like occludin, zonula occludens-1, and E-cadherin.[20] Treatment with cyclophosphamide, especially at high doses, was found to induce intestinal permeability by reducing the expression of tight and adherens junctions' proteins in the intestinal epithelium. Moreover, Viaud and colleagues' experiments evidenced the disruption of the intestinal barrier and the subsequent bacterial translocation, through the detection of several gram-positive bacteria belonging to Firmicutes phylum, including *Lactobacillus johnsonii* and *Enterococcus hirae*, into mesenteric lymph nodes and the spleen.[22] Furthermore, these authors highlighted the impact of intestinal flora on chemotherapy-elicited anticancer immune responses. In particular, gram-positive bacteria detected in lymphoid organs were found to be indispensable for gearing the conversion of CD4-positive T cells into type 17 T helper cells (Th17) and type 1 T helper cells, which contribute to

control cancer overgrowth. Furthermore, this specific set of gram-positive bacteria is able to stimulate the accumulation of specific Th17 within the spleen. Interestingly, the tumor growth inhibitory effect of cyclophosphamide decreased in GF or antibiotic-treated mice, whereas the efficacy of chemotherapy was restored by Th17 cells adoptive transfer. Viaud and colleagues demonstrated through this study that gut microbiota enhances the anticancer immune response.[22]

Recently, Sougiannis and colleagues conducted *in vivo* experiments, which highlight that gut microbiota modified by fluorouracil has an effect on circulating immune cells.[23] In particular, fecal microbiota of mice without the tumor and treated with the antitumoral drug were transplanted in control mice. Gene expression analysis on colon tissue of transplanted mice evidenced a decreased expression of genes connected with macrophages profile, such as monocyte chemoattractant protein 1, IL-10, IL-1β, and epidermal growth factor-like module containing mucin-like hormone receptor 1, compared with controls. In addition, fewer CD68-positive cells were detected in the transplanted mice colon tissue by immunohistochemical analysis, indicating that the engrafted microbiota, previously modified by fluorouracil chemotherapy, contributes to reduce the macrophage population.

Moreover, *Bacteroides fragilis* (Bacteroidetes) plays a role in immunomodulation, facilitating checkpoints of T-lymphocyte-associated protein 4 blockage response by monoclonal antibodies, such as ipilimumab.[24] Comparing the therapeutic efficacy in specific pathogen-free and GF mice after treatment, Vetizou and colleagues[24] evidenced a control of tumor progression only in specific pathogen-free mice and a reduction of splenic CD4-positive T cell activation and lymphocytes infiltration in GF or antibiotic-treated mice. The anticancer activity and the immune cells function reappeared after feeding GF and antibiotic-treated mice with *Bacteroides fragilis*, after immunization with its polysaccharides or after bacterial-specific T cells adoptive transfer.

Enzymatic drug modification and metabolism

Enzymatic drug modification by the microbiota has been studied both *in vitro*, looking for the drug activity after incubation with bacterial cultures and/or bacteria lysates, and *in vivo*, looking for the effect of co-administration of antimicrobials during chemotherapy, leading to the precise identification of some drug-modifying enzymes and of the reactions they catalyse.

Azoreductases produced by many enteric species, mostly belonging to genera *Clostridium* and *Eubacterium* (Firmicutes), play a fundamental role in the activation of the azo-bonded prodrugs of 5-aminosalicylic acid used to treat patients with ulcerative colitis and Crohn's disease, as they cleave the azoic bond, releasing the active moiety 5-aminosalicylic acid. As these bacteria are most abundant in the colon, the therapeutic activity of the drug is mainly triggered at this level.[25]

The involvement of gut microbiota in modulating doxorubicin toxicity at the intestinal level has long been known and was mainly ascribed to an increase of epithelial permeability, as described above. However, the different effects exerted by gut bacteria at different levels of the intestinal tracts remained unexplained: apparently protective for the colonic epithelium, damaging for the small intestine.[21] The identification of some doxorubicin inactivating enzymes produced by different bacterial species recently helped to explain this difference. *Raoultella planticola*, a gut isolate belonging to the *Enterobacteriaceae* family (Proteobacteria), inactivates doxorubicin by a deglycosylation pathway that needs strictly anaerobic conditions typical of the colon; also, *Klebsiella pneumoniae* and *Escherichia coli*, belonging to the same family, degrade doxorubicin if incubated in anaerobic conditions, through a yet undefined molybdopterin-dependent pathway. On the contrary, in aerobic conditions, bacterial metabolisms degrading the drug by an oxidative phosphorylation pathway, which leads to the production of reactive oxygen species toxic for the tissues, have been described. At the moment, the latter has been found only in environmental species[26] and there is no evidence that it is expressed by gut microbiota. However, the hypothesis that bacterial degradation of doxorubicin might be either protective or damaging to the mucosal epithelium depending on the aerobic (small intestine) or anaerobic (colon) conditions is intriguing.[27]

In some cases, modification of drugs by the microflora metabolism causes the release of secondary metabolites that are toxic for the host, leading to the need of reducing the doses or even modifying the therapy. This is the case of irinotecan, an antineoplastic drug used for the treatment of colorectal and pancreatic cancers, which is metabolized by the host hepatic carboxylesterases to its active moiety, SN-38, part of which is converted to an inactive glucuronide and eliminated by the biliary route. The severe diarrhea observed in 30–40% of patients, initially considered simply the consequence of a drug-induced dysbiosis, was recently explained with the production of bacterial β-glucuronidases by many species of the microbiota (e.g., *Bacteroides* (Bacteroidetes), *Clostridium* (Firmicutes), and *Bifidobacterium* (Actinobacteria)), which release active SN-38 directly in the gut causing epithelial damage and consequently diarrhea. This hypothesis was confirmed by the addition to the therapy of specific inhibitors of bacterial β-glucuronidases, which significantly reduced the incidence of diarrhea with no modification of the microflora composition in mice.[28,29]

Besides intestinal biotransformation of drugs by gut bacteria, the activity of several nucleoside analogues, widely used in cancer therapy, is decreased in some patients due to the presence of intratumoral bacteria that produce inactivating enzymes: the thymidine phosphorylase produced by *Mycoplasma* species (Tenericutes), detected in different carcinoma tissues (e.g., ovarian, cervical, gastric, and colon), degrades 5-fluoro-deoxyuridine, 5-trifluorothymidine, and 5-halogenated-2'-deoxiuridines, causing therapeutic failure; restoration of chemotherapy after co-administration of a thymidine phosphorylase inhibitor confirmed the involvement of this enzyme.[30] Another example is the cytidine deaminase production detected in different species of *Enterobacteriaceae* (Proteobacteria) that were found in pancreatic carcinomas, which appeared significantly related to gemcitabine (2',2'-difluorodeoxycitidine) resistance exhibited by some patients during treatment[31]: in this case, the

efficacy of the treatment was restored by co-administration of an antibiotic.

