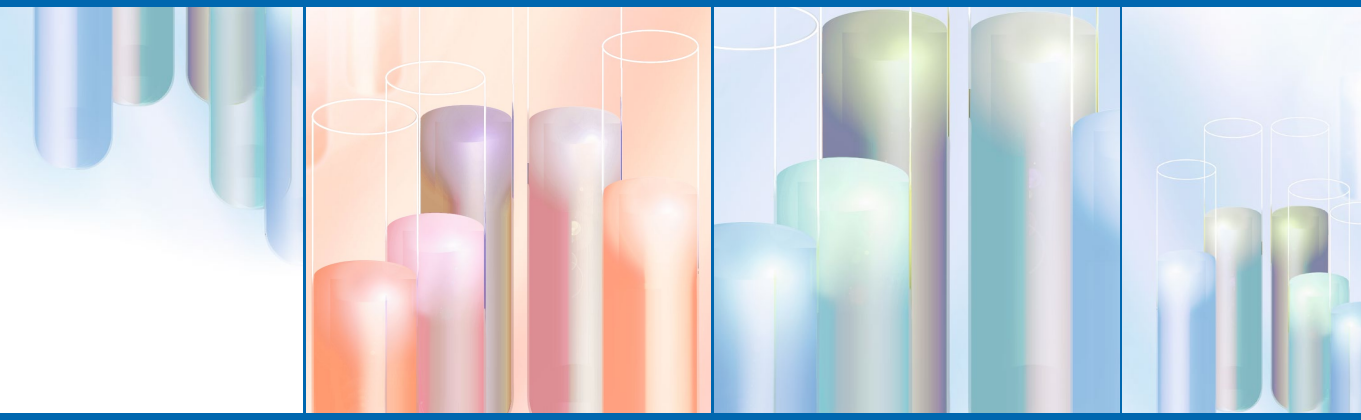
Inflammatory Bowel Diseases

Laboratory Diagnostics



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Publisher

FALK FOUNDATION e.V.

Leinenweberstr. 5
79108 Freiburg
Germany

www.falkfoundation.org

20th revised edition 2022

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Contents

I Introduction

The past two decades have brought major changes in the treatment of inflammatory bowel disease (IBD). For example, complications that were once treated almost exclusively by surgery are increasingly becoming accessible by endoscopic procedures, while the emergence of biologics has revolutionized pharmacological care. Guidelines from national (German Society for Gastroenterology, Digestive and Metabolic Diseases, DGVS) and international (European Crohn's and Colitis Organisation, ECCO) societies provide evidence-based diagnostic and treatment recommendations allowing optimal implementation. Nonetheless, it must be emphasized that there is no such thing as the hypothetical "ideal guideline patient", and hence each treatment option must be tailored to each individual patient.

Just as there is no "ideal guideline patient", there is also no "one laboratory parameter" that adequately reflects all phases of a condition from diagnosis to recurrence (fig. 1).

Several new serological and fecal biomarkers have become established in recent years. Measurement of ASCA and pANCA in serum and calprotectin in stool have been integral lab-based components of IBD diagnosis for years. New methods to measure the concentration of biologics and their corresponding anti-drug antibodies (ADA) in blood are now providing the first opportunity to optimize these new drugs to the pharmacokinetics of each individual patient and provide hints about the possible need to shorten dosage intervals, increase dosage, or switch to a new antibody.

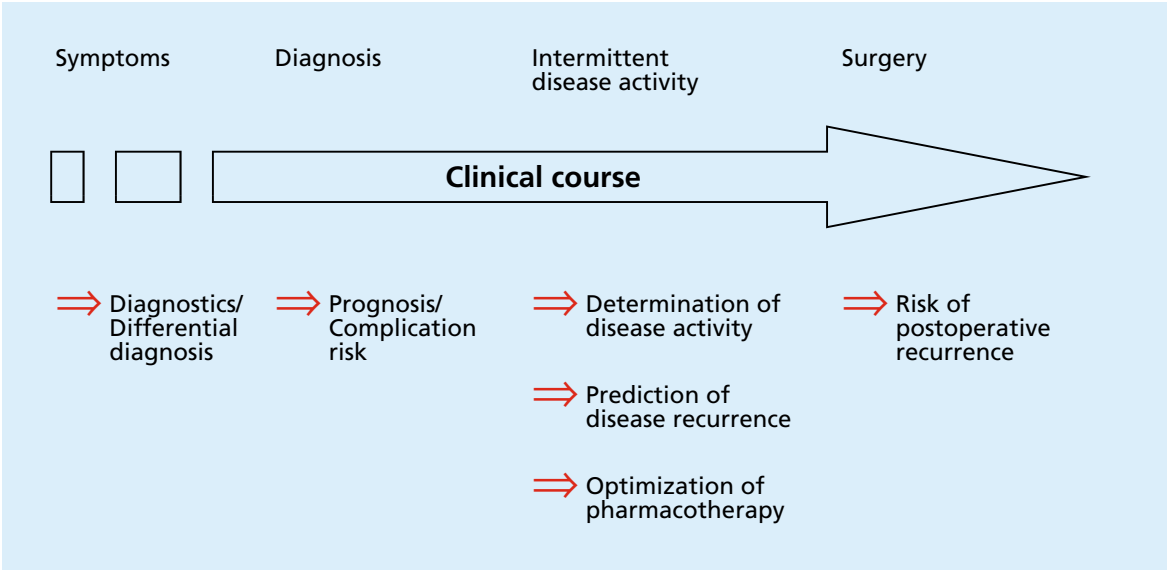


Figure 1:
Clinical course of inflammatory bowel disease and potential diagnostic value of laboratory parameters

In contrast, the fecal neutrophil markers calprotectin and lactoferrin allow timely detection of a flare following remission induced by medication or surgery. These markers also allow for non-invasive monitoring of treatment response in the form of mucosal healing.

A complete arsenal of tests for autoantibodies also helps predict disease forms with mounting complications and allows treatment escalation at earlier time points.

II Diagnosis and differential diagnosis of inflammatory bowel disease (IBD)

1 Differential diagnosis: IBD vs. infectious gastroenteritis

Patients with a primary symptom of persistent bloody, mucous diarrhea may have either IBD or a gastrointestinal infection, the latter of which thus represents the most important differential diagnosis both for initial onset and clinical relapse of IBD.

It is often not possible to reliably distinguish the endoscopic pathologies caused by infectious colitis from those caused by ulcerative colitis or Crohn's disease. For example, edema, mucosal reddening, increased susceptibility to injury, aphthoid lesions, and ulcers may be observed in patients with infectious colitis. These infections typically initially present with diffuse mucosal changes. After 2–3 weeks, this transitions into a discontinuous spread with discrete erosions and ulcers. In rare cases, infectious colitis may also be chronic and persist for months (e.g. *Entamoeba*, *Yersinia*).

Laboratory tests to diagnose microbial etiologies of infectious gastroenteritis require that sensitive and specific techniques be available at short notice. The range of available tests must also account for the fact that the spectrum of diarrheal pathogens is continuously expanding in the age of global tourism (table 1). Consequently, there is an increasing demand for complex and costly laboratory work with special culture media and special incubation conditions, special time-consuming staining procedures, and expensive immunological and molecular genetic techniques for the identification of toxins, antigens and molecular virulence factors [1].

Pathogen	Culture	Toxin	Antigen ELISA	PCR	Microscopy	Specific stain
Clostridioides difficile	Stool	Stool	–	Culture[a]	–	–
Other bacteria[b]	Stool	–	Stool[c]	–	–	–
EPEC/EaggEC*	Stool	–	–	Culture	–	–
EHEC**	Stool	Stool	–	Culture	–	–
Cryptosporidia	–	–	Stool	–	–	Stool
Cyclospora	–	–	–	–	–	Stool
Giardia	–	–	Stool[d]	–	Stool	–
Entamoeba	–	–	Stool[d]	–	Stool	–
Microsporidia	–	–	–	Stool	–	Stool
Isospora belli	–	–	–	–	Stool	Stool
Blastocystis hominis	–	–	–	–	Stool	–
Strongyloides	–	–	–	–	Stool[e]	–
Helminth eggs	–	–	–	–	Stool	–
Rotaviruses	–	–	Stool	–	–	–
Adenoviruses	–	–	Stool	–	–	–
Noroviruses	–	–	Stool	Stool	–	–
Cytomegaloviruses	–	–	–	Biopsy	–	–

[a] cultured bacteria
[b] *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia*, *Vibrio*, *Aeromonas*
[c] *Campylobacter jejuni/coli*
[d] fresh stool needed to detect cysts
[e] fresh stool needed to detect larva
* EPEC/EaggEC = Enteropathogenic/enteroaggregative *Escherichia coli*; ** EHEC = Enterohemorrhagic *E. coli*

Table 1:
Microbiological testing methods for bacterial, parasitic, and viral pathogens of the GI tract (from Kist[1])

Stool samples are most suitable material for microbiological testing of patients with acute and chronic diarrhea (table 2). Unfixed colonic biopsies are needed to detect cytomegalovirus (CMV) infection. Fresh small bowel biopsies can be useful for testing for microsporidia by histology or molecular biology, while small bowel or bile duct biopsies may be helpful for *Cryptosporidium* spp. in addition to stool testing.

For bacteriological examination on suspicion of shigellosis or campylobacteriosis or for the detection of *Clostridioides difficile* toxin, stool samples should be tested promptly, and at the latest, within 24 hours of collection.

Examination for parasites requires fresh stool samples (< 1 hour) in order to microscopically detect vegetative forms of *Entamoeba histolytica* and *Giardia lamblia*. Likewise, infestation with *Strongyloides stercoralis* can only be diagnosed via microscopic detection of living larval stadia in fresh stool, since helminth eggs are not present in stool. In stool samples that are parasitologically examined more than 1 hour after collection, only cysts and spores of the relevant protozoa, as well as microsporidia and helminth eggs remain detectable. These samples are also still suitable for ELISA-based detection of protozoan antigens (*Entamoeba*, *Giardia*, *Cryptosporidium*).

A single stool sample is usually sufficient for microbial testing and toxin detection. In order to rule out parasitic infections with adequate certainty, three suitable stool samples must be analyzed, preferably from three consecutive days[1]. The utility of "dysbiosis tests" remains unconfirmed and hence they are not recommended. The conventional methods used for the detection of individual pathogens and the appropriate test materials are summarized in table 1[1].

Test for	Test material	Transport conditions
Vibrio cholerae	Stool (in peptone water)	By courier and register with lab
Other enteric pathogens and/or their toxins	Stool (in Cary-Blair medium if needed)	Do not transport longer than 24 hours, shorter better
Parasites (cysts) and *Strongyloides* larvae	Stool	Do not transport longer than 1 hour, shorter better
Parasites (cysts and spores)	Stool (in SAF fixative if needed)	Do not transport longer than 24 hours before test
Microsporidia spp.	Plus small intestinal biopsy for histology and NAAT[a]	Fixed and unfixed
Cryptosporidia spp.	Plus small intestinal biopsy and bile duct biopsy[a]	Fixed for histology
Viral antigens	Stool	Do not transport longer than 24 hours, shorter better
Cytomegaloviruses	Colonic biopsy	For PCR in water or alcohol

NAAT = Nucleic acid amplification test
[a] in addition to stool samples

Table 2:
Test materials and transport conditions (from Kist[1])

2 Differential diagnosis: IBD vs. food intolerance

Non-toxic and non-immune-mediated food intolerances that have functional and structural causes (fig. 2) are much more prevalent (15–20%) than immune-mediated allergies (2–5%) or toxic pathologies. For this reason, non-immune-mediated food intolerance such as carbohydrate malabsorption, neurodermatitis, or exocrine pancreatic insufficiency should first be ruled out as a differential diagnosis whenever the trigger of the symptoms is not known. This approach is also appropriate in light of the frequent association of carbohydrate malabsorption, histamine intolerance, or infections with atopic disorders or food allergies.

Stepwise diagnosis of a food intolerance begins with the patient's dietary history, a food diary, skin tests (e.g. prick test) for food extracts, environmental antigens, molds, spices, etc., and measurement of total immunoglobulin (Ig)E and the suspected allergen-specific IgE antibodies in serum, in order to find indications of an IgE-specific hypersensitivity.

If the normal differential diagnoses have been ruled out in this situation and routine diagnostic parameters (history, skin test, antigen-specific IgE) do not provide any specific indication, a local (seronegative) allergy or a non-IgE-mediated allergy must be kept in mind. The diagnostic repertoire is much smaller for type II–IV hypersensitivity reactions which are not IgE-mediated than for type I hypersensitivity reactions, which can be detected systemically. Potential markers of a delayed type II–IV food intolerance include a prick test read out after 24–48 hours, measurement of C3 and C4 complement factors (consumption), measurement of immune complexes (IgG, IgA, IgM, and IgE), and cytokine analysis[2].

Although the German Society for Allergology and Clinical Immunology calls for structured oral challenge tests, these are not performed in clinical practice due to their complexity. For this reason, *urine levels of methylhistamine* should be measured (normal < 6.5 µg methylhistamine/mmol creatinine x m^2 body surface area) as a screening measure. If the condition cannot be identified by routine IgE and/or non-IgE-based tests in patients with elevated methylhistamine excretion, internal

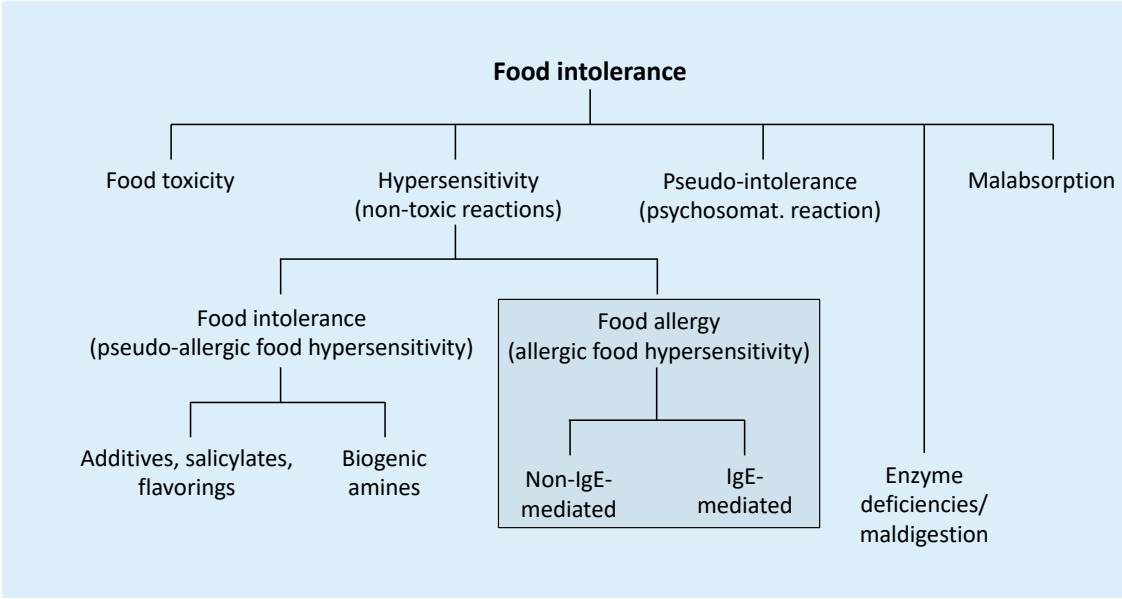


Fig. 2:
Differential diagnosis of food intolerance[3]

differential diagnoses should be examined, and targeted allergy diagnosis should be performed by endoscopy.