Finally, a recent study, carried out with a high-throughput approach, extensively analyzed the drug metabolism by the gut microbioma *in toto*, evaluating 76 different bacterial species, belonging to different phyla, able to metabolize 271 orally administered drugs, and suggesting that direct modification of drugs by the microbiota could have a much higher impact on chemotherapy than thought so far.[32]

Reduced diversity

There is a growing consensus that diseases affecting the immune cells, such as acute lymphoblastic leukemia (ALL), inflammatory bowel disease (IBD), and juvenile idiopathic arthritis (JIA), are related to changes in gut microbiota composition (dysbiosis) occurring as a decrease in the bacterial diversity.[33–35] Although more studies are needed to investigate the role of intestinal microflora in the onset and exacerbation of autoimmune diseases, it is well known that chemotherapeutic agents can modify gut microbiota composition, usually reducing bacterial diversity and, therefore, improving pathological conditions of patients.[19] Evaluation of the gut microbiota after cyclophosphamide treatment evidenced changes in gut colonization, especially in the small intestine.[20,22] One week after cyclophosphamide administration, the mucosal microflora of mice bearing subcutaneous cancers showed a reduction of phylum Firmicutes (mainly for genera *Clostridium*, *Roseburia*, and *Coprococcus*, and for the family of *Lachnospiraceae*) and a decrease of *Lactobacilli* and *Enterococci*.[22] Another study on mouse models highlighted the reduction in bacterial diversity after intraperitoneal administration: cyclophosphamide induced the predominance of potentially pathogenic bacteria groups belonging to *Enterobacteriaceae*, *Pseudomonadaceae* (Proteobacteria), and *Enterococcaceae* (Firmicutes).[20] Sougiannis and colleagues noticed that fluorouracil chemotherapy alters gut microbiota richness and evenness, as already mentioned.[23] The authors showed that the chemotherapeutic agent increased the amount of Actinobacteria and Verrucomicrobia phyla in mice without colorectal cancer, whereas differences in the abundance of Firmicutes phylum was found to be more evident in treated mice bearing the tumor.

Furthermore, recent studies showed a different gut microbiota composition in patients with melanoma who respond to antiprogrammed cell death protein 1 immunotherapy. Metagenomic analysis of stool samples showed a correlation between antiprogrammed cell death protein 1 efficacy and the amount of *Rumicoccaceae* (Firmicutes),[36] *Bifidobacterium longum*, and *Collinsella aerofaciens* (Actinobacteria), *Enterococcus faecium* (Firmicutes),[37] and *Akkermansia muciniphila* (Verrucomicrobia).[38]

Zhou *et al.*[39] investigated the role of methotrexate in modifying gut microbiota composition and found out that the immunosuppressive drug dramatically alters the diversity of gut microbiota as we shall examine in depth afterward.

Interactions and effects of microbiome represented by the TIMER scheme, described above, could also be interpreted in a pharmacokinetic (PK) and pharmacodynamic (PD) framework. In particular, translocation and immunomodulation could be considered PD factors, affecting mainly the efficacy and adverse effect of drugs, whereas metabolism and enzymatic modification determined by bacteria on therapeutics are clearly PK parameters, which may alter their bioavailability and metabolite profiles. Reduced diversity may act both as a PD or PK determinant, depending on the bacterial strains involved: for instance, if a pathogenic strain prevails, increased drug-induced infections may be observed, whereas, if a strain involved in drug activation is lacking, bioavailability may be reduced. The study of the microbiome would, thus, provide additional information beyond traditional PK/PD studies, allowing to predict PK patterns before drug administration, providing insights on mechanisms of resistance that may not be evident by standard PD consideration, describing tissue-specific effects that may be still relevant for drug response and providing innovative targets and mechanisms for drug development.

Hereafter, this review will be focused on the effect of the microbiome on the efficacy of pharmacological therapy of agents that have immunomodulating and antileukemic effects, used in particular to treat IBD, ALL, and JIA, pathological conditions affecting the pediatric population. A better understanding of factors affecting susceptibility and therapeutic outcomes in this special population of patients is particular important because these diseases are particularly severe and may lead to permanent negative outcomes compromising life quality also in adulthood or even be fatal. The pharmacomicrobiome has received particular attention and emerging preliminary evidences are being collected and are presented in this review.

ROLE OF MICROBIOTA AND EFFICACY OF THERAPEUTICS IN IBD

IBD includes two different pathologies, Crohn's disease and ulcerative colitis, that show common clinical characteristics, such as a chronic immune-mediated inflammation at different levels of the digestive tract, and alternation between active and inactive phases. Although these diseases can manifest at any age, approximately one-quarter of patients are diagnosed in childhood or early adulthood, when the disease course and subsequent outcomes can be particularly severe.[40] No definitive cure for IBD is currently available and the treatment is mainly focused on the control of inflammation, through immunomodulators capable of inducing and maintaining remission. The main therapies include aminosalicylates and glucocorticoids used as first-line agents, whereas thiopurines are efficacious for maintaining remission; more recently, monoclonal antibodies directed toward TNF-α (infliximab and adalimumab), the common p40 subunit of IL-12 and IL-23 (ustekinumab) and the integrin (vedolizumab) have been introduced.[41,42] The effects of all these drugs are characterized by a high interpatient variability, associated with a significant number of side effects.[43]

Microbiota and IBD susceptibility

Although IBD etiology remains unresolved, a general consensus supports the important role of gut dysbiosis in promoting and determining chronic intestinal inflammation.[44–46]