Endoscopy-guided segmental lavage is the most effective method for detecting intestinal IgE antibodies and other mediators of allergies (e.g. eosinophil cationic protein, ECP). This method is carried out with 50–100 ml of saline and can generally be performed across the entire GI tract, but is particularly effective in the terminal ileum, cecum, and rectosigmoid junction (detailed description in Raithel[2]).

3 Differential diagnosis: IBD vs. celiac disease

Celiac disease is a permanent gluten-sensitive enteropathy. Gluten is an adhesive protein in wheat and related grains (spelt, barley, rye, etc.). Advanced stages of the disease are characterized by villous atrophy and crypt hypertrophy in the small intestinal mucosa, both of which are, however, not sufficiently specific for a diagnosis. The disease is most often clinically silent (reviewed in German by Stein[4]). There have been many reports of an association between celiac disease and IBD, especially ulcerative colitis. Celiac patients are at a 10-fold greater risk of developing IBD. Hence, celiac disease is an important differential diagnosis during the initial diagnosis of IBD, particularly in patients with elevated transaminases of unclear origin, but also with abdominal symptoms of unknown cause[5-7].

The value of antibody testing in the diagnosis of celiac disease has increased in recent years with the introduction of anti-endomysial antibodies (table 3). Testing for specific anti-endomysial IgA antibodies is of great importance for diagnosing celiac disease in adults. However, anti-tissue transglutaminase antibodies (anti-tTG) levels are of equal diagnostic value, as their sensitivity and specificity has been reported to be 95–99% in adults. In both cases, it must be kept in mind that approximately 2–5% of all celiac patients have an IgA deficiency, which leads to negative results in IgA tests. Given that individuals with IgA deficiency have a 10-fold higher risk of developing celiac disease than the general population, the possibility of IgA deficiency must always be excluded. Antibody levels can also be determined for IgG

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
IgA AGA	85 (57–100)	90 (47–94)	18	99
IgG AGA	85 (42–100)	80 (50–94)	31	99
EMA	95 (86–100)	99 (97–100)	83	99
IgA anti-tTG[a]	98 (78–100)	98 (90–100)	72	99
IgG anti-tTG[b]	70 (45–95)	95 (94–100)	42	99
IgA anti-DGP	88 (74–100)	95 (90–99)	44	99
IgG anti-DGP	80 (63–95)	98 (90–99)	68	99
IgA/IgG anti-DGP	97 (75–99)	95 (87–100)	51	99

AGA = Anti-gliadin antibodies; DGP = Deamidated gliadin peptide; EMA = Anti-endomysial antibodies; tTG = Tissue transglutaminase
[a] Only anti-human tTG-based assays; older tests based on guinea pig antibodies have a much lower sensitivity and specificity.
[b] The sensitivity is significantly higher among the IgA-deficient population (approx. 90–95%) but lower in the total population of celiac patients.

Table 3:
Antibodies for the differential diagnosis of celiac disease (adapted from Stein[4], Leffler and Schuppan[8])

class antibodies for the two antigens described above, even though the sensitivity is lower in both cases. The same antibodies can also be measured to diagnose dermatitis herpetiformis.

Anti-gliadin antibodies (AGA) targeting all types of gliadin (α, β, γ, ω) can also be detected in the serum of celiac patients. However, determination of these antibodies in adults is now obsolete due to their limited diagnostic value[4].

In contrast, recently developed assays based on deamidated gliadin peptide (DGP) exhibit characteristics equivalent to those of the tTG-IgA assay.

4 Markers for differential diagnosis of ulcerative colitis and Crohn's disease

Even after all clinical, endoscopy, and histological findings have been evaluated, differentiation between Crohn's disease and ulcerative colitis nonetheless proves difficult in about 10% of patients (→ indeterminate colitis), particularly in the initial stages of the diseases. In many cases, the correct diagnosis can be made with the help of (auto-) antibodies.

Antibodies targeting goblet cells (anti-intestinal goblet cell autoantibodies, GAB) were identified in ulcerative colitis patients as early as 1959[9]. A protein of about 40 kDa in size was identified in inflamed colonic mucosa as a target antigen. This antigen was later determined to be human tropomyosin (isoform 5) and has since also been detected in the skin, joints, and bile duct epithelia[10]. The diagnostic sensitivity and specificity of GAB depend greatly on the method of detection used. Specificities of up to 100% have been reported with optimal test conditions, although the sensitivity of these tests is only about 15–46% (table 4).

Anti-neutrophil cytoplasmic antibodies (ANCA)

Anti-neutrophil cytoplasmic antibodies (ANCA) are found in many autoimmune disorders. pANCA, which targets myeloperoxidase, is often found in patients with idiopathic glomerulonephritis. These antibodies must be differentiated from atypical pANCA (fig. 3), which react with lysosomal enzymes in the cytoplasm of neutrophils and monocytes and were first described in ulcerative colitis patients by Saxon et al.[11] in 1990. The molecular

			Prevalences				
Serological markers	**Epitopes**	**Iso-types**	**CD**	**UC**	**Healthy**	**Sensi-tivity***	**Speci-ficity***
ASCA	Carbohydrate epitope (Man-α-1,3 Man-α-1,2) found in phospho-peptidomannans in the cell wall of *Saccharomyces cerevisiae*, also expressed by *Candida albicans*	IgG/IgA	50–70%	5–15%	0–5%	50–70%	80–85%
pANCA	As-yet unidentified lysosomal en-zymes in neutrophils and monocytes		6–20%	50–70%	0–2,5%	65–70%	80–85%
Anti-OmpC	*Escherichia coli* outer membrane porin C	IgG/IgA	20–55%	10%	5%	20–55%	88.5%
Anti-I2	*Pseudomonas fluorescens* associated bacterial sequence	IgA	54%	10%	4%	42%	76%
Anti-CBir	Flagellin CBir (*Clostridium subphylum*)	IgG	50%	15%	8%		
ALCA	Carbohydrate laminaribioside (Glc-β-1,3 Glc)	IgG	17.7–27%	4–7%	2%	18%	93%
ACCA	Carbohydrate chitobioside (GlcNAc-α-1,4 GlcNAc)	IgA	20.7–25%	5–15%	12–15%	21%	85%
AMCA	Anti-mannobioside (Man-α-1,3 Man)	IgG	28%	18%	8%	28%	82%
GAB	Intestinal goblet cells (tropomyosin isoform 5)	IgG	ND	ND	ND	46%	98%
PAB	Exocrine pancreas	IgG	27–41%	0–8%	0–5%	30%	100%

* at differentiating between Crohn's disease (CD) versus ulcerative colitis (UC); ND = Not determined

Table 4:
Antibodies used for differential diagnosis between Crohn's disease and ulcerative colitis

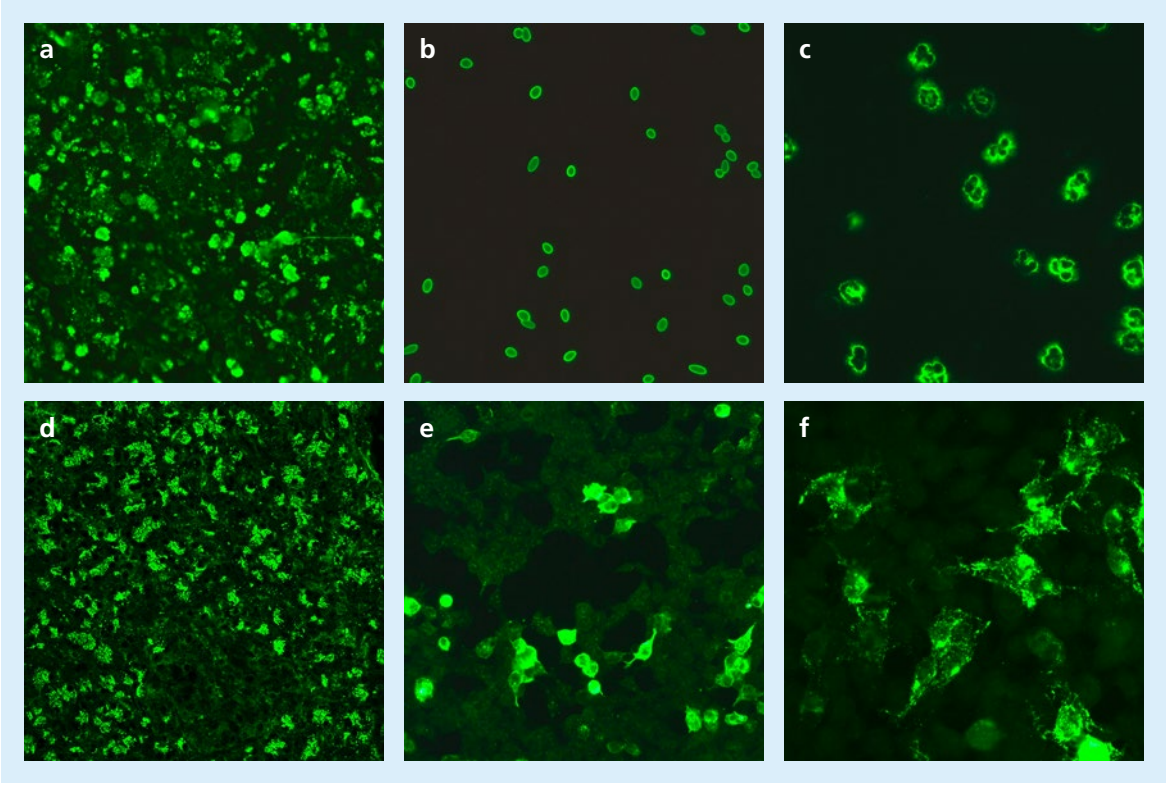


Fig. 3:
Immunofluorescence images of (a) antibodies against intestinal goblet cells (GAB), (b) anti-*Saccharomyces cerevisiae* antibodies (ASCA), (c) perinuclear anti-neutrophil cytoplasmic antibodies (pANCA), (d) antibodies against the exocrine pancreas (PAB), (e) antibodies against glycoprotein 2 (GP2), and (f) antibodies against CUB and zona pellucida-like domain-containing protein 1 (CUZD1). Used with kind permission of Prof. Dr. Stöcker and Dr. Teegen, Klinisch-immunlogisches Labor, Lübeck, Germany.

target antigen for these antibodies has yet to be definitively identified[12]. In addition to cathepsin G and elastase, DNA-bound lactoferrin has also been proposed as a marker[13].

pANCA can be detected in 67–88% of ulcerative colitis patients but in less than 7% of Crohn's patients and up to 50% of patients with primary sclerosing cholangitis (PSC)[14]. The sensitivity and specificity of pANCA depend greatly on the detection method and the quality of the neutrophils used. For example, the specificity for ulcerative colitis can be increased from 72% to 92% if the neutrophils are incubated with DNAse before detection by IFA (indirect immunofluorescence assay). The typical perinuclear staining pattern is absent in positive cases[15].

Cytoplasmic ANCA (cANCA) specific for proteinase 3 (PR3) is an autoantibody marker for granulomatosis with polyangiitis (GPA, previously known as Wegener's granulomatosis). PR3 is a serine proteinase localized to neutrophil granules that activates interleukin (IL)-1β. Anti-PR3 can also be detected in 15–40% of patients with ulcerative colitis but in no more than 10% of patients with Crohn's disease[16].

Anti-Saccharomyces cerevisiae antibodies (ASCA)

Anti-*Saccharomyces cerevisiae* (brewer's yeast) antibodies (ASCA) which have primarily been reported in Crohn's disease, are antibodies that target the oligomannosidic epitopes in the cell membrane of this yeast species[17]. Specific antibodies from both IgA and IgG classes can be identified by ELISA. A specificity of 92–97% (sensitivity 40–72%) has been reported variously in the literature (reviewed in Bossuyt[18]).

Since pANCA is rarely found in Crohn's disease and ASCA is rarely found in ulcerative colitis, combining these two tests can increase their specificity for sub-classifying IBD even further[18]: The combination ASCA+/pANCA- is highly specific for Crohn's (92–97%) and, vice versa, ASCA-/pANCA+ is highly specific for ulcerative colitis (88–98%). Conversely, the moderate sensitivities of 38–64% and 44–58%, respectively, are a drawback of this method[19-25]. A study in children with indeterminate colitis reported that this combination allowed 80% of Crohn's cases and 63.6% of ulcerative colitis cases to be successfully diagnosed. Neither ASCA nor pANCA antibodies were detected in 48.5% of

children, a finding that was confirmed through additional follow-up and which suggests that this pattern may represent a stand-alone clinical/serological entity[26,27].

In contrast, Ruemmele et al.[15] reported a high rate of discrimination in another pediatric study. In this study, children with indeterminate colitis were primarily pANCA-positive and ASCA-negative, which supports the notion that clinically indeterminate colitis in children is in fact a variant of ulcerative colitis.

On the other hand, a European study on a large population of adults with indeterminate colitis detected neither ASCA nor pANCA in 85% of subjects. The authors, Joossens et al.[26], speculated that this phenotype of IBD might represent a clinical/serological subgroup that was previously undefined.

There is still no consensus about whether detection of pANCA in ulcerative colitis patients is associated with more severe outcomes of left-sided colitis or treatment-refractory forms of the disease[28]. It is, however, clear that chronic pouchitis is significantly more common in patients with pANCA titers > 100 U/ml[29,30], with reports of an even greater risk of pouchitis in patients who are also anti-CBir1-positive[31].

Anti-exocrine pancreas antibodies in Crohn's disease

Antibodies targeting exocrine pancreatic tissue (pancreatic antibody, PAB) were first reported in IBD patients in 1984. They were detected in 29–39% of Crohn's patients but only in 0–4% of ulcerative colitis patients[32-34]. The major antigens recognized by PAB were identified as glycoprotein 2 (GP2) as well as CUB and zona pellucida-like domain-containing protein 1 (CUZD1)[35].

Antibodies against GP2 are promising serum markers for distinguishing between ulcerative colitis and Crohn's disease. GP2 is a membrane receptor that can be detected in several types of cells including the M cells of intestinal Peyer's patches. It interacts with FimH-positive bacteria and mediates bacteria-specific immune reactions of the intestinal mucosa[36]. Because M cells are more numerous in the small intestine than in the colon, the rate of anti-GP2-positive patients is much higher in Crohn's than in ulcerative colitis[37]. Crohn's colitis patients also have significantly lower titers of anti-GP2 than

patients whose disease is restricted to the ileum[38,39]. Moreover, high levels of anti-GP2 were associated with early onset of disease, L3 localization, and longer disease duration[40,41]. In contrast to GP2, the biological function of CUZD1 remains mostly unknown. It has been postulated to be involved in regulating the homeostasis between immune response and immune tolerance[42]. Anti-CUZD1 is particularly common among Crohn's patients with ileocecal disease, perianal lesions, and stricturing disease[43].

Anti-microbial antibodies

In recent years, numerous other antibodies such as the anti-outer membrane porin C (OmpC) of *Escherichia coli*, anti-*Pseudomonas*-associated sequence I2 (anti-I2) and the antibacterial flagellin CBir1 (anti-CBir1), as well as antibodies targeting specific carbohydrate chains of bacterial membranes (anti-glycan antibodies), have been detected in IBD patients, especially those with Crohn's disease. These include anti-mannobioside carbohydrate antibodies (AMCA), anti-laminaribioside carbohydrate antibodies (ALCA), anti-chitobioside carbohydrate antibodies (ACCA), anti-laminarin carbohydrate antibodies (anti-L) and anti-chitin carbohydrate antibodies (anti-C)[44-53]. The identification of these and other antibodies supports the hypothesis that IBD leads to a dysfunction of the non-specific (local) immune system with a loss of immunological tolerance to a fraction of the commensal microbiota. Remarkably, up to 44% of ASCA-negative Crohn's patients have detectable anti-glycan antibodies[52].

Antibody titers do not correlate with either disease activity or response to treatment. Even though the serum concentrations of some antibodies increase slightly over years of prolonged disease, they are generally observed to be stable across the duration of the disorder[48,54]. However, a significant increase in complications or requirement for surgery is associated with titer levels and the number or levels of cumulative serum titers[46,48-51,53].

III Markers of disease activity

Suitable methods for non-invasive evaluation of inflammation in IBD patients include both well-known imaging modalities (ultrasound, MR/CT enterography) and the measurement of serum levels of acute phase proteins and stool levels of neutrophil markers.

1 Acute phase proteins

Acute phase proteins (APPs) are defined as proteins whose serum concentration increases (positive APPs) or decreases (negative APPs) by more than 25% during inflammatory disease. Their synthesis occurs primarily in the hepatocytes, but also extrahepatically to differing degrees in different species. They may engage up to 20% of the protein synthetic capacity of the liver during an intense inflammatory reaction (sepsis)[55]. The synthesis and secretion of APPs are not regulated by the direct effects of toxic agents on hepatocytes but instead indirectly mediated by cytokines, which in turn are produced by activated immune cells at the site of injury (fig. 4). Depending on the trigger, APPs are sub-classified as type 1 APPs (primarily IL-1β and TNFα) and type 2 APPs (primarily IL-6). They are also classified by the magnitude and response time for upregulation into major (10- to 1,000-fold), moderate (2- to 10-fold) and minor (< 2-fold) (table 5).

Each APP has a different and unique function, including inhibiting proteinases, regulating the coagulation and fibrinolysis systems, modulating immune functions, binding radical oxygen species, and more (table 5).

C-reactive protein (CRP)

C-reactive protein (CRP) is a classic example of an APP. It is a 105 kDa annular pentameric protein comprised of five identical subunits. The term "C-reactive" refers to its ability to bind the C-polysaccharide of *Streptococcus pneumoniae* in a calcium-dependent manner, as was first described in 1930 by Tillet and Francis Jr.[56].

One of its key functions is related to the high affinity between CRP and the phosphocholine-rich membrane structures of infectious pathogens, which leads to aggregation with consecutive opsonization of the foreign agents by activation of the complement system[57,58].

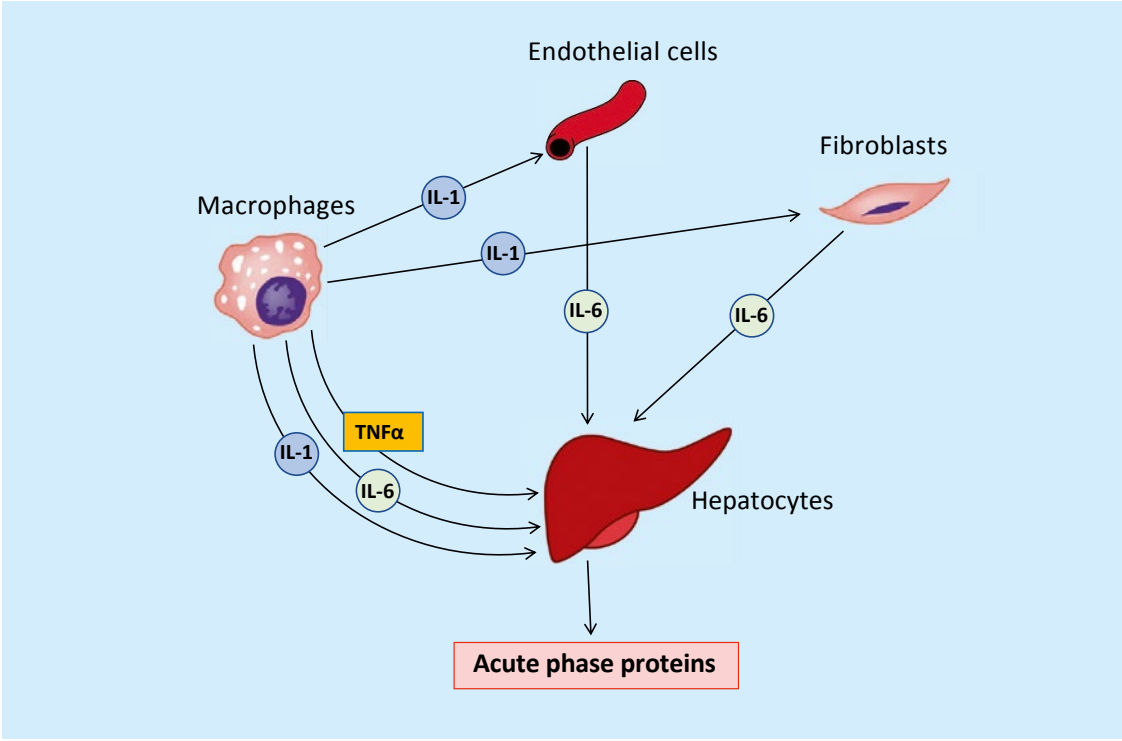


Fig. 4:
Acute phase reaction in humans

Acute phase proteins	Normal concentrations (mg/l)	Response time (h)	Increase (fold normal)	Function during inflammation			
				Inhibiting proteases	Clotting/ fibrinolysis	Opsonization	Immune modulation
CRP	< 5	6–10	10- to 1,000-fold			x	x
Procalcitonin	< 0.0005	2–4				x	
Serum amyloid A	< 0.01	6–10					
α_1-Antichymotrypsin	0.3–0.6	10		x			
α_1-Acid glycoprotein	500–1,400	24–48	2- to 10-fold				x
α_1-Antitrypsin	1,900–3,500	24–48		x			x
Fibrinogen	2,000–4,500	24–48			x		
Ferritin	20–200						x
Haptoglobin	700–3,800	24–48					
Hepcidin							
Ceruloplasmin	150–600	48–72	1- to 2-fold				
C1 inhibitor	0.15–0.35			x			
α_2-Antiplasmin	0.04–0.08				x		
Complement C3	500–1,200	48–72					
Complement C4	200–500	48–72				x	
Albumin	35–53*		Decrease				
Pre-albumin	250		Decrease				
Transferrin	2.9*		Decrease				
α_1-Lipoproteins			Decrease				

* adults up to age 65, g/l

Table 5:
Acute phase proteins in humans

CRP is measured in serum using nephelometric or turbidimetric immunoassays following addition of highly specific antibodies. The limit of quantitation is 3–5 mg/l. Latex-enhanced assays have a limit of quantitation of 0.1–0.3 mg/l (called highly sensitive [hs]-CRP).

The diagnostic value of CRP testing in IBD patients lies in its sensitivity and its comparatively short response time (6–10 h) and half-life (18 h), which permits therapeutic monitoring. A satisfactory correlation between CRP levels and (mucosal) inflammation exists only in Crohn's disease, not in ulcerative colitis. This is assumed to be due both to the fact that the inflammation in ulcerative colitis is restricted to the mucosa and the fact that rectal inflammation is drained via the vena cava, leading to lower systemic concentrations of IL-6. The potential importance of polymorphisms in the CRP gene is still the subject of debate[59,60].

Because the hepatic synthesis and clearance of CRP varies greatly from person to person, its levels are not reliably predictive of disease activity or spread. The low specificity of the test is another drawback, since it is not possible to distinguish between Crohn's disease and ulcerative colitis or infectious colitis. There is also no added diagnostic value to the determination of hs-CRP at values of < 5 mg/l.

Procalcitonin (PCT)

Procalcitonin (PCT) is a 13 kDa precursor of the hormone calcitonin that is normally only produced by the C cells of the thyroid. Unlike calcitonin, it is not released into the bloodstream. However, during bacterial, fungal, and protozoan infections, the calcitonin gene is expressed and PCT is produced by organs other than the thyroid (liver, kidneys, fatty tissue, and muscles) and released into the blood. Endotoxin-producing, gram-negative bacteria are thought to be the most potent stimuli of PCT release, with interleukins such as tumor necrosis factor (TNF)α and IL-6 being much weaker stimuli. PCT levels also increase during infections by gram-positive bacteria and by fungi – but not by viruses – as well as in autoimmune and allergic disorders.

In contrast to CRP, PCT can be detected in serum as early as after 2–4 hours, and reaches a plateau after 6–8 (12) hours. Its half-life is approx. 24 hours but may be prolonged by 30–40% in patients with end stage kidney disease.

PCT is measured quantitatively in plasma (EDTA, citrate, heparin plasma) or serum using a chemiluminescent immunoassay or semi-quantitatively using a solid-phase immunoassay[61].

The normal level for children and adults is < 0.5 µg/l. Systemic inflammation of bacterial origin can lead to levels above 1,000 µg/l depending on the severity of inflammation, with a strong correlation between PCT levels, inflammation severity, and mortality[62].

The diagnostic value of PCT measurement lies in its high specificity for inflammation caused by bacterial infection[63]. It is currently considered to be the only marker of sepsis suitable for modern clinical requirements in critical care owing to its high specificity and sensitivity for early and differentiated diagnosis[64]. Only two small studies are presently available on the benefit of PCT in the differential diagnosis between inflammatory bowel disease and bacterial enterocolitis. Both of these studies confirm the high specificity of PCT in the differential diagnosis between infectious gastroenteritis and IBD but also demonstrate that CRP is vastly superior for longitudinal monitoring of inflammatory activity[65,66]. Interestingly, recent studies have reported a quite good correlation between PCT, CRP, and endoscopic/radiographic markers of IBD activity as well as response to intravenous steroid therapy for severe acute ulcerative colitis[67,68].

α_1-Acid glycoprotein (orosomucoid)

α_1-Acid glycoprotein (orosomucoid) is an approx. 42 kDa protein with a high degree of glycosylation (45%) which represents the major component of the seromucoid fraction. Due to its much longer response time (24–48 h) and even longer half-life (120–144 h)[69] than CRP, orosomucoid currently plays no role in the monitoring of inflammation in IBD patients[60] despite claims by earlier publications[70,71].

Lipopolysaccharide-binding protein

Lipopolysaccharide-binding protein (LBP) is a 58 kDa, type 1 acute phase protein that mediates endotoxin-induced activation of mononuclear cells via the CD14 receptor. It is primarily synthesized by the liver but also by intestinal and pulmonary epithelia. At 36 hours, its induction time is much longer than those of PCT and CRP. Like CRP, LBP levels are elevated during inflammation of both infectious and non-infectious origin. However, due

to the very small number of publications on this serological marker, it has not been possible to date to justify an indication for its routine use in IBD. Indeed, the first larger prospective study in IBD patients found no diagnostic benefit of LBP over hs-CRP[72].

Hepcidin

Hepcidin is a 25-amino-acid-long antimicrobial peptide first described by Nemeth et al.[73] in 2003 as a type 2 APP. Measurement of hepcidin is primarily suitable for the differential diagnosis of iron deficiency anemia in patients with inflammatory bowel disease owing to its role as the negative "master regulator" of iron absorption[74].

α_1-Antitrypsin

Although serum and stool levels of the approx. 60 kDa acute phase protein α_1-antitrypsin correlate well with endoscopic and clinical disease activity indices in IBD patients, they have been demonstrated to be inferior to fecal leukocyte markers (calprotectin, lactoferrin) at evaluating inflammation. A further drawback is the need to collect a 24-hour sample for the more sensitive measurement of α_1-antitrypsin clearance. However, it remains valuable as a laboratory marker for primary and follow-up diagnosis of enteric protein loss in patients with protein-losing enteropathy[75].

Erythrocyte sedimentation rate (ESR, Westergren method)

Although an elevated ESR correlates quite well with inflammation activity in Crohn's colitis, the correlation is inadequate for Crohn's terminal ileitis or for ulcerative colitis[76]. The main disadvantages of this marker are a delayed response time versus CRP and a half-life of several days[60].

2 Platelets

Reactive thrombocytosis with platelet counts > 600,000/µl is often detected during an acute flare of ulcerative colitis, less often in Crohn's colitis, and rarely in patients with Crohn's terminal ileitis[77]. While the European Crohn's study found no direct correlation to the Crohn's Disease Activity Index (CDAI), this study did not evaluate patients with colonic involvement separately[78].

There is no consensus on the role of elevated thrombopoietin levels[79,80]. Shen et al.[81] postulate that thrombocytosis is a reflection of a globally elevated (plasma and cellular) homeostasis resulting from inflammation. Studies by the Gasché group also point to a potential link with iron deficiency anemia secondary to inflammation, which might lead to thrombocytosis via stimulation of megakaryopoiesis[82]. On the other hand, it is also conceivable that this same stimulation of megakaryopoiesis is a consequence of erythropoietin (EPO) resistance secondary to inflammation, resulting in elevated EPO levels. It remains unclear whether and to what extent measurement of the mean platelet volume may represent a superior diagnostic alternative[83].

3 Leukocyte markers

Detection of indium-labeled granulocytes in stool

The excretion of ^{111}In-labeled leukocytes by scintigraphy gained major prominence in the early 1980s[84]. This method allows the topographic site of leukocyte extravasation through the intestinal wall into the lumen to be precisely determined. Quantitative measurement of fecal excretion of ^{111}In correlates well with inflammation parameters such as orosomucoid, α_1-antitrypsin, CRP, ESR, leukocyte counts, platelets, albumin, iron, α_1- and α_2-globulin, but not with CDAI.