Hundreds of research papers have described the composition of IBD intestinal microbiota and its poor bacterial diversity in respect to healthy controls; however, it has not yet been clarified whether the gut microbial perturbations are a cause or an effect of disease.[34,47] The results of these studies involving pediatric and adult patients with IBD have highlighted a common reduction of several bacterial taxa, such as *Clostridium groups IV* (*C. leptum*) and *XIVa* (*C. coccoides*), *Roseburia*, *Faecalibacterium prausnitzii* (Firmicutes), Bacteroidetes, *Sutterella* (Proteobacteria), *Bifidobacterium* (Actinobacteria), and an increase of Proteobacteria (in particular *Escherichia coli*), *Pasteurellaceae*, *Veillonellaceae*, *Fusobacterium*, and *Ruminococcus gnavus* (Firmicutes).[48–50] Moreover, shifts in microbial composition in patients with IBD are implicated in disease severity and progression over time and seem to be more pronounced in patients with Crohn's disease than in ulcerative colitis.[51,52]

Microbiota as IBD therapy response biomarkers

Growing evidence suggests that the efficacy of immunomodulators used to treat pediatric and adult patients with IBD is associated with modifications of gut microbiota, as well as by the microbial metabolic activities.[53] The effect of 5-aminosalicyclic acid on the mucosal microbiota composition of 57 patients with ulcerative colitis[54] has been investigated. In particular, the higher abundance of Proteobacteria, such as *Escherichia–Shigella* and Bacteroidetes, including *Bacteroides*, *Prevotella*, and *Parabacteroides*, was reduced after 5-aminosalicylic acid treatment. Additionally, the authors observed a parallel change between the decrease in disease activity and bacterial alteration (e.g., decreased *Escherichia–Shigella*) after 5-aminosalicylic acid treatment, indicating a relationship between the efficacy of the therapy and the bacterial load. Swidsinski and collaborators also described the effect of 5-aminosalicylic acid on the mucosal flora of 20 patients with ulcerative colitis or indeterminate colitis: the anti-inflammatory agent significantly reduced the concentrations and adherence of mucosal bacteria (in particular *Bacteroides* and *Enterobacteriaceae*) in comparison to the untreated patients.[55]

Mercaptopurine treatment was shown to be correlated with a reduction of the microbial richness and diversity in seven patients with IBD,[51] indicating that the immunomodulator may contribute to dysbiosis in patients. Different results were obtained following administration of thiopurines in colitis mouse models: the drug caused caecal mucosa-associated microbiota alteration mainly manifesting as a decrease in Bacteroidetes and an increase in Firmicutes.[56]

Microbiota analysis of fecal samples derived by 56 patients with ulcerative colitis responsive or poorly responsive to anti-TNF therapy (infliximab and adalimumab) revealed a separate profile of gut microbiota before treatment start.[57] In particular, a lower dysbiosis index and higher amount of *Faecalibacterium prausnitzii* (Firmicutes) in responders compared with nonresponders at baseline was found. *F. prausnitzii* increased also during induction therapy in responders, indicating this species as a biomarker of treatment effectiveness.[57] Consistent with several studies, *F. prausnitzii* contributes to the reduction of disease activity by producing butyrate, a SCFA, which inhibits the differentiation of Th17 cells and increases Treg cells.[58]

Recently, Yilmaz and collaborators published a similar paper focused on the role of microbiota composition in two independent long-term IBD cohorts (cohort 1 = 346 patients; cohort 2 = 156 patients).[59] Interestingly, patients with Crohn's disease responsive to infliximab or adalimumab therapy showed an increase of Actinobacteria, including *Bifidobacterium*, *Eggerthella*, and *Collinsella*, and Firmicutes, such as *Lachnospira* and *Roseburia*, and reduced *Phascolarctobacterium* (Firmicutes). Most of these taxa, in particular *Lachnospiraceae* and *Roseburia*, produce SCFA metabolites, which have important anti-inflammatory properties.

Furthermore, in Crohn's disease, the abundance of *Eggerthella*, *Clostridiales*, and *Oscillospira* (Firmicutes) increased in patients with quiescent disease, whereas *Enterobacteriaceae* (among which *Klebsiella*) were associated with a more severe clinical course. No significant differences were highlighted comparing patients with ulcerative colitis responding to anti-TNF therapy with patients failing to respond to treatment.[59]

To determine whether the gut microbiome can predict responses to the anti-integrin monoclonal antibody vedolizumab in IBD, Ananthakrishnan *et al*.[60] conducted a prospective study enrolling 85 patients at the beginning of the therapy (baseline microbiota), and at weeks 14, 30, and 54 after therapy initiation. Only patients with Crohn's disease achieving remission at week 14 showed a significantly more diversified microbiome composition and *Roseburia inulinivorans* and *Burkholderiales* species were more abundant.[60]

Similarly, Doherty *et al.*[61] evaluated the differences in 232 patients' gut microbiota between patients with anti-TNF refractory Crohn's disease responders and nonresponders to ustekinumab, a monoclonal antibody directed against the common p40 subunit of IL-12 and IL-23. Patients in remission after ustekinumab induction presented a higher abundance of Firmicutes, in particular *Faecalibacterium*, *Blautia*, *Clostridium XIVa*, *Ruminococcaceae*, and *Roseburia*. The microbiota diversity of responders increased over the 22 weeks of the study in comparison to nonresponsive patients. The most predictive elements at baseline were related to a high abundance of *Faecalibacterium* and low amount of *Escherichia/Shigella*. The differences observed in fecal microbiota before the start of therapy and its modification due to therapeutic effectiveness encourages the potential role for the microbiota as a response biomarker.

Microbiota species involved in IBD therapeutics metabolism

Drug modifications by gut microbes can lead to their activation as known for sulfasalazine, an anti-inflammatory drug used in the treatment of IBD.[62] Sulfasalazine, the prodrug of 5-aminosalicylic acid, can be activated through cleavage of the azo bond by bacterial azoreductases. The recent paper published by Zimmermann and colleagues described in detail the microorganisms capable to metabolize sulfasalazine among 76 different bacterial species that represent the major phyla of the human gut microbiome.[32] Interestingly, Bacteroidetes (e.g., *Alistipes* and *Prevotella*)

and Firmicutes (e.g., *Clostridium* and *Ruminococcus gnavus*) are the phyla with the higher ability to metabolize sulfasalazine, whereas this activity seems to be reduced or absent among Proteobacteria (e.g., *Salmonella typhimurium* and *Escherichia coli*). Considering that most of these strains are under-represented or over-represented in IBD, a change in effectiveness of sulfasalazine, dependent on the patient-specific gut microbiota could be important to consider.