The method requires that stool be collected over 4 days[85]. The absence of a link to CDAI may also reflect the lack of a link between the CDAI and endoscopic findings as reported by Modigliani et al.[86]. Excretion of ^{111}In appears to reflect colonic inflammation better than small intestinal inflammation[87].

Leukocyte proteins

The detection of calprotectin and lactoferrin – degradation-resistant leukocyte proteins – in stool in the early 1990s and the resulting development of much more sensitive and specific immunoassays[88,89] led to a boom in this sector of laboratory diagnostic tests over the following years. Other diagnostic candidate markers followed: myeloperoxidase, lysozyme, eosinophil cationic protein (ECP), eosinophil protein X (ECX) and human neutrophil lipocalin (table 6).

Fecal marker	Origin	Correlation with IBD	Correlation with disease activity (Crohn's disease)	Correlation with disease activity (ulcerative colitis)	Stability at RT (half-life)
^{111}In-labeled leukocytes	Leukocytes/ granulocytes	Yes	Yes	Yes	2.8 days
Calprotectin	Neutrophil cytoplasm	Yes	Yes	Yes	7 days
Lactoferrin	Neutrophil (secondary) granules	Yes	Yes	Yes	4 days
Lysozyme	Leukocytes/ macrophages	ND	ND	ND	1 day
PMN elastase	Neutrophil azurophilic granules	Yes	Yes	Yes	1 day
Neutrophil lipocalin	Neutrophil (secondary) granules	ND	NS	NS	7 days
Myeloperoxidase	Neutrophil (primary) granules	ND	NS	NS	7 days
Eosinophil cationic protein	Eosinophil granules	ND	NS	NS	3 days
Eosinophil protein X	Eosinophil granules	ND	ND	ND	7 days
α_1-Antitrypsin	Serum macrophages, intestinal epithelia	NS	Moderate	Moderate	7 days

ND = Not determined; NS = Not significant

Table 6:
Correlation of fecal inflammation markers in IBD (adapted from Sutherland et al.[75])

Calprotectin (36 kDa) and lactoferrin (76 kDa) are proteins that comprise up to 60% of the total protein content of neutrophils. These proteins have chemotactic and antimicrobial properties and are primarily expressed by granulocytes but also at lower levels by macrophages, monocytes, and intestinal epithelial cells. The diagnostic value of these markers in primary and follow-up diagnosis was first described in studies by Tibble et al.[90,91] and has since been confirmed by numerous other studies and is now considered proven[75,92]. Calprotectin appears to be a better validated diagnostic marker than lactoferrin, at least in adults, with the result that lactoferrin testing no longer plays a notable role in routine clinical practice (table 7).

One drawback of this marker is its low specificity, as any disorder associated with inflammation-related (infectious or non-infectious) mucosal pathology leads to elevated levels of fecal calprotectin[98,99]. On the other hand, the negative predictive value of these markers, particularly calprotectin, for excluding a somatic disorder of the lower gastrointestinal tract is unmatched at > 95% and has proven very helpful in distinguishing IBD from irritable bowel syndrome (IBS) and individuals without bowel disorders[100,101].

One of the greatest challenges in diagnosing IBD is the non-invasive assessment of inflammatory activity. The studies published on this topic in recent years demonstrate that fecal calprotectin is superior to other non-invasive biomarkers for predicting intestinal activity, particularly colonic involvement. A meta-analysis published in 2018 calculated a pooled sensitivity of 85% and specificity of 75%. A subgroup analysis in this study also showed slightly better diagnostic accuracy for ulcerative colitis versus Crohn's disease. The optimal cut-offs were reported to be concentrations of < 50 µg/g for the best sensitivity (91%) and > 100 µg/g for the best specificity (78%)[102].

In summary, the published data demonstrate that measurement of fecal calprotectin is currently the gold standard for non-invasive diagnosis of inflammatory bowel disease[103] (fig. 5). The recent development of (semi-quantitative) rapid tests with easy handling also allow self-monitoring or monitoring in outpatient settings by patients themselves or other medical staff without the need for expensive equipment[97,104-106].

		Sensitivity (%)		Specificity (%)		Accuracy (%)		PPV (%)	
	Patients (n)	Lact	Cal	Lact	Cal	Lact	Cal	Lact	Cal
D'Incà et al. (2007)[93]	144	80	78	85	83	87	86	81	80
Schröder et al. (2007)[94]	88	82	93	100	100	NE	NE	100	100
Langhorst et al. (2008)[95]	139	86.7	81.7	77.2	83.5	80.7	82.1	NE	NE
Sipponen et al. (2008)[96]	77 (CD)	71	81	83	79	NE	NE	89	94
Otten et al. (2008)[97]	114	78.3	95.7	90.1	88.8	NE	NE	66.7	65.7
Total	**562**	78.8 (71–87)	**85.9 (79–93)**	83.5 (67–100)	**85.1 (74–100)**	77.4 (77–87)	**80 (82–86)**	86.4 (66.7–100)	**87.9 (65.7–100)**

Lact = Lactoferrin; Cal = Calprotectin; NE = Not evaluated; PPV = Positive predictive value

Table 7:
Sensitivity and specificity of lactoferrin and calprotectin in stool in IBD from direct comparative studies

In contrast, PMN elastase, lysozyme, neutrophils, lipocalin, and myeloperoxidase are of secondary diagnostic importance due to their lack of stability and/or much lower degree of correlation with inflammation. This also applies to ECP and ECX, although these are certainly more important as screening markers for food allergies (table 6).

New kids on the block

Osteoprotegerin

Osteoprotegerin (OPG) is a soluble receptor secreted by osteoblasts that can bind RANKL (receptor activator of NF-κB ligand), a protein which is also secreted by osteoblasts. This interaction inhibits the differentiation of monocytic osteoclast precursor cells to mature osteoclasts that is induced by RANKL. Hence, synthesis of OPG allows osteoblasts to counteract differentiation to osteoclasts and the associated bone turnover (reviewed in German by Jakob et al.[108]).

In addition to this central role of OPG in bone metabolism, there is also mounting evidence that it plays a role in IBD, during which OPG is expressed primarily by colonic dendritic and epithelial cells in response to inflammatory stimuli[109,110]. Its function remains mostly unknown at present. OPG may promote perpetuation of inflammation by binding to TRAIL (TNF receptor apoptosis-induced ligand), which is associated with a decrease in apoptosis by dendritic cells and T cells[110,111].

According to preliminary findings in pediatric cohorts, fecal excretion of OPG is only significantly elevated in ulcerative colitis and Crohn's colitis but not in Crohn's disease with small intestinal involvement, in contrast to the neutrophil markers described above[112-114].

S100A12 (calgranulin C)

Like calprotectin (S100A8/A9), S100A12 belongs to the family of S100 proteins whose name is derived from their 100% solubility in ammonium sulfate[107,115]. In contrast to all of the inflammation markers described above, S100A12 (also known as calgranulin C) is reportedly only released by activated (intestinal) neutrophils. Like calprotectin, it has chemotactic and antimicrobial properties[116]. The first study comparing S100A12 versus calprotectin observed a better ability of the former to

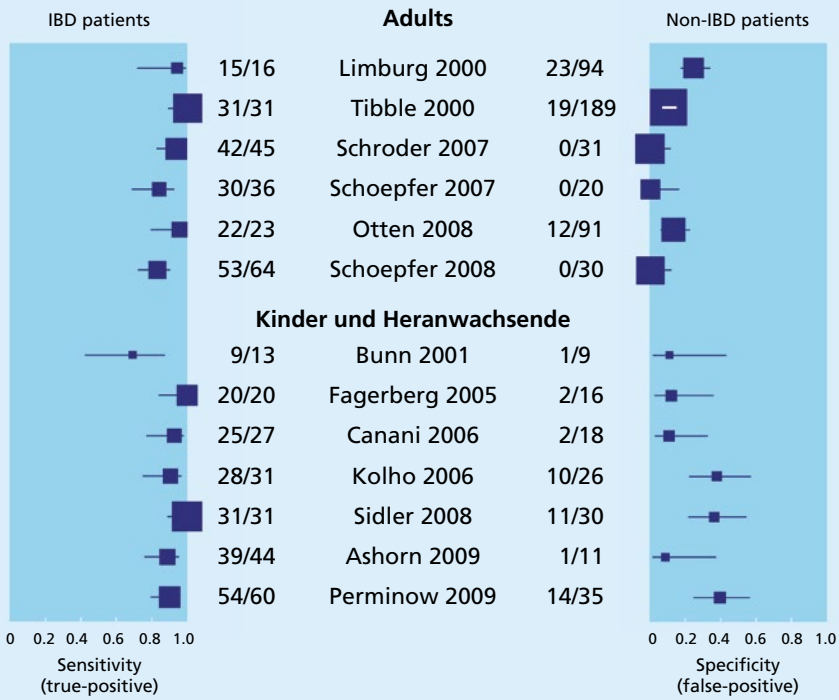


Fig. 5:
Meta-analysis of the diagnostic value of calprotectin in IBD (source: 103 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2904879/); licensed under CC BY-NC 2.0 (https://creativecommons.org/licenses/by-nc/2.0/); Falk Foundation e.V. has no rights to this figure.

discriminate between IBD and IBS and/or viral and bacterial intestinal infections than the latter. However, this finding could not be confirmed to a similar extent in subsequent studies[117-120]. It remains unknown whether S100A12 will provide a crucial improvement over calprotectin in the non-invasive monitoring of IBD disease activity.

Pyruvate kinase M2 (PKM2)

A reduced ability to fulfill cellular energy needs by glycolysis is considered to be a specific property of tumor cells that has been attributed to increasing expression of a form of dimeric pyruvate kinase (PKM2) during the process of malignant transformation. Hence, detection of PKM2 was initially thought to represent a novel, highly specific biomarker for nascent malignancies in the intestinal tract. However, preliminary studies by our group[121] and other groups[122-124] have suggested that this test may be positive in up to 100% of IBD patients depending on inflammation activity. Nonetheless, the clinical value of this marker versus calprotectin has not been conclusively determined (table 8).

Study	Cut-off	Controls	Patients	Pouchitis	Crohn's disease	Ulcerative colitis	Sensitivity (CD/UC)	Specificity (CD/UC)
Walkowiak et al. 2005[124]		70	27	25	–	–		
Czub et al. 2007[122]	4 U/g	45	107	–	32	75	94.4	
Chung-Faye et al. 2007[125]	3.7 U/g	43 (IBS)	81	–	50	31	73	74
Shastri et al. 2008[121]	4 U/g		276	–	182	92	87.7/83.3	62.9/71.9
Jeffery et al. 2009[126]	4 U/g	91	10	–	9	1	67	88
Johnson et al. 2009[123]	4 U/g	94	46	–		Pouch	80	71
Turner et al. 2010[119]			101	101				

Table 8:
Sensitivity and specificity of pyruvate kinase M2 (PKM2) in the stool of IBD patients

IV Markers for predicting complications and recurrence

Multiple prospective case-control studies as well as independent cross-sectional studies have repeatedly demonstrated not only the high potential of antibodies in differential diagnostics (see above), but also their value as tools to predict complications (fistulas, strictures, resections, etc.)[44,46,48-51,53,127,128].

For example, an association has been observed between anti-I2 and fibrosing Crohn's disease, between anti-OmpC and penetrating Crohn's disease, and between anti-CBir1 and both phenotypes[45,129].

Moreover, numerous studies have reported a close link between ASCA titers and complications of Crohn's terminal ileitis[44-46,51,127].

According to a meta-analysis published in 2014, the best evidence by far points to an association between anti-OmpC positivity and complications and/or surgery[130].

Antibodies detected after surgery have also been postulated to have a potential diagnostic value. For example, preliminary data suggest that there may be a correlation between pre-operative ASCA status and the risk of early recurrence after surgery[131,132].

Determination of fecal neutrophil markers has repeatedly been shown to be superior to CRP at predicting recurrence. A meta-analysis reported a pooled sensitivity of 78% and specificity of 73% for calprotectin[133].

The best predictive values for ulcerative colitis and Crohn's colitis are consistently found over a period of 3 months (table 9). Calprotectin also appears to be a better biomarker than lactoferrin in this situation. Calprotectin concentrations > 150–250 µg/g are usually proposed as a risk factor for a disease flare[134-137].

Several promising studies have recently been published on the predictive value of fecal calprotectin during treatment monitoring. For example, one study reported that a calprotectin cut-off concentration of 139 µg/g could predict the risk of clinically active disease one year after induction therapy with TNFα antagonists with a sensitivity of 72% and a specificity of 80%[138].

More than 80% of patients with Crohn's disease require surgery within 10 years of their diagnosis. Many patients also develop endoscopic recurrence within 1 year after surgery, before clinical symptoms become apparent. Multiple studies have repeatedly observed a link between elevated levels of calprotectin after ileocecal resection and a risk of post-

Author	**Marker**	**Patients** CD/UC (n)	**Cut-off** (µg/ml)	**Sensitivity** CD/UC (%)	**Specificity** CD/UC (%)	**PPV*** CD/UC (%)	**NPV*** CD/UC (%)
Costa et al. 2005[141]	Cal	38/41	150	87/89	43/82	50/81	83/90
D'Inca et al. 2008[142]	Cal	65/97	130	65/70	62/70	44/60	80/79
Walkiewicz et al. 2008[143]	Cal	76/21 (children)	400	65/70	62/70	44/60	80/79
Gisbert et al. 2009[144]	Cal Lact	89/74	150 qualitative	28/31 77/46	93/91 68/61	30** 25	92** 90
Ho et al. 2009[145]	Cal	0/90	431/ 1,923***	96 24	21 97		
Garcia-Sanchez et al. 2010[146]	Cal	66/69	CD: 200 UC: 120	80 81	65 63	46 49	88 88
Kallel et al. 2010[147]	Cal	53/0 (colon)	340	80	91		

* PPV = Positive predictive value; NPV = Negative predictive value
** Total population
*** Colectomy rate

Table 9:
Sensitivity, specificity, and positive and negative predictive values of calprotectin (Cal) and lactoferrin (Lact) for predicting recurrence of IBD

operative disease recurrence. In the POCER study, calprotectin concentrations of > 100 µg/g were indicative of endoscopic recurrence with a sensitivity of 89% and a negative predictive value of 91%[139].

Accordingly, international recommendations propose a calprotectin test 3 months after surgery and immediate clarification by endoscopy in the event of elevated levels[140].

V Extraintestinal manifestations and complications

Up to 50% of IBD patients experience extraintestinal manifestations (EIM), which are thus a major contributor to the morbidity and mortality of the disease. While some EIM occur primarily during flares (e.g. erythema nodosum), others exhibit no relationship to the underlying disease (e.g. type 2 arthritis). While there is currently no clear consensus of the definition of EIM for IBD, they should still be distinguished from complications that are not specific to the disease, adverse effects of IBD drugs, and autoimmune disorders associated with IBD.

1 Anemia

Anemia is the most common systemic complication of IBD, with a prevalence ranging in studies from 6 to 74%[148,149]. Anemia is most often asymptomatic or presents with non-specific symptoms, such as fatigue and reduced stamina. In contrast, typical symptoms such as dizziness, tachycardia, or dyspnea are rare and occur only in the context of severe cases. IBD patients with anemia have a reduced quality of life and ability to work as well as higher rates of hospitalization and health care costs compared with IBD patients without anemia.

1.1 Causes of anemia in IBD patients

Anemia is defined in IBD patients according to the guidelines of the World Health Organization (WHO) (table 10) and is caused by multiple factors (table 11). The most common cause by far is thought to be iron deficiency due to blood loss caused by ulceration of the intestinal mucosa or decreased iron intake, in addition to anemia of chronic disease (ACD), first described by Cartwright in 1996. ACD is characterized by normal or reduced mean corpuscular volume (MCV), reduced serum iron levels, reduced total iron binding capacity (TIBC), normal to elevated levels of serum ferritin, and an elevated ratio of iron stores in the reticuloendothelial system (RES) to total iron in the body[149].

Age and/or sex	Hemoglobin		Hematocrit (%)
	(g/dl)	(mmol/l)	
Children 6 months to 5 years	11.0	6.83	33
Children 5–11 years	11.5	7.14	34
Children 12–13 years	12.0	7.45	36
Women > 18 years	12.0	7.45	36
Pregnant women	11.0	6.83	33
Men > 18 years	13.0	8.07	39

Table 10:
Cut-offs used to define anemia (WHO 1988)

Common	Uncommon	Rare
Iron deficiency Anemia of chronic disease (ACD)	Vitamin B_{12}/folic acid deficiency (drug-induced → sulfasalazine, thiopurines)	Hemolysis Myelodysplastic syndrome Chronic kidney disease Aplasia (usually drug-induced) Congenital hemoglobinopathies or erythropoietic disorders

Table 11:
Etiology of anemia in IBD

1.2 Diagnosis of anemia in IBD patients

1.2.1 Diagnosis of vitamin B_{12} deficiency

Vitamin B_{12} levels in serum exhibit only limited specificity and sensitivity for the diagnosis of vitamin B_{12} deficiency. For example, many patients whose serum levels are within the specified reference range already show symptoms of a vitamin B_{12} deficiency. Like iron (and most vitamin) deficiencies, a vitamin B_{12} deficiency develops in stages with different clinical and subclinical symptoms:

- **Stage 1:** negative vitamin B_{12} balance with increasing depletion of vitamin B_{12} stores, usually asymptomatic. Decreased holo-transcobalamin (holoTC, reference range: 37–171 pmol/l), which is the metabolically active vitamin B_{12} fraction (stage 1a). Holo-haptocorrin also decreases over time (stage 1b).
- **Stage 2:** functional vitamin B_{12} deficiency. Cells are no longer able to compensate for vitamin B_{12}-dependent metabolism due to depleted stores. In addition to low levels of holoTC and holo-haptocorrin, levels of the metabolic parameters homocysteine and methylmalonic acid (MMA) are increased, with MMA (reference range: 73–271 nmol/l) being a more sensitive but less specific biomarker than homocysteine[150].
- **Stage 3:** This is the stage of clinical manifestation of typical megaloblastic anemia with elevated MCV and low hemoglobin levels as well as hypersegmented neutrophils.

Diagnostic procedures

If a vitamin B_{12} deficiency is suspected, holoTC levels should be measured as a first step. A reduced serum concentration of < 37 pmol/l is considered to be the earliest marker of vitamin B_{12} deficiency and can be an indication of both metabolic vitamin B_{12} deficiency and depleted stores secondary to a negative vitamin B_{12} balance. No clinical or hematological symptoms are observed yet at this stage. In contrast, low holoTC levels together with elevated MMA and homocysteine are indicative of a metabolically active vitamin B_{12} deficiency which may or may not be accompanied by clinical signs[150] (table 12; fig. 6). However, the once-common vitamin B_{12} absorption test (Schilling test) is now considered obsolete.

1.2.2 Diagnosis of iron deficiency anemia

It usually makes little sense to refer to an "iron deficiency". Iron deficiency can be sub-classified into three stages based on severity (fig. 7): low iron stores, iron-restricted erythropoiesis (functional iron deficiency), and iron deficiency anemia. In stage 1, low iron stores do not result in any functional impairment. However, as it transitions to stage 2 (iron-deficient erythropoiesis), iron deficiency becomes a disorder since the body's cells can no longer be adequately supplied with iron. In stage 3, the reduced iron supply to cells is so severe that hemoglobin drops below the normal levels[151].

	Stage 1	**Stage 2**	**Stage 3**
Vitamin B_{12} level (156–762 pmol/l)	156–300 pmol/l	156–300* pmol/l	< 156 pmol/l
HoloTC level	< 37 pmol/l	< 37 pmol/l	< 37 pmol/l
Holo-haptocorrin level	Normal	Low	Low
Methylmalonic acid (MMA)**	< 271 nmol/l	> 271 nmol/l	> 271 nmol/l
Homocysteine level (HCyst)**	< 12 µmol/l	> 12 µmol/l	> 12 µmol/l
Vitamin B_{12} store	Starts to deplete	Depleted	Depleted
Symptoms	Cognitive disorders?	**Functional vitamin B_{12} deficiency** → neurological disorders → cognitive disorders	**Clinical manifestations** → macrocytic anemia → neutrophil hyper-segmentation

* Initial vitamin B_{12} levels > 300 pmol/l reliably rule out deficiency; ** MMA and HCyst are metabolic parameters

Table 12:
Symptoms of deficiency based on vitamin B_{12} status (from Herrmann and Obeid[150])

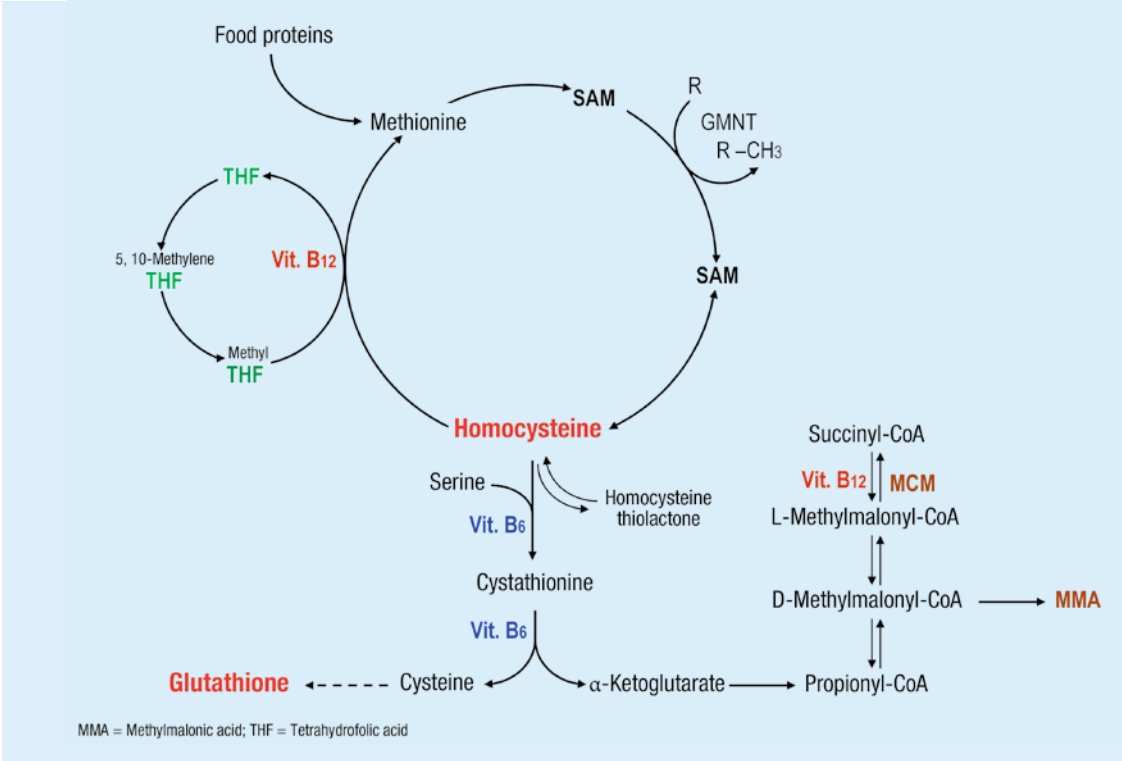


Fig. 6:
Role of vitamin B_{12} in the metabolism of homocysteine

Fig. 7:
Classification of the stages of iron deficiency and individual laboratory parameters (from Hastka et al.[151])

Theoretically, iron status can be evaluated quite easily by monitoring all compartments of iron metabolism in routine diagnosis:

- iron stores via serum ferritin,
- iron transport via transferrin saturation,
- iron utilization via erythropoiesis, e.g. the percentage of hypochromic RBCs or reticulocytes.

Serum iron levels are subject to a circadian rhythm and can also be low in patients with anemia of chronic disease (ACD). Therefore, the measurement of serum iron as a sole marker is obsolete in the diagnosis of iron deficiency.

Reticulocytes

The term reticulocyte denotes the fraction of newly produced red blood cells (RBCs) that still contain enough ribonucleic acid (RNA) to be detected by microscopy after staining with specific alkaline dyes. This parameter is provided by all major laboratories (flow cytometry) with reasonable turnaround time and expense.

In chronic anemia, the steady-state relative reticulocyte count is inversely correlated (non-linearly) with a decrease in RBC lifespan. Conversely, the absolute reticulocyte count is a measure of effective RBC production by bone marrow.

The reticulocyte production index (RPI) describes the relative reticulocyte count (Retic, in %) corrected by the degree of anemia (current hematocrit [Hct] to ideal Hct [0.45] and the reticulocyte lifespan in blood)[152]:

$$\text{RPI} = \text{Retic (\%)} \times \text{Hct}/\text{reticulocyte lifespan} \times 0.45$$

The RPI thus reflects an increase or decrease in RBCs as a factor of the normal level.

Serum ferritin

Serum ferritin is a marker of iron stores in the reticuloendothelial system. Measuring ferritin helps detect disorders in iron stores (reserve iron). Low concentrations are a sign of iron deficiency. The normal levels are 15–100 µg/l for women and 30–200 µg/l for men, with a serum ferritin concentration of 100 µg/l representing about 1,000 mg of iron stores. Serum ferritin levels < 15 µg/l are considered to be a sign of absolute iron deficiency. However, this no longer applies to patients

Parameter	Reference range
BM iron stores*	2
BM sideroblasts	15–50%
Hemoglobin	
Women:	12.3–15.3 g/dl
Men:	14.0–17.5 g/dl
MCV	80–96 fl
MCH	28–33 pg
Hypochromic RBCs	< 2.5%
Reticulocyte hemoglobin	> 26 pg
Serum iron	
Women:	6.6–26 µmol/l
Men:	11–28 µmol/l
Ferritin	
Women:	15–150 µg/l
Men:	30–400 µg/l
Transferrin	200–400 mg/dl
Transferrin saturation	16–45%
sTfR**	0.81–1.75 mg/dl
TfR index***	
Women:	0.9–3.7
Men:	0.9–3.4
ZPP	< 40 µmol/mol heme

* Scale from 0–4
** Reference levels are test-dependent, here Dade Behring, Marburg, Germany
*** Tinaquant sTfR assays from Roche Diagnostics, Mannheim, Germany

Table 13:
Reference levels of individual iron parameters (from Hastka et al.[151])

with active inflammatory bowel disease since ferritin, like transferrin, is an acute phase protein. In these patients, ferritin levels < 100 µg/l are considered to be indicative of iron deficiency[153] (table 13). Therefore, inflammation parameters should be continuously measured for differential diagnosis, as these are not dependent on iron metabolism (ESR, CRP)[149,153].

Transferrin/transferrin saturation

Measuring transferrin allows detection of iron transport disorders. Iron deficiency is usually associated with decreased transferrin saturation. Transferrin saturation, reported in %, is the quotient of iron concentration (µmol/l) divided by transferrin concentration (mg/dl) in serum or plasma multiplied by 70.9 (fasting blood draw).

Transferrin saturation is a measure of iron bound to circulating transferrin, which is the plasma protein responsible for transporting iron from its stores to bone marrow. As such, measurement of transferrin saturation does not provide any insight into the status of iron stores, and even its value as a reflection of iron utilization in bone marrow is only an indirect estimation.

Under physiological conditions, 16–45% of the transferrin molecules in plasma are saturated with iron (3–4 mol iron per mol transferrin). Values < 16% are thought to indicate a suboptimal supply of iron for erythropoiesis. Low levels of transferrin saturation (< 20%) have a relatively good sensitivity (90%) for detecting iron deficiency but a relatively low specificity at only 40–50%[154]. Because measurements of serum iron and serum transferrin may be subject to considerable circadian fluctuations, these tests should always be performed at the same time of day and should be repeated several times. Transferrin levels are elevated by oral contraceptives and decreased by inflammation (negative APP), meaning transferrin saturation may be low in patients with acute and chronic inflammation despite normal iron stores.

Soluble transferrin receptor

While every cell in the body expresses transferrin receptor (TfR), the majority (80%) of TfR is found in the bone marrow. TfR is considered to be an indicator of iron requirements. In any instance of functional iron deficiency, i.e., insufficiency in the supply of iron for erythropoiesis, the production of TfR on the membrane is upregulated. Since TfR is continuously secreted from the cell membrane and converted to soluble transferrin receptor (sTfR) in plasma, serum levels of sTfR correlate with the supply of iron available for erythropoiesis[155,156]. In addition to iron deficiency, elevated concentrations of sTfR are also observed at any expansion of erythropoiesis, for example in cases of hemolytic anemia, thalassemia, and polycythemia. The sTfR concentration is reduced in aplastic anemia and other disorders with hypoproliferative anemia, such as renal anemia.

Unlike ferritin and transferrin, chronic inflammation and liver damage have no impact on TfR[74,149].

TfR-F index

The sensitivity and specificity of sTfR as a marker of iron-restricted erythropoiesis can be enhanced by measuring sTfR and ferritin in parallel and by determining the TfR-F index[74]. The TfR-F index represents the quotient of sTfR (mg/l) divided by log serum ferritin (µg/l). Calculating this ratio yields a parameter that is dependent on iron stores, the supply of iron available for erythropoiesis, and erythropoietic activity. The TfR-F index is higher

in individuals with low iron stores. The inconsistent reference values (normal values are assay-dependent) and the relatively high cost of the tests are hindrances to the diagnostic use of the TfR-F index.