Bacterial metabolism of thiopurines was first investigated *in vitro* using the representative gut bacteria *Escherichia coli* (Proteobacteria), *Enterococcus faecalis* (Firmicutes), and *Bacteroides thetaiotaomicron* (Bacteroidetes).[56] Thioguanine (TG) nucleotides (TGNs), the active products of thiopurine metabolism, were detected in the bacterial pellets following incubation of log phase cultures of all three bacterial strains with 1 mM TG for 120 minutes. Moreover, TGNs were also detected by liquid chromatography-double mass spectrometry when fecal slurries derived from mice were incubated with 5 or 10 μM TG for 6 hours, demonstrating that generation of TGN can occur via bacterial activation too. The intestinal delivery of the drug and the conversion of thiopurines to active compounds by microbiota could significantly provide a more rapid therapeutic action avoiding unwanted toxicity. Furthermore, microbiota profiles associated with side effects in patients treated with thiopurines could provide interesting insights on potential targeted intervention, comprising treatments useful to modulate microbiota composition.

Thiopurine S-methyltransferase (TPMT) is an enzyme involved in thiopurine metabolism that reduces the conversion of these drugs into active TGNs. An orthologue of human TPMT was found and characterized also in bacteria (in particular Proteobacteria).[63] Interestingly, the bacterial enzyme similarly catalyzes the S-adenosylmethionine-dependent transmethylation of thiopurines and shares 45% similarity with the human enzyme; however, no data about the role of bacterial TPMT on the efficacy of thiopurines *in vitro* or *in vivo* have been reported.

ROLE OF MICROBIOTA AND EFFICACY OF THERAPEUTICS IN ALL

Microbiota and ALL susceptibility

The interest of microbiota in ALL has been growing in recent years. ALL is an oncohematological disorder due to malignant transformation and proliferation of lymphoid progenitor cells in the bone marrow, followed by their release in the peripheral blood stream and impairment of the mature immune system.[64] Several studies highlighted the ALL-induced gastrointestinal dysbiosis possibly due to an interaction between the immune system and host microbiota, in particular in the gut immunological niche.[65] Rajagopala and collaborators extracted DNA from the stool samples of 28 pediatric and adolescent patients at diagnosis (before chemotherapy) and 23 matched healthy siblings to compare the gastrointestinal microbiota composition by 16S rRNA gene sequencing.[66] They demonstrated that, in both groups, microbiota profiles were dominated by members of the Bacteroidetes (*genera Bacteroides* and *Prevotella*) and Firmicutes phyla (*genus Faecalibacterium*), although with changes in the mean abundances (lower percentages of *Bacteroides*, and higher percentages of *Prevotella*, and *Faecalibacterium* in ALL). Major differences between the two groups occurred in the remaining less represented *taxa* with lower diversity in patients: *genera Anaerostipes*, *Coprococcus*, *Roseburia*, and *Ruminococcus* (Firmicutes phylum) had relatively lower abundance in the patient group.[66] In agreement with Rajagopala's results, Bai and coworkers[67] also found that the amount of Firmicutes decreased and that of Bacteroidetes increased in 10 children with ALL compared with 17 healthy controls: *Lachnospiraceae*, in particular *Blautia genus*, and *Erysipelotrichiaceae* families (Firmicutes) were less abundant in children with ALL; *Bacteroidales* (Bacteroidetes) were more abundant in children with ALL and *Porphyromonadaceae* (Bacteroidetes) and *Enterococcaceae* (Firmicutes) families were effective in distinguishing children with ALL from controls.

Patients with ALL have often been treated with antibiotics before diagnosis to treat infections, which are a major symptom of the disease. The study of Bai and coworkers[67] demonstrated that antibiotics reduced the microbiota diversity, indeed, the administration of antibiotics for < 1 month in healthy children (n = 20) reduced the number of species and decreased the diversity of their gastrointestinal microbiota compared with those without antibiotics (n = 17). However, changes caused by ALL cannot be eliminated or neutralized by antibiotics: indeed, with regard to diversity, samples from children with ALL always diverged from the controls, regardless of antibiotic use.[67]

Microbiota as ALL chemotherapy response biomarkers

ALL polychemotherapeutic treatment is long-lasting (overall length ~ 2–3 years) and is organized in an initial remission induction phase (~ 2 months) followed by consolidation (~ 2 months) and a long continuation afterward. Hakim and coworkers[68] analyzed the gastrointestinal microbiota changes over therapy phases in 199 children with ALL treated at St. Jude Children's Research Hospital (Memphis, TN) according to the clinical trial Total XVI (NCT00549848). Induction treatment began with prednisone, vincristine, daunorubicin, PEG-asparaginase, and triple intrathecal treatment (methotrexate, cytarabine, and corticosteroids), followed by cyclophosphamide, cytarabine, and thioguanine. Consolidation included high doses of methotrexate and mercaptopurine plus triple intrathecal therapy every other week. Continuation therapy varied week by week and comprised two periods of more intensive chemotherapy (re-induction I in weeks 7–9 and re-induction II in weeks 17–20). Microbiota diversity significantly decreased after higher intensive therapeutic phases: indeed, it decreased from diagnosis to the end of induction, reverted to the baseline level after less-intensive consolidation and continuation treatment before re-induction I, and significantly decreased again after intensive re-induction II.[68] Compared with baseline, Bacteroidetes, Actinobacteria, and Verrucomicrobia phyla, as well as Firmicutes families *Faecalibacterium* and *Ruminococcaceae* significantly decreased in at least one of the postchemotherapy

samples. In contrast, other Firmicutes families, including *Clostridiaceae*, *Streptococcaceae*, *Lactobacillaceae*, and *Enterococcaceae* significantly increased after chemotherapy.[68] In addition, in the Rajagopala study, the increased differences of gut microbial communities were observed during the maintenance phase compared with prechemotherapy baseline.[66]

Prior chemotherapy may have a lasting effect on microbial ecosystems, affecting the success of therapy coming afterward. Dysbiosis was studied in 20 patients with leukemia, 13 after induction therapy, and 7 after repeat (salvage) therapy due to persistent or relapsed disease, measuring the similarity of microbiota in longitudinal samples collected throughout induction or repeat therapy. Compared with induction samples, bacterial composition of the repeat therapy samples showed less similarity to their baseline pretherapy samples and a more prominent *Enterococcus* (Firmicutes) outgrowth over time, suggesting that the major microbiota instability observed at salvage therapy potentially resulted in greater vulnerability of microbiota to enterococcal pathogenic genera.[69]