Hypochromic RBCs/ reticulocyte hemoglobin

Measurement of the cellular hemoglobin content of reticulocytes (CHr) and the percentage of hypochromic RBCs (%HYPO) has a high predictive value for the time-dependent differential diagnostics of iron deficiency anemia. Since reticulocytes take 3–5 days to mature in bone marrow and 1 day in peripheral blood, a drop in CHr represents an indication of acute iron deficiency. In contrast, a drop in %HYPO based on the 120-day lifespan of RBCs indicates a more chronic deficiency in iron supply. CHr and %HYPO are thus comparable to measuring blood glucose and HbA1c levels in diabetic patients[74,149].

Some blood testing devices are readily able to measure the hemoglobin content of each individual RBC and calculate the percentage of hypochromic RBCs and can also determine the volume and hemoglobin content (CHr) of individual reticulocytes. The percentage of hypochromic RBCs (hemoglobin content < 28 pg) is < 2.5% in individuals with no iron deficiency or stage 1 iron deficiency. Values > 10% are considered proof of iron-restricted erythropoiesis. %HYPO increases prior to microcytic changes in blood tests. CHr values < 26 pg are considered proof of iron-restricted erythropoiesis[74,149].

Zinc protoporphyrin (ZPP)

A lack of iron available for erythropoiesis results in a compensatory incorporation of zinc into the protoporphyrin complex (fig. 8) and thus to increasing formation of zinc protoporphyrin (ZPP). Owing to its greater fluorescence, this complex can be easily measured in whole blood by high performance liquid chromatography (HPLC)-coupled fluorescence detection[157]. Individuals with low iron stores have normal ZPP levels as long as there is an adequate supply of iron for erythropoiesis. The ZPP concentration increases continuously from the start of iron-restricted erythropoiesis. Values < 40 µmol/mol heme are considered normal, values 40–80 µmol/mol heme indicate latent iron deficiency (normal hemoglobin), values > 80 µmol/mol

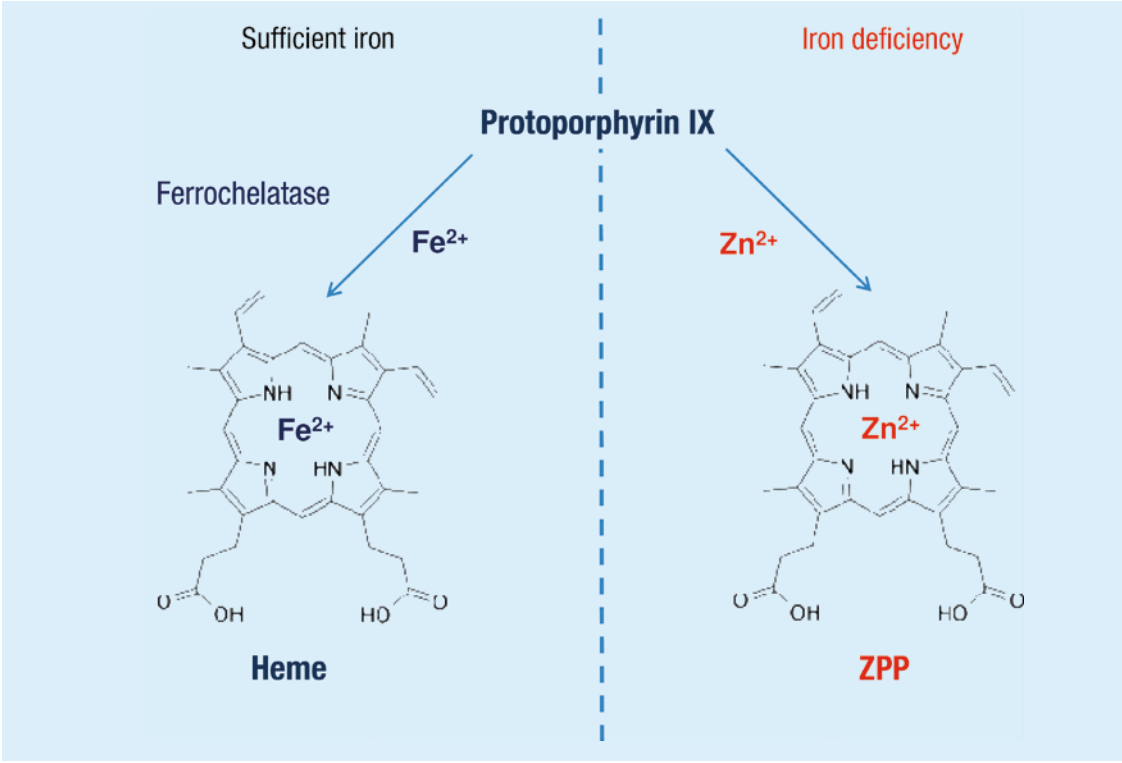


Fig. 8:
Formation of zinc protoporphyrin (ZPP)

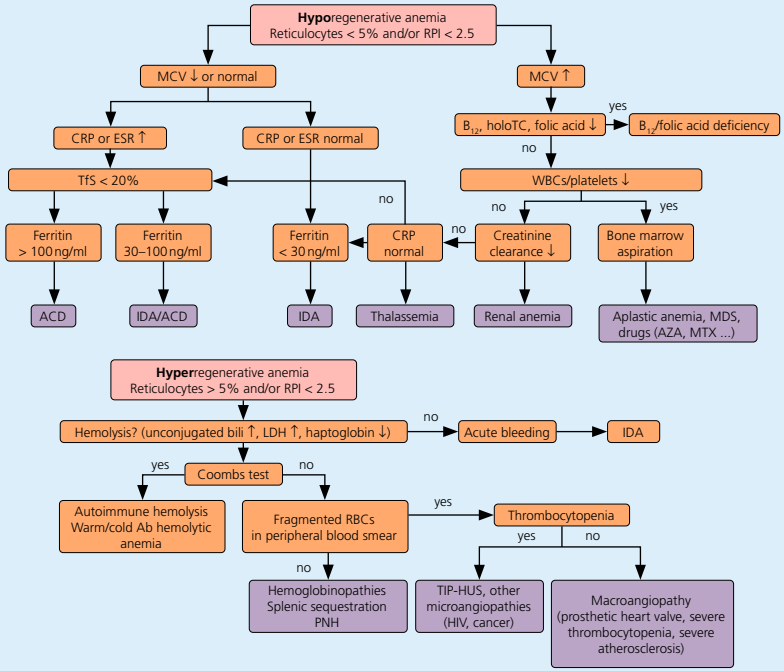
heme indicate manifest iron deficiency; in severe cases, values up to 1,000 µmol/mol heme may be observed[74,149,151]. ZPP measurement thus allows iron-restricted erythropoiesis to not only be detected but also quantified.

Conclusion

The choice of which parameter to use depends on the clinical question and the availability of laboratory tests. Serum ferritin should be used in clinical practice as a first-line parameter for iron metabolism. The correlation between serum ferritin and iron stores makes it the most sensitive test for iron metabolism, which, in contrast to other laboratory parameters, can also detect low iron stores (stage 1). Nonetheless, when interpreting ferritin values, it must always be kept in mind that ferritin may be false-normal or elevated in patients with inflammation, cancer, or liver disease and in pregnant women, which may mask existing iron deficiency. A diagnostic panel comprising ferritin, transferrin saturation, hemoglobin, and CRP allows sufficiently reliable interpretation of iron deficiency as the cause of anemia in most cases (fig. 9). If there is any doubt, sTfR, ZPP, %HYPO, and CHr should also be measured, whenever available (table 14). While in contrast to ferritin, these parameters can only detect iron deficiency after the onset of iron-restricted erythropoiesis, their predictive value is not impacted in patients with inflammation and/or cancer. In other words, ZPP detects not only genuine iron deficiency, but also disorders of iron metabolism in patients with chronic inflammation, cancer, myelodysplastic syndrome (MDS), or lead intoxication and can thus be used as an inexpensive screening parameter for iron metabolism. However, few data are currently available on ZPP in IBD patients[158].

2 Joint disorders

Musculoskeletal disorders are the most common organ system EIM of IBD; up to 40% of patients experience non-specific arthralgia[160]. These are distinct from peripheral and axial arthropathies (spondyloarthropathies), the latter of which in particular can develop concurrently with IBD but also beforehand or even many years after diagnosis[161]. Peripheral arthropathies are more common in ul-



ACD = Anemia of chronic disease; AZA = Azathioprine; CRP = C-reactive protein; holoTC = Holo-transcobalamin; IDA = Iron-deficiency anemia; MCV = Mean corpuscular volume; MDS = Myelodysplastic syndrome; MTX = Methotrexate; PNH = Paroxysmal nocturnal hemoglobinuria; RPI = Reticulocyte production index; TfS = Transferrin saturation

Fig. 9:
Algorithm for the diagnosis and treatment of iron deficiency anemia in IBD[159]

Laboratory parameter	Normal	IDA	ACD	IDA/ACD
BM iron**	2–3	0–1	2	1–2
Hemoglobin	♀ ≥ 12 (g/dl) ♂ ≥ 13 (g/dl)	↓	↓	↓
Serum iron	40–165 µg/l	↓ or normal	↓	↓
Mean corpuscular volume	80–96 fl	↓	↓ or normal	↓ or normal
Serum ferritin	16–350 µg/l	↓	↑	↑ or normal
Transferrin	↑	↑	↓ or normal	↓
Transferrin saturation	20–50%	↓	↓	↓
sTfR*	0.8–2.2 mg/l	↑	normal or ↓	↑ or normal
TfR-F index*	> 2	> 2	< 1	> 2
CHr	≥ 29 pg	↓↓	normal or ↓	↓
%Hypo	1–5%	> 5%	< 5%	
ZPP*	≤ 40 (µmol/mol heme)	> 80	> 40	> 40
C-reactive protein	< 5 mg/l	normal	↑↑	↑
Hepcidin*	≤ 4 nmol/l	↓↓	↑↑	normal or ↓

* Values fluctuate depending on assay; CHr = Cellular hemoglobin content of reticulocytes; %Hypo = Percentage of hypochromic RBCs; sTfR = Soluble transferrin receptor; sTfR-F = sTfR/log serum ferritin; ZPP = Zinc protoporphyrin

** Iron content of bone marrow (Prussian blue staining of bone marrow) based on 6-point scale: 0 = none, 1 = reduced, 2–3 = normal, 4 = greatly elevated, 5 = massively elevated

Table 14:
Laboratory values in patients with iron deficiency anemia (IDA), anemia of chronic disease (ACD), and both (typically IBD patients)

cerative colitis than in Crohn's disease (5–10% vs. 10–20%)[160]. Although comparatively rare, axial skeleton involvement can manifest as sacroiliitis or ankylosing spondylitis.

Joint involvement can be diagnosed anamnestically, by clinical examination, and in cases with axial involvement, by radiographic examination, according to rheumatological criteria. Cardinal laboratory parameters do not exist for either Crohn's disease or ulcerative colitis[162].

HLA-B27

The HLA (human leukocyte antigen) system is another term for the human major histocompatibility complex (MHC) and comprises a group of genes that play crucial roles in the etiology of countless immune disorders. The HLA system is located on the short arm of chromosome 6 and is divided into three regions (classes I–III)[163].

An association between HLA-B27 (belonging to MHC class I) and ankylosing spondylitis (AS) has been reported in up to 90% of patients with this gene[164,165]. In contrast, while HLA-B27 has been identified in 25–78% of IBD patients, only 7–15% of HLA-B27-positive patients actually suffer from isolated sacroiliitis[165,166]. Furthermore, there appears to be no association between HLA-B27 and isolated sacroiliitis in Crohn's disease[164,167]. Conversely, AS patients who are HLA-B27-negative have a much higher risk of developing IBD[164].

Antibodies against cyclic citrullinated peptides (anti-CCP)

Citrullinated proteins and cyclic citrullinated peptides contain arginine residues that have been deiminated to citrulline (a process called citrullination) by a peptide-arginine deiminase (PAD). Approx. 75% of patients with rheumatoid arthritis are positive for anti-CCP (96% of rheumatoid factor-positive patients) versus 0.5% of the healthy population. The prevalence of these antibodies in other inflammatory disorders has been reported to range between 2% (polymyalgia rheumatica) and 8% (psoriatic arthritis)[168]. Highly specific anti-CCP assays have increasingly become established as important tools for differential diagnosis in recent years[169]. Two prospective studies reported that detection of CCP antibodies is not specific for joint involvement in IBD[170,171].

Glycoprotein 39 (HC gp39)

Punzi et al. were the first group to report an association between arthritis and serum levels of the joint glycoprotein HC (human cartilage) gp39[172]. Since then, no further studies have been published on this topic.

3 Primary sclerosing cholangitis

Primary sclerosing cholangitis (PSC) is a chronic fibrosing, sclerosing cholangitis of the intrahepatic and/or extrahepatic bile ducts with an incidence of 0.9–1.3/100,000 in Northern Europe (< 0.1 in

Antibodies	Prevalence (%)	Number of publications
Anti-BEC	63	1
pANCA	26–94	19
AMA	0–9	10
Anti-LKM	0	7
Anti-SLA/LP	0	4
Anti-cardiolipin	4–63	3
ANA	8–77	13
SMA	0–83	10
ASCA	44	1
RF	15	1
AECA	35	1

Anti-BEC = Antibodies against biliary epithelial cells (measured by flow cytometry); pANCA = Perinuclear anti-neutrophil cytoplasmic antibodies; AMA = Anti-mitochondrial antibodies; Anti-LKM = Liver-kidney microsomal antibodies; Anti-SLA/LP = Antibodies against soluble liver antigen/liver pancreas; ANA = Anti-nuclear antibodies; SMA = Smooth muscle antibodies; ASCA = Anti-*Saccharomyces cerevisiae* antibodies; RF = Rheumatoid factor; AECA = Anti-endothelial cell antibodies

Table 15:
Autoantibodies in primary sclerosing cholangitis (adapted from Hov et al.[177])

Southern Europe and Asia) and a prevalence of 8–14/100,000 in Northern Europe (1.1–1.4 in Southern Europe and Asia). It afflicts men about twice as frequently as women. The prevalence of PSC is 2–7% among IBD patients, while conversely 70–80% of PSC patients also have IBD (approx. 85% ulcerative colitis and 1–14% Crohn's colitis), although this prevalence is only about 20% in Asia[173,174]. PSC can be detected by laboratory tests in its early stages through elevated cholestasis parameters (alkaline phosphatase [AP], gamma-glutamyltransferase [GGT]), while transaminase levels rarely reach concentrations of > 300 U/l. Analogous with the undulating course of IBD, PSC-specific laboratory parameters also fluctuate over time. While several different antibodies (table 15) may be detected, there is no single laboratory parameter that is conclusive evidence of PSC in the way that, for example, anti-mitochondrial antibodies (AMA) demonstrate primary biliary cholangitis (PBC). Although up to 80% of patients are positive for perinuclear anti-neutrophil cytoplasmic antibodies (pANCA), they are neither specific (they can also be detected in over 40% of patients with autoimmune hepatitis [AIH] or ulcerative colitis) nor do titers correlate with any prognosis. Hypergammaglobulinemia is observed in about 30% of PSC patients, which should raise suspicion of an overlap with AIH, which afflicts up to 10% of PSC patients[175,176].

4 Acute pancreatitis

Serum levels of the typical pancreatic enzymes amylase and lipase elevated more than three-fold over normal, together with the typical clinical symptoms, represent confirmation of acute pancreatitis. Acute pancreatitis can develop in IBD patients not only in response to classical triggers, such as gallstones or alcohol abuse, but also as a consequence of adverse reactions to drugs such as mesalazine products or purine analogues, as well as in the form of autoimmune pancreatitis (AIP) – especially type 2 AIP – in the context of a systemic immune reaction. Patients with ulcerative colitis are at a higher risk of developing AIP than are Crohn's patients. Because type 2 AIP is not accompanied by elevated IgG4 levels, measurement of this IgG subfraction is not useful in diagnosing

IBD-associated AIP. In this situation, elevated serum levels of IgG4 are much more suggestive of inflammatory or neoplastic entities[178].
Amylase and lipase levels rise asymptomatically in 8–21% of patients without any detectable link to disease activity or medications. Proposed causes of these elevations include a latent extraintestinal pancreatic involvement in IBD (PAB = pancreatic antibodies!) or extrapancreatic release of amylase and lipase in the gastrointestinal tract[179].

5 Disorders of bone metabolism

Osteoporosis is a systemic skeletal disorder which is characterized by low bone mass and abnormal bone architecture, and is associated with higher bone fragility and an elevated risk of fractures. The prevalence of osteoporosis among IBD patients varies between 7% and 18%, while 34–67% of all patients have osteopenia. Patients with ulcerative colitis are at a 1.4-fold increased risk of spinal or hip fractures compared with the general population. Elevated bone turnover is considered to be an independent risk factor for osteoporotic fractures as well as a predictive factor for response to antiresorptive agents. Several sensitive and specific markers of bone formation (e.g. bone-specific AP, osteocalcin) and bone resorption (e.g. pyridinium crosslinks, telopeptides) have recently been identified and can assist in evaluating osteoblast/osteoclast activity (table 16). However, insufficient data are available to determine the value of these markers, especially for predicting the course of IBD[180,181], and there are also no validated genetic markers.
With the exceptions of alkaline phosphate (AP), calcium, phosphate, and 25-hydroxyvitamin D levels – all of which can be measured easily and rapidly in normal laboratory tests – laboratory parameters currently play only a minor role in the diagnosis and monitoring of bone turnover disorders in patients with IBD.
The current standard method for detecting osteoporosis is bone densitometry (DEXA) of the lumbar spine. Other diagnostic procedures are currently not recommended due to high levels of radiation and low availability (quantitative computed tomography [CT]) or due to a lack of standardization (osteosonography)[162].

Bone formation	Bone resorption
– Bone-specific isoform of alkaline phosphatase (BAP) – Osteocalcin (OC) – Type I procollagen propeptide (PICP, PINP)	– Pyridinium crosslinks – Pyridinoline (PYD) – Deoxypyridinoline (DPD) – Crosslinked type I collagen telopeptides – C-terminal (CTX) – N-terminal (NTX) – CTX generated by MMP* – Tartrate-resistant acid phosphatase (TRAP) – OC fragments (mid-regional) in urine

*MMP = Matrix metalloproteinases

Table 16:
Biochemical markers of bone turnover

VI Monitoring of complications and sequelae

1 Opportunistic infections

Infections (bacterial, viral, or parasitic) are not only important as differential diagnoses during the initial diagnosis of IBD, but increasingly relevant as opportunistic infections in an age of intensive immune therapies and as a potential trigger for flares and/or cause of therapy-refractory disease activity. Accordingly, the odds ratio for opportunistic infections is 2.9 in patients taking steroids, azathioprine, methotrexate, or TNFα antibodies alone, but increases to 14.5 (!) once two or more of these medicines are combined[182]. Table 17 summarizes the spectrum of opportunistic infections in IBD and their risk factors, with CMV and *Clostridioides difficile* colitis playing the most important roles[183,184].

CMV colitis

Human cytomegalovirus (CMV) is an opportunistic pathogen which is nearly ubiquitous. The seroprevalence for CMV is about 70% in the general population and even higher under poor socioeconomic conditions or with advanced age. Because IBD patients have the same seroprevalence as the general population, general screening regardless of clinical situation is not recommended. Multiple studies have demonstrated higher rates of CMV in patients taking systemic steroids[185-187]. The data for other immunosuppressants are contradictory and/or insufficient. However, diagnostic tests for CMV should also be performed for patients taking these drugs if they show inadequate improvement in ulcerative colitis activity and/or signs of systemic CMV infection (especially fever and leukopenia).

CMV can be diagnosed using whole blood, serum, or tissue. Methods that directly detect CMV in inflamed mucosa have gained prominence in recent years (CMV tissue PCR, CMV immunohistochemistry), while serological methods (serum CMV IgM) or detection in whole blood (CMV-PCR, pp65) have been studied less. Samples intended for direct detection by immunohistochemistry or molecular biology should be collected from ulcerated tissue whenever possible – ideally from basal tissue or the margin of the ulcer[188-190].

Detection of CMV alone does not indicate a need for treatment. As with all CMV end-organ diseases, CMV colitis must always be diagnosed from a combination of clinical factors (immunosuppressant

Predisposing factors for opportunistic infections in IBD	**Viral infections**	**Bacterial infections**	**Parasitic and fungal infections**
IBD type (ulcerative colitis > Crohn's colitis > small intestinal Crohn's), extent of disease (pancolitis > left-sided colitis), duration of disease	Cytomegalovirus	*Clostridioides difficile*	*Candida* spp.
Malnutrition	Varicella zoster virus	*Escherichia coli*	*Pneumocystis jirovecii* (*carinii*)
Immunosuppressants	Herpes simplex virus	*Salmonella* spp.	*Aspergillus* spp.
Leukopenia under immuno-suppressants	Epstein-Barr virus	*Streptococcus pneumoniae*	Histoplasmosis
Comorbidities	Human papillomavirus	*Staphylococcus* spp.	*Cryptococcus* spp.
		Mycobacterium tuberculosis	*Isospora belli*
		Legionella pneumophila	*Coccidioides immitis*
		Listeria monocytogenes	*Leishmania donovani*
		Mycobacterium avium spp. or *xenopi*	Blastomycosis
		Nocardia	

Table 17:
Opportunistic infections in immunosuppressed IBD patients (adapted from Viget et al.[183] and Irving and Gibson[184])

therapy, "refractory" colitis) and detection of the virus by histology or immunohistochemistry.

Clostridioides difficile colitis

Clostridioides difficile (*C. difficile*) is a gram-positive, spore-forming, rod-shaped, anaerobic bacteria that was first described in the intestinal microbiota of healthy newborns in 1935. Pseudomembranous colitis triggered by *C. difficile* toxins was first described in 1978 by Bartlett et al.[191]. Today, *C. difficile* is the most important cause of nosocomial diarrhea. IBD patients are at a much greater risk of acquiring a *C. difficile* infection, with an odds ratio of 2.9 (Crohn's disease: 2.1; ulcerative colitis: 4)[192]. Moreover, *C. difficile* is known to be by far the most common cause of opportunistic infections in IBD patients, with an incidence that has doubled to tripled in the past 5–7 years according to recent studies[183,193].

Sensitive diagnostic tests should be used to detect *C. difficile* as early as possible[194]. A soft/liquid stool sample is usually sufficient for this purpose, whereas solid, formed stools cannot be used for diagnostic testing. Rectal swabs may also be used in exceptional circumstances, for example patients with ileus.

A multi-step algorithm[195] that combines a sensitive screening test with a confirmatory test for toxigenic infection is recommended for diagnosis (table 18). Rapid antigen tests and genome detection using nucleic acid amplification tests (NAAT) have gained a key role in routine diagnosis due to their short turn-around time (TAT: 15 min to 2 h). Toxigenic culture, i.e., anaerobic cultivation on special media combined with toxin detection in the cell culture supernatant, is the diagnostic gold standard and prerequisite for special tests like antibiotic resistance testing or ribotyping. However, this method is less suitable for diagnosing acute cases due to its long turn-around time (TAT > 72 h)[196].

For immunosuppressed patients, lower toxin levels appear to be sufficient to trigger pseudomembranous colitis. Because this can lead to a potential diagnostic gap, repeated toxin detection by stool testing or *C. difficile* culture with subsequent toxin detection are recommended for patients in this situation[197]. Alternatively, sigmoidoscopy may also provide further insights in these cases, especially for treatment-refractory patients with negative stool cultures[198]. Negative findings still do not rule out the possibility of infection in these cases, es-

Test method	Indication
Glutamate dehydrogenase (GDH)-EIA (TAT < 2 h)	Initial screening test with high sensitivity and high negative predictive value; GDH-positive samples always require confirmation of toxigenic infection.
Toxin A- and toxin B-EIA (TAT < 2 h)	Confirmatory test for toxigenic infection after GDH-positive samples (two-step algorithm); good correlation with severe infections but only limited sensitivity; NAAT is recommended if toxin detection is negative (three-step algorithm).
Cell culture cytotoxicity test, cytotoxin neutralization assay (CTNA) (TAT < 24 h)	Reference test for detecting toxins in stool; however, CTNA is rarely used for routine diagnosis due to longer TAT and greater difficulties with standardization and automation.
NAAT for toxin genes (TAT < 4 h)	Confirmatory test for toxigenic infection; NAAT (e.g. PCR) is not recommended as a screening test since it also frequently detects asymptomatic carriers of *C. difficile* who do not need to be treated or isolated.
Anaerobic toxigenic culture (TAT > 3 days)	The diagnostic gold standard for a confirmatory test of toxigenic infection; limited relevance for early diagnosis of *C. difficile* infection due to long TAT; the culture is a requirement for ribotyping and antibiotic resistance testing in critically ill patients and during outbreaks.

TAT = Turn-around time; EIA = Enzyme immunoassay; NAAT = Nucleic acid amplification test (e.g. polymerase chain reaction [PCR])

Table 18:
Diagnostic methods used to detect *Clostridioides difficile*-associated diarrhea and their importance[196]

pecially considering that the typical endoscopic presentation with pseudomembrane formation is often not observed[199].

2 Small bowel dysfunction: steatorrhea

Fat absorption disorders that result in steatorrhea and consequent deficiencies in fat-soluble vitamins (A, D, E, K) primarily afflict Crohn's patients as sequelae of bile acid deficiency. Bile acid deficiency arises either as a result of pathogenic bacterial colonization (e.g. strictures) of the small intestine (bile acid cleavage by bacteria results in levels dropping below the critical micelle concentration of 1.5–2.0 mM = qualitative bile acid deficiency) or due to reduced reabsorption in patients with ileal dysfunction or resection (quantitative bile acid deficiency). Ileal resections of a mean of 50 cm or more result in fatty stools; steatorrhea can frequently be detected in patients with a distal ileum resection > 90 cm. Today, steatorrhea is rarely detected by quantitative measurement of the fat content in stool using the traditional fecal fat analysis method of *van de Kamer*[200]. Semi-quantitative determination of β-carotene in serum has now become established in clinical practice instead. β-carotene represents an indirect parameter for detecting steatorrhea. This method uses spectrophotometry and is simple, reliable, cost-effective, and provides rapid results. It also does not present the practical hurdles of stool collection and preparation, causes of resistance against the van de Kamer method. Therefore, semi-quantitative measurement can be used as a clinically practical alternative to fecal fat analysis when quantitative determination of the extent of steatorrhea is not required.

The diagnostic use of this method is based on the behavior of β-carotene with regard to partitioning and absorption. Fat malabsorption disorders increase the solubility potential of β-carotene and other fat-soluble substances (e.g. vitamins), resulting in decreased uptake of β-carotene from food. Because humans can only store minute levels of β-carotene, its serum concentration begins to drop after 1 to 4 weeks of malabsorption.

The decrease in the serum concentration of β-carotene allows steatorrhea to be rapidly detected by simple means, but provides no insight into the

etiology of fat malabsorption or the quantitative extent of steatorrhea.

Steatorrhea can almost definitively be ruled out at β-carotene concentrations of > 100 µg/100 ml, while levels < 47 µg/100 ml indicate increased excretion of fat in stool[201]. Values < 100 µg/100 ml should raise suspicion (diagnostic sensitivity 88%) of steatorrhea (fecal fat analysis or fecal fat monitoring is indicated).

Protein-losing enteropathy

Measuring fecal protein excretion is not generally suitable for directly diagnosing protein-losing enteropathy, since the proteins secreted into the lumen of the gut are reabsorbed as amino acids and thus escape detection.

In 1959, Gordon introduced the first inert marker molecule for the detection of protein-losing enteropathy using ^{131}I-polyvinylpyrrolidine (^{131}I-PVP) (Gordon test)[202]. In the following years, several modifications to this molecule were reported using various radioactively-labeled macromolecules such as the ^{99m}Tc-albumin test. However, none of these nuclear medicine techniques plays a role in contemporary clinical practice.

α_1-Antitrypsin

The use of the protease inhibitor α_1-antitrypsin as an endogenous marker for detecting protein-losing enteropathy was first described in 1977 by Crossley and Elliott[203]. α_1-Antitrypsin is a large acute phase protein with a molecular weight of 50,000 (albumin: 67,000) that is primarily synthesized in the liver and comprises about 4% of total serum protein (serum concentration 2–5 g/l). Its half-life is about 4 days. Its polypeptide component comprises 85% of its mass, with sialic acid comprising 3–4% and carbohydrates 12%. At least 24 alleles are currently known which exhibit co-dominant behavior. Type M is the most prevalent at 80%. Due to its antiproteolytic activity (it is the most potent known inhibitor of serine proteases such as trypsin, chymotrypsin, elastase, and proteinases of the coagulation system), only a small fraction is degraded in the intestine and nearly all of it is excreted unmodified in stool.

Fecal α_1-antitrypsin clearance (ATC) is calculated using the following equation:

$$\alpha_1\text{-ATC (ml/day)} = \text{V (ml/day)} \times \text{F (mg/100 ml)/S (mg/100 ml)}$$

where V = mean stool volume over 3 days, F = mean fecal concentration of α_1-antitrypsin over 3 days, and S = mean serum concentration of α_1-antitrypsin over 3 days.
Healthy individuals have an intestinal ATC of < 35 ml/day; values of > 400 ml/day can be detected in patients with severe protein-losing enteropathy[201].