Altogether, the above-mentioned studies on patients are descriptive of alterations happening in the gastrointestinal microbiota during and following chemotherapy regimens. Major interest in the ALL field is to get more detailed information on microbial profiles that are associated with clinical response. Currently, the predictive value of the minimal residual disease assessed on bone marrow on clinical outcome is very well known, whereas the role of microbiota still needs to be well-established. In the ALL field, microbiota could be especially useful to predict drug-induced gastrointestinal side effects, particularly the development of various infections and persistent diarrhea. To our knowledge, the only study assessing this issue in ALL is the one of Hakim *et al*.[68] A baseline gut microbiota characterized by Proteobacteria phylum and some components of the *Lactobacillales* families (Firmicutes phylum) was associated with severe side effects during chemotherapy. Proteobacteria had been correlated to an increased incidence of febrile neutropenia, *Enterococcaceae* (Firmicutes, relative abundance $\geq$ 30%) predicted significantly greater risk of subsequent febrile neutropenia and diarrheal illness, whereas *Streptococcaceae* (Firmicutes) dominance predicted significantly greater risk of subsequent diarrheal illness.[68]

Small animal models have been used to investigate the mechanism of gastrointestinal toxicity induced by a single antileukemic drug, and the effect on the resident microbiome, as already highlighted for cyclophosphamide.[20,22] Methotrexate is a gold-standard drug during ALL therapy and is used throughout all therapeutic phases (i.e., intrathecally in induction, at high doses (2–5 g/m^2 i.v. infusion) during consolidation and at low doses (20 mg/m^2 *per os*) in maintenance). Although methotrexate is safely administered to most patients, it can cause significant multi-organ toxicities, including intestinal toxicity typically accompanied with nausea, bloating, abdominal pain, and diarrhea.[70] Mechanisms by which methotrexate induces intestinal impairment are multiple and include a direct cytotoxic effect on intestinal epithelial cells, a dysregulation of the intestinal mononuclear phagocyte system, comprising dendritic cells and macrophages, and consequent imbalance in the host gut microbiota. Mice intraperitoneally injected with 1 mg/kg of methotrexate for 14 days developed mucosal damage accompanied by inflammatory cells' infiltration and significant changes in macrophages in the colon. By analyzing gut microbiota by high throughput pyrosequencing of 16S rRNA gene amplicons over the treatment period, a gradually reduced diversity of the total microbiota was observed: *Bacteroidales* order (Bacteroidetes) were dramatically reduced in the feces, whereas *Lachnospiraceae* family (Firmicutes) underwent a sharp increase after methotrexate treatment in a time-dependent manner.[39] In another rat mucositis model, methotrexate infusion induced also an overall decrease (705-fold) in most bacteria compared with controls. Reduced bacterial presence was related with diarrhea and a reduced villus length. After 4 days of treatment, there was an absolute and relative decrease of commensal protective intestinal anaerobes (13-fold and –58%, respectively), including *Ruminococci* species, and of oxygen-tolerant Firmicutes *streptococci* (296-fold and –1%, respectively).[71] However, in contrast to the previous study, a relative increase of enteropathogenic anaerobic *Bacteroides genera* compared with controls (+49%) was observed.

Microbiota species involved in ALL chemotherapeutic metabolisms

Being the oncologic approach polychemotherapeutic, it is not easy to understand which drug is most altered by the microbiota. A recent already mentioned outstanding study measured the ability of 76 human gut bacteria to metabolize 271 orally administered drugs (including agents used in antileukemic therapy, such as the corticosteroids prednisone and dexamethasone, cyclophosphamide, and the tyrosine kinase inhibitors, imatinib and dasatinib), and combined high-throughput genetic analyses with mass spectrometry to systematically identify microbial gene products that metabolize drugs.[32] In this screen, dexamethasone was metabolized by *Clostridium scindens* (Firmicutes) in axenic bacterial cultures and was metabolized (side chain cleavage) in the intestine, particularly in the cecum, in mice mono-colonized with *Clostridium scindens* compared with GF mice. Notably, anaerobic incubation of dexamethasone with fecal cultures from 28 healthy human donors showed substantial interpersonal variation in metabolite production, suggesting that other bacterial taxa may also metabolize dexamethasone. A similar pattern was reported also for prednisone. Several *Bacteroides* species (Bacteroidetes), also significantly reduced (> 20%) corticosteroids: in particular, *Bacteroides dorei* acts on both dexamethasone and prednisone. Cyclophosphamide is metabolized mainly by Firmicutes taxa, although the main metabolizer was *Alistipes indistinctus* (Bacteroidetes). Among tyrosine kinase inhibitors, imatinib was decreased after incubation with several *Bacteroides* species (*Parabacteroides johnsonii*, *Bacteroides eggerthii*, *Bacteroides vulgatus*, and *Bacteroides stercoris*), whereas dasatinib was affected by *Clostridium* species (including *Clostridium bolteae*, phylum Firmicutes) and also by *Bifidobacterium ruminatum* (Actinobacteria phylum) and *Bacteroides fragilis* (Bacteroidetes).

ROLE OF MICROBIOTA AND EFFICACY OF THERAPEUTICS IN JIA

Rheumatological diseases, such as rheumatoid arthritis or JIA, are a clinically heterogeneous group of conditions characterized by chronic arthritis, synovial inflammation, and erosion of bone and cartilage, and significant associations with variations in the microbiota have been described. In particular, for spondyloarthritis, a rheumatological disease that affects both the joints and entheses, gut inflammation is present,[72] which may be associated with abnormalities in the composition of the gastrointestinal microbiota.

Microbiota and JIA susceptibility

Specific bacterial species potentially associated with JIA have been identified. Among major environmental factors that can impact the composition of microbiota, delivery mode and early exposure to antibiotics have been demonstrated to be relevant for the incidence of these pathologies. A population and national register-based cohort study performed in Denmark indicated that, among all children born in the country from January 1997 to December 2012, those delivered by cesarean section were at increased risk of disease associated with immune function. In particular, elective cesarean section was associated with an increased risk of JIA.[73] For antibiotic use, in a nested case-control study in a large pediatric population database from the United Kingdom, antibiotic administration was associated with newly diagnosed JIA, with a dose-dependent and time-dependent effect.[74] Both these associations may be mediated through alterations in the microbiota.

An analysis of the composition of the fecal microbiota of 30 children with new-onset JIA compared with 27 healthy controls by 16S region-based sequencing profiling, identified a lower proportion of bacteria belonging to the phylum Firmicutes in children with JIA compared with controls, whereas bacteria belonging to Bacteroidetes were significantly more abundant in JIA than in control samples.[75] These results are in agreement with previous analyses performed in other rheumatic diseases, such as spondyloarthritis.[76,77] This study also described that phyla Actinobacteria and Fusobacteria were present only in patients with JIA, whereas phylum Lentisphaerae was present only in controls. No differences between patients with JIA and controls in the diversity indexes was found in this study.[75] Another study considered children with a JIA subtype, enthesitis-related arthritis (JIA-ERA). This study enrolled 25 children with JIA-ERA and 13 controls. Patients with JIA-ERA had less *Faecalibacterium prausnitzii*, a bacterial species that is considered to have anti-inflammatory effects through production of SCFAs, such as butyrate.[78] Moreover, increased abundance of *Bacteroides* and of the Verrucomicrobia *Akkermansia muciniphila* was evident.[79]

Metabolomic profiling of fecal samples of children with JIA-ERA was performed by nano-liquid chromatography-mass spectroscopy, combined with sequencing of the 16S ribosomal DNA on the same stool specimens. These analyses revealed an under-representation of multiple metabolic pathways, including the tryptophan metabolism pathway. Significantly fewer microbial genes associated with metabolic processes in the patients compared with the controls were observed, indicating diminished metabolic diversity as a potential feature of JIA-ERA.[80]

A study in an Italian cohort of 29 patients with JIA (19 with JIA-ERA and 10 with JIA-non ERA) in comparison to 29 healthy controls, identified differences in various bacterial families belonging to the Firmicutes phyla.[81] In particular, in comparison to healthy subjects, *Ruminococcaceae* resulted as increased in patients with JIA, *Veillonellaceae* in JIA-non ERA, whereas *Clostridiaceae* and *Peptostreptococcaceae* were decreased in JIA-ERA. This study, therefore, illustrates clearly a more complex pattern of association of the Firmicutes phylum abundance, than a more general pattern of reduction described by previous studies in JIA[75] and other rheumatic or immune-mediated diseases.[82] Authors underline in particular that the enrichment of gram-positive *Clostridium* cluster XIVb in patients with JIA-ERA suggests a causal relation with inflammation. Indeed, previous studies have indicated that cell wall peptidoglycans of *Clostridium* and other anaerobic gram-positive species can induce articular damage if the peptidoglycan has lysine as the third amino acid of the stem peptide.[83] Also, this study identified a reduction in *Faecalibacterium*, considered to be an anti-inflammatory microorganism, in JIA-non ERA.[84] A study in Asian patients considered 33 patients with JIA-ERA and 14 age matched controls. All patients had active arthritis and were receiving exclusively nonsteroidal anti-inflammatory drug (NSAID) therapy (no immunosuppressive drugs, disease modifying anti-rheumatic drugs, or biologicals). Gut microbiota showed dysbiosis in patients with JIA-ERA, which presented a wider dispersion than controls. *Bacteroidaceae* (Bacteroidetes) and *Enterobacteriaceae* (Proteobacteria) were more abundant and *Prevotellaceae* (Bacteroidetes) were less abundant than in controls. In addition, the genera *Bacteroides* (Bacteroidetes), *Enterococcus* (Firmicutes), and *Klebsiella* (Proteobacteria) were over-represented and the genus *Prevotella* (Bacterioidetes) was under-represented in patients with JIA-ERA.[85] This study also evaluated, in eight patients, the effect of 12 weeks of probiotic therapy (VSL#3; Sun Pharmaceuticals, Mumbai, India) twice daily orally, with each capsule containing around 100 billion bacterial cells belonging to eight species, namely *Streptococcus thermophilus* (Firmicutes), *Bifidobacterium breve*, *B. longum*, *B. infantis* (Actinobacteria); *Lactobacillus acidophilus*, *L. plantarum*, *L. paracasei*, and *L. delbrueckii* (Firmicutes) as part of a clinical trial. However, no significant difference was noticed between the fecal specimens collected before and after probiotic intake, considering diversity and abundances of various bacterial taxa at all levels. The authors discussed this negative result as in line with comprehensive analyses that reveal a lack of evidence for an impact of probiotics on fecal microbiota composition in healthy adults[86] and children.[87]

A recent study attempted to clarify the association of alteration of Bacteroides and reduction in Firmicutes, in particular of *Faecalibacterium prausnitzii* in a cohort of adult and pediatric patients with spondyloarthritis and JIA-ERA. This cohort was composed of 11 adult and 30 pediatric patients, compared with, respectively, 10 and 19 age-matched controls. Recent exposure to systemic antibiotics was an

exclusion criterion. Adult patients could be treated with immunomodulators (classic disease modifying anti-rheumatic drugs or biologics), whereas all pediatric patients were treatment naïve at the moment of enrollment. Pairwise comparisons at most of the phylogenetic levels failed to identify any groupwise differences. The only exception was that patients with JIA-ERA demonstrated decreased abundance of the Actinobacteria phylum as compared with controls. A candidate analysis focused on *Faecalibacterium prausnitzii* and *Bacteroides fragilis* (Bacteroidetes) indicated a decreased abundance of the anti-inflammatory *F. prausnitzii* A2-165 strain and an increased abundance of the neutral *F. prausnitzii* L2/6 strain. However, the abundance of *F. prausnitzii* as a whole was nominally higher in the patients, in contrast to previous results from the same group already discussed above.[79] Shotgun metagenomics sequencing of the fecal DNA in the pediatric subjects confirmed diminished coverage of the anti-inflammatory butyrate pathway. Similar trends were observed in adults with longstanding spondyloarthritis. In contrast, the fecal abundance of *Bacteroides fragilis* (Bacteroidetes) was increased in patients with JIA-ERA, whereas resulted as diminished in adult subjects compared with controls. This difference may be related to a role of *Bacteroides fragilis* in the development of the immune system,[88] but may be also affected by the fact that, in this study, > 80% of adult patients were under immunomodulating therapy.[89]

A recent large multicentric study, enrolling 78 Italian and 21 Dutch treatment-naive patients with JIA compared with 107 geographically matched samples from healthy children, also did not confirm in patients with JIA a protective effect of *Faecalibacterium prausnitzii*, that actually resulted as increased, together with *Erysipelotrichaceae* (Firmicutes); *Allobaculum* (Firmicutes) was instead reduced. Forty-four follow-up samples from patients with inactive disease and 25 follow-up samples from patients with persistent activity were also analyzed. Microbiota richness, in terms of rare species, was reduced in patients with JIA compared with healthy controls, at baseline, during inactive disease, and during persistent disease activity, especially in Italian subjects. However, when comparing baseline samples with paired inactive disease samples, no differences in relative abundance were found. This finding may indicate that the gut microbiota profile is specific to the individuals with JIA, rather than the disease activity status.[90]

Microbiota as JIA therapy response biomarkers

Regarding the effect of pharmacological therapies on gut microbiota of patients with JIA, one of the first studies to describe potentially significant associations considered a group of patients with JIA-ERA treated with NSAIDs, alone or associated with sulfasalazine/methotrexate/biologics in different combinations, and a group of patients with JIA-non ERA, mainly treated with biologic drugs, such as etanercept and abatacept. Despite the low number of patients stratified by pharmacological treatment, authors observed enrichment in *Collinsella* (Actinobacteria), associated with exacerbation of joint disease, in patients with JIA-ERA treated with combined NSAIDs and sulfasalazine therapy. The abundance of *Collinsella* correlated strongly with inflammation markers as well as production of the proinflammatory cytokine IL-17A also in adult patients with rheumatoid arthritis and a role for *Collinsella* in altering gut permeability and disease severity was confirmed in experimental arthritis.[91] Among the patients enrolled, 19% of patients with JIA-ERA and 41% of patients with JIA-non ERA presented clinically active disease: *Sutterella* (Proteobacteria) was increased in samples collected during active disease, whereas *Clostridium* cluster IV and XVIII (Firmicutes), *Parasutterella* (Proteobacteria), and *Odoribacter* (Bacteroidetes) were more abundant in samples collected from patients in remission. Prediction analysis of metabolic functions indicated that JIA-ERA metagenome presented enrichment in bacterial functions associated with cell motility and chemotaxis, suggesting selection of potential virulence traits.[81]

A recent study attempted to develop algorithms to predict inactive disease according to Wallace criteria at 6-month intervals in the first 2 years of JIA. Potential predictors were baseline clinical variables, joint status, gut microbiota composition, and a panel of inflammation-related compounds in blood plasma. The study considered 152 treatment naïve patients with JIA, who could be treated with standard therapies, categorized in the study as intra-articular joint injections with or without NSAIDs, methotrexate with or without oral steroids, and biological agents. Inactive disease could not be predicted with satisfactory accuracy in the whole cohort, likely due to disease heterogeneity. However, a gut microbiota-associated predictor in a subgroup of patients was identified: in particular, inactive disease was predicted by a lower relative abundance of *Mogibacteriaceae* (Firmicutes) for patients with oligoarthritis.[92] Authors discuss that nothing is known about *Mogibacteriaceae* in the context of autoimmune diseases, whereas this bacterium was less present in obese Japanese subjects, in comparison to lean subjects.[93]

In 18 French adult patients with spondyloarthritis, modifications of the microbiota composition were observed after 3 months of anti-TNF treatment (15 patients received etanercept, 2 received adalimumab, and 1 received infliximab), but no specific taxon was modified, whatever the clinical response. Authors performed an analysis to identify predictors of anti-TNF treatment after 3 months of therapy, selecting two subgroups of patients, five with good response (change in disease activity score > 2), and eight with limited efficacy (change in disease activity score < 1). Before anti-TNF administration, a higher proportion of *Burkholderiales* (Proteobacteria) in future responder patients was identified by this study. This suggests an interaction between anti-TNF treatment and intestinal microbiota even if further studies are needed.[94]

A recent study, also performed in adult patients with rheumatoid arthritis, enrolled 42 patients divided according to current therapy: 11 patients were naïve to immunosuppressants, 11 patients were receiving methotrexate, 10 patients were receiving the anti-TNF agent etanercept, and 10 patients were receiving etancercept plus methotrexate. Ten healthy subjects were used as controls. The relative abundance of the microbial phyla was almost unchanged between patients and controls. When compared with naïve patients, significant changes were recorded in patients

treated with etanercept: phylum Cyanobacteria significantly increased, in particular, in the *Nostocophycideae* class. In the group of patients treated with methotrexate, a decrease in the amount of Enterobacteriales (Proteobacteria) was described, whereas no significant variations were seen in patients treated with etanercept and methotrexate.[95]

Microbiota species involved in JIA therapeutics metabolism

Studies on the effect of drug therapy on microbiota are still limited, especially in children. Methotrexate is a very important medication also for the treatment of JIA and it is known that intestinal microbiota in rodent models can modify this drug, but the implication of this finding in patients still needs to be explored.[96]

DISCUSSION AND CONCLUSIONS

The microbiota is clearly altered in diseases involving immune system cells, such as IBD, ALL, and JIA, even if generalization of the findings described are still limited by the small sample sizes and by the influence of confounding environmental factors, such as patients' age or geographic areas of the studies. Moreover, besides the presence of reproducible associations, such as alterations of the *Faecalibacterium*/Bacteroidetes ratio, demonstration of the causality between differences observed and diseases still needs to be provided. This is particularly true also for the effects of microbiota on the activity of immunomodulatory and antileukemic agents (**Figure 1**). The needs of research in this field are larger studies in pediatric patient populations to identify bacterial strains associated with disease subtype and drug responses, with better control of environmental factors, such as diet, hygiene, and geographic location.

The overall utility of characterizing microbial composition in dosing drugs still has to be determined. For some chemotherapeutics, such as irinotecan, a clear contribution of the microbiota in determining drug-induced gastrointestinal toxicity has been proved and the use of inhibitors of bacterial glucuronidases, which release in the intestine the cytotoxic form, may be important to reduce adverse effects. Emerging evidence seems to indicate that, in patients with IBD, a higher abundance of Firmicutes may be important for the efficacy of biologic agents, such as anti-TNF drugs, whereas in ALL and JIA more research is needed before drawing specific patterns of association between bacterial strains and therapeutic response.

Current evidence seems to indicate that bacterial composition before or during treatment may affect drug effects and an application of the microbiome analysis could be the identification of bacterial strain present, putatively predictive both in terms of efficacy or adverse effects, and its modification by interventions, such as antibiotic administration. Because infections could be a consequence of all immunosuppressive therapies, microbioma profiling could be proposed as an infection risk stratification tool and used to propose personalized treatment approaches. In this regard, the modification of microbiome composition seems to be promising in applying the pharmacomicrobiome to

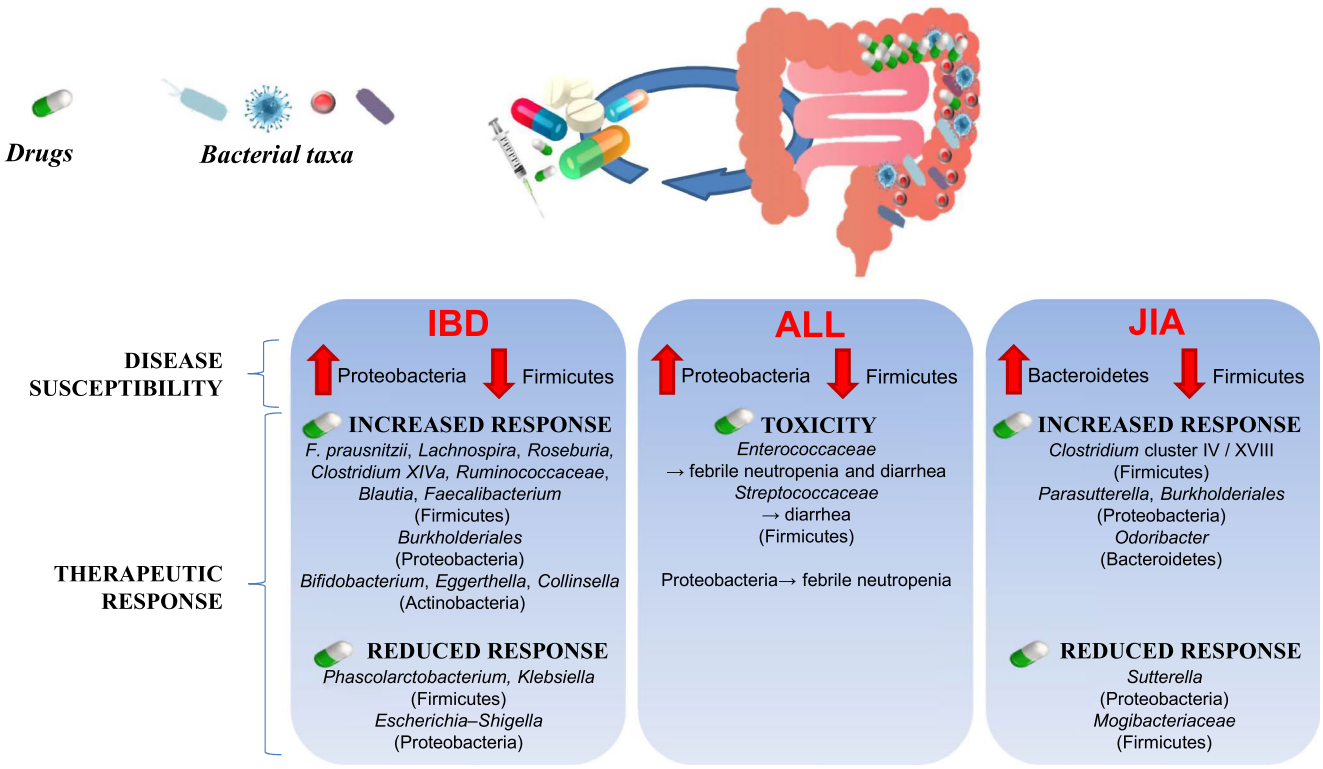

Figure 1 Representative scheme of possible interactions between drugs and gut-associated microbioma. ALL, acute lymphoblastic leukemia; IBD, inflammatory bowel disease; JIA, juvenile idiopathic arthritis.

improve therapeutic outcomes. Besides the use of antibiotics, additional customized innovative interventions could be developed, such as probiotics, fecal microbiota transplant, and modified diet. The efficacy of these strategies to modify the microbioma in the clinical setting still needs to be fully demonstrated. In ALL, the hypothesis that the relative abundance of species at diagnosis could be predictive of chemotherapy-induced toxicities, given their severity, is particularly intriguing, and deserves further studies.

Although different models demonstrate the role of specific bacteria on the biotransformation and modulation of the effects of these drugs, evidence of their role in interindividual variability in patients is still lacking. However, to really understand the relevance of microbiota composition for the interindividual variability in drug responses mechanistic studies using innovative experimental approaches are also important. Combination of high-throughput genetics with mass spectrometry to systematically identify drug-metabolizing microbial gene products could be relevant. As already mentioned in this review, a recent study applied this approach, validating 30 microbiome-encoded enzymes that collectively convert 20 drugs to 59 candidate metabolites and providing a mechanistic understanding of microbiome drug biotransformation.[32] Moreover, a paradigmatic study separating host and microbiome contributions to drug PKs and toxicity has been published combining gut commensal genetics in gnotobiotic mice carrying no microbiota, genetically manipulated gut commensals, or a complex microbial community, to measure drug metabolism in various tissues.[97]

These mechanistic studies will be particularly important for drug development: knowing the interaction between the microbiota and approved drugs could favor the development of computational models for the identification of new molecules, such as inhibitors or prodrugs activated by specific bacterial enzymes, or optimization of existing drugs and formulations. In this regard, bacteria may provide targets that could be pharmacologically modified to improve therapeutic outcomes. Agents addressing bacterial targets not present in human cells may have limited side effects. Moreover, particularly important, as already mentioned, will be studies to evaluate innovative pharmacological strategies to modify the microbiota. Reducing specific bacterial strains related to therapeutic efficacy may indeed be a promising approach.

The research on the interactions between drug effects and the microbioma presents several challenges: from an *in vitro* experimental point of view, a feasible approach is to study the biotransformation and testing candidate strains on specific drugs; however, bacterial communities are heterogenous, presenting several strains interacting in complex patterns. Therefore, innovative microbiological tests should be developed to evaluate the effects of microbial communities, besides individual strains. The complexity of microbiota, in terms of changes occurring between several locations in the body, also at the intestinal level, constitutes an additional challenge. Thus, experimental design needs to be accurate in order to collect the most informative samples from patients. Finally, the great amount of data generated by microbiota metagenomic analysis represents an issue common to all -omic approaches. In conclusion, there are several implications of microbial alterations for the pharmacological therapy mentioned in this review that need further exploration. In particular, it remains to be demonstrated whether microbial alteration can explain the inter/intra-individual variability or drug levels or responses across demographics or comorbidities. Moreover, the importance of the contribution of the microbiota on standard PK/PD factors, like liver/kidney clearance or conjugation, also remains to be investigated.

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