3 Hyperoxaluria/nephrolithiasis

Oxalate nephropathy may develop in Crohn's patients with marked ileal involvement or post-ileal resection as a consequence of a secondary hyperoxaluria. In this condition, free fatty acids and deconjugated bile acids form complexes with calcium, thereby increasing the proportion of free, easily diffusible oxalate, while calcium oxalate is very poorly absorbed. Deconjugated bile acids also lead to increased uptake of oxalate in the colon through increased paracellular permeability.
Hyperoxaluria is detected by *direct measurement* of the concentration of oxalate in 24-hour urine using enzymatic, colorimetric, or chromatographic (HPLC; gas chromatography [GC]) methods. Depending on the method used, the reference ranges for oxalate excretion in 24-hour urine are 42–71 mmol/24 h (colorimetric) or 7–44 mmol/24 h (enzymatic). Levels > 45 mmol/24 h are suggestive of elevated absorption of oxalate[201].

4 Determination of the functional integrity of the small intestine

D-xylose test

The D-xylose test examines the functional integrity of the small intestine, thereby allowing a (semi-) quantitative inference about its absorptive surface. Orally administered D-xylose (pentose) is absorbed actively but very slowly by the upper small intestine. About 50% of the D-xylose is absorbed by the small intestine, with one half being metabolized and the other half excreted with the urine.
Diseases which impair the absorptive surface of the small intestine excrete less D-xylose into the urine and lead to low post-absorption serum concentrations of D-xylose. The classical indication for the D-xylose test is celiac disease. However, the

D-xylose test is relevant in many other settings, since other disorders of the small intestine (e.g. partial small bowel resection, drug toxicity, small intestinal bacterial overgrowth due to blind loop syndrome, small bowel diverticula, or motility disorders) lead to reduced absorption of D-xylose[201,204].

After emptying his or her bladder, the fasting patient drinks 25 g D-xylose in 600 ml water or weak tea. The entire 5-hour urine sample must be saved after the start of the test. Venous blood is collected after 0, 15, 30, 60, 90, and 120 minutes. D-xylose is measured in serum and urine by spectrophotometry[201,205].

Measurements of D-xylose excretion in urine are not only subject to collection errors, but also influenced by patient age (decreased kidney function), inadequate hydration, acetylsalicylic acid, and hyperthyroidism. The normal level of D-xylose excretion is > 4 g/5 h (26.6 mmol/5 h), i.e., 16% of the 25 g dose administered, and increased D-xylose serum concentration is defined as > 10 mg/dl (after 15 min) or > 20 mg/dl (after 30 min) or > 30 mg/dl (after 60 min).

Pathological results of a D-xylose test indicate a reduction in the absorptive surface area of the small intestine. A false-negative test can be caused by malabsorption resulting from disorders of the distal small intestine (e.g. ileal Crohn's disease) or by maldigestion resulting from exocrine pancreatic insufficiency, cholestasis, or bile acid deficiency. Liver disorders with ascites, kidney failure, and small intestinal bacterial overgrowth may lead to a false-positive test result.

Citrulline test

Citrulline is an amino acid that is produced from glutamine only in enterocytes and is not subject to hepatic clearance (fig. 10). The serum concentration of citrulline thus correlates with the mass of active enterocytes in the small intestine but not with the extent of intestinal inflammation, and varies depending on the underlying disorder[206-208]. Citrulline concentration is measured by HPLC-UV (cut-off 23 µmol/l) and takes relatively less time and is more cost-effective than the classical xylose test.

5 Diagnosis of suspected small intestinal bacterial overgrowth

Prerequisites for bacterial overgrowth of the small intestine, defined as colonization of the jejunum with > 10^5 mixed bacteria (usually containing anaerobic bacteria) per milliliter aspirate, is an anatomical abnormality (blind loop, diverticulum) or disorder of the migrating motor complex (MMC), as occurs in scleroderma, chronic intestinal pseudoobstruction, or diabetic enteropathy. Moreover, the secretory and cellular immune functions of the small intestine are assumed to play a role in preventing overgrowth (reviewed in German by Stein and Schneider[209]).

Small intestinal bacterial overgrowth (SIBO) disproportionately afflicts patients with Crohn's disease (about 25%) versus those with ulcerative colitis[28] – especially Crohn's patients who have undergone ileocecal resection (35–65%). Small intestinal strictures and enteroenteric fistulas are additional causes. SIBO may mimic an acute flare (weight loss, increase in stool frequency, flatulence) and thus represents an important differential diagnosis upon recurrence of Crohn's disease[210,211].

Direct, quantitative diagnosis of SIBO is performed by stepwise or direct jejunal aspiration and adequate microbial diagnostic testing, which is time-consuming and presents considerable technical challenges. Consequently, this method is restricted to only a few research institutions and is not performed routinely.

Indirect tests are based on detection of bacterial metabolic products in exhaled air[212]. The glucose H_2 and the lactulose H_2 breath tests are used. However, the value of these tests is limited and cannot unconditionally justify their intended role as confirmatory detection methods or screening tests.

Glucose H_2 breath test

The glucose H_2 breath test is the preferred method in routine clinical practice due to its acceptable accuracy, simple procedure, and low substrate costs. Glucose is normally completely absorbed by the small intestine and does not lead to H_2 production. However, bacterial overgrowth of the upper small intestine leads to premature bacterial fermentation of glucose to hydrogen. End-expiratory H_2 concentrations are measured before the start of the test and at 30, 60, 90, 120, 150, and 180

minutes after oral administration of 80 g glucose dissolved in 400 ml water.

An increase in the end-expiratory H_2 concentration of > 20 ppm following glucose administration indicates that the sugar has been metabolized by bacteria. Because glucose malabsorption only occurs after gastric resection (Billroth II) or small bowel resection, a premature increase in H_2 concentration in exhaled air demonstrates bacterial overgrowth of the upper small intestine. The sensitivity (65–93%) and specificity (approx. 90%) of the bacterial overgrowth test are satisfactory[209].

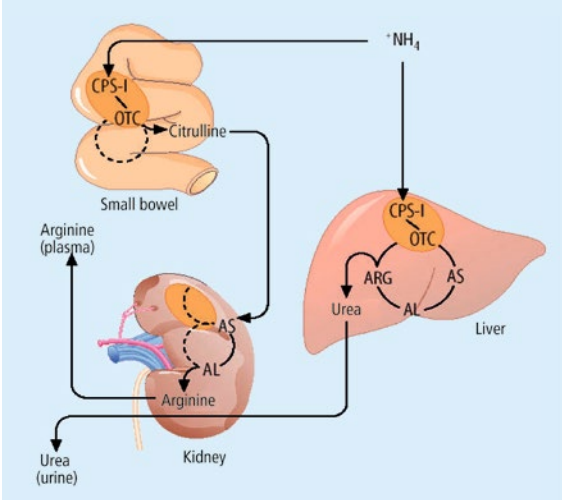


Fig. 10:
Synthesis and metabolism of citrulline (adapted from Crenn et al.[206])

Lactulose H_2 breath test

The rapid formation, absorption, and exhalation of H_2 from lactulose can be used both to measure the orocecal transit time and to detect bacterial overgrowth in the proximal small intestine[201].

Lactulose, which is not absorbed by the digestive tract, is administered orally and the H_2 concentration in exhaled air is measured sequentially at brief intervals (5 min). An increase in the H_2 concentration after 150–180 minutes correlates with the arrival of the test solution in the cecum (validated for 10 g lactulose in 150 ml water). An increase after 60–90 minutes indicates bacterial overgrowth of the proximal small intestine[209]

VII Therapeutic drug monitoring

Purine analogues

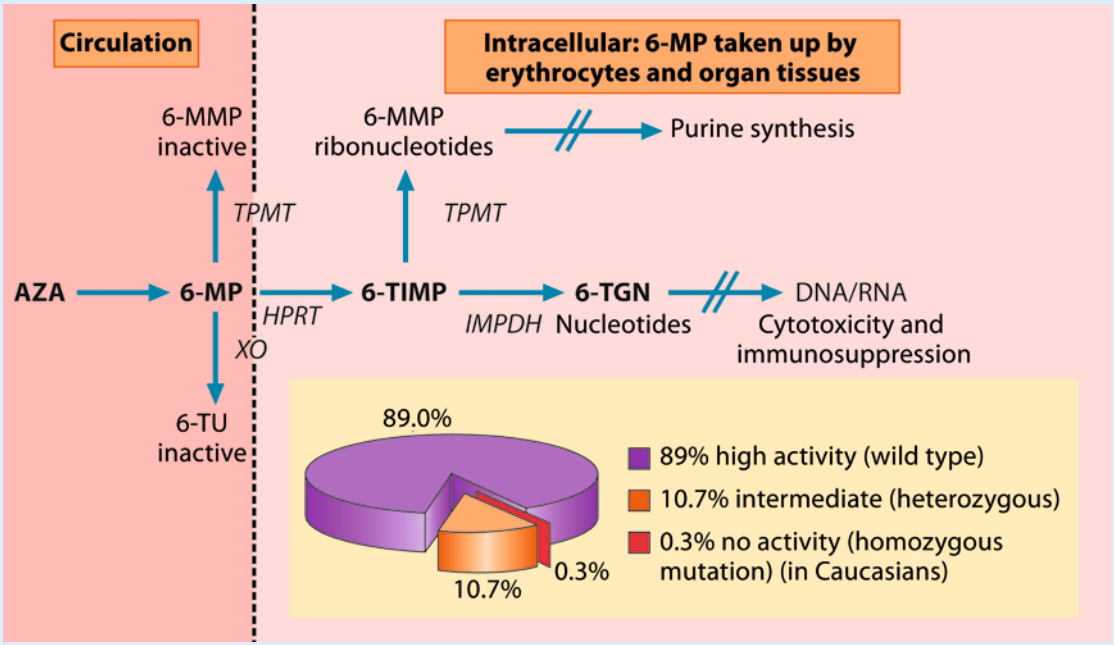
Neither azathioprine (AZA) nor its metabolite 6-mercaptopurine (6-MP) have immunomodulatory effects per se. Both drugs are initially metabolized via multiple competitive pathways to their pharmaceutically-active metabolite 6-thioguanine nucleotide (6-TGN), by xanthine oxidase (XO) to the inactive 6-thiouric acid (6-TU), and by thiopurine S-methyltransferase (TPMT) to 6-methylmercaptopurine (6-MMP), which is thought to be the main driver of hepatic adverse effects (elevated transaminase levels) (fig. 11). With half-lives of 1.7 and 1.2 hours, respectively, AZA and 6-MP are unsuitable for therapeutic monitoring, in contrast to the metabolites, which are stored intracellularly and degrade much more slowly.

Levels of the active metabolite 6-TGN are dependent on the relative, i.e., competitive, activities of all of the enzymes. For example, inhibition of XO by allopurinol or a genetic TPMT deficiency can lead to a toxic accumulation of 6-TGN, resulting in leukopenia or even pancytopenia.

The correlation between treatment response and 6-TGN levels > 235 pmol/8 x 10^8 RBCs, which has been demonstrated in numerous studies together with the observation that adverse effects begin to increase at serum levels > 450 pmol/8 x 10^8 RBCs, has led to proposals to use 6-TGN concentration as a predictor of clinical response to AZA therapy. However, the studies conducted on this hypothesis led to disappointing results[213], and consequently determination of 6-TGN levels is not generally recommended.

Studies on 6-TGN serum levels (> 235 pmol/8 x 10^8 RBCs) and simple blood count parameters have demonstrated that the predictive value of an MCV:leukocyte ratio of ≥ 12 or an increase in mean corpuscular volume (MCV; Δ > 10) is equivalent to drug level measurement in terms of sensitivity for responders or non-responders. An increase in eosinophil count is a sign of a treatment-related drift toward Th2[214,215].

However, measurement of 6-TGN and 6-MMP levels (usually by HPLC) has been shown to be helpful in patients without adequate clinical response after 3 (2–4) months of body weight-adjusted AZA therapy[216,217], as this allows non-compliance (6-TGN and 6-MMP levels low or undetectable), underdosage (6-TGN levels normal or high), and 6-MMP "shunters" (who preferentially metabolize



6-MMP = 6-Methylmercaptopurine; 6-TGN = 6-Thioguanine (active metabolite); 6-TIMP = 6-Thioinosine 5´-monophosphate; 6-TU = 6-Thiouric acid; HPRT= Hypoxanthine phosphoribosyltransferase; IMPDH = Inosine-5′-monophosphate dehydrogenase; TPMT = Thiopurine S-methyltransferase; XO = Xanthine oxidase

Fig. 11:
Metabolism of azathioprine (AZA) and 6-mercaptopurine (6-MP) (from: Stein J, Farrag K, eds. *Diagnostik und Therapiekontrolle bei chronisch-entzündlichen Darmerkrankungen*. UNI-MED Science, 2nd revised ed. Bremen, London, Boston: UNI-MED Verlag AG; 2019)

AZA to 6-MMP) to be identified and clinical response to be improved by appropriate measures (fig. 12; table 19).

It must be kept in mind that the 6-TGN concentration will decrease by 10–15% per day when the sample is shipped at room temperature[218] since 6-TGN is unstable in whole blood (→ maintain the cold chain when shipping!).

It remains unclear whether measurement of thioguanine triphosphate might represent a superior alternative for drug monitoring[219,220], as there are presently no cost-effective assays available for this parameter.

Normal thiopurine doses cause severe myelosuppression in patients with homozygous TPMT deficiency, which afflicts approx. 0.3% of all Caucasians. However, due to the rarity of this deficiency and the fact that two-thirds of patients with severe purine analogue-associated neutropenia have normal TPMT activity[221], routine screening of TPMT activity before treatment with purine analogues has not become established in practice (fig. 13). Should TPMT activity nonetheless be measured and found deficient, AZA or 6-MP treatment should not be initiated. On the other hand, if intermediate TPMT activity (2.8–9.9 nmol/ml RBC x h) is detected – which is typically caused by a heterozygous mutation in TPMT – it is recommended to start with 30–50% of the standard dose and increase the dose weekly if well tolerated (→ blood count) until the target dose is achieved. Most centers recommend or practice incremental dose increases in all patients regardless of TPMT status. Because changes in the blood count may occur many months later, regular blood testing must remain an integral component of thiopurine therapy (table 20).

Methotrexate

Measurement of methotrexate (MTX) or its primary metabolite 7-hydroxy-MTX has been shown to provide little value in monitoring drug effectiveness and/or toxicity due to the fact that these compounds have very short half-lives of 5–8 hours. While a link has been proposed to the 129C mutation in methylenetetrahydrofolate reductase (MTHFR), the data are contradictory and inconsistent, and hence pharmacogenetic testing is also not currently recommended (reviewed by Bruns and Stallmach[224]) (fig. 14).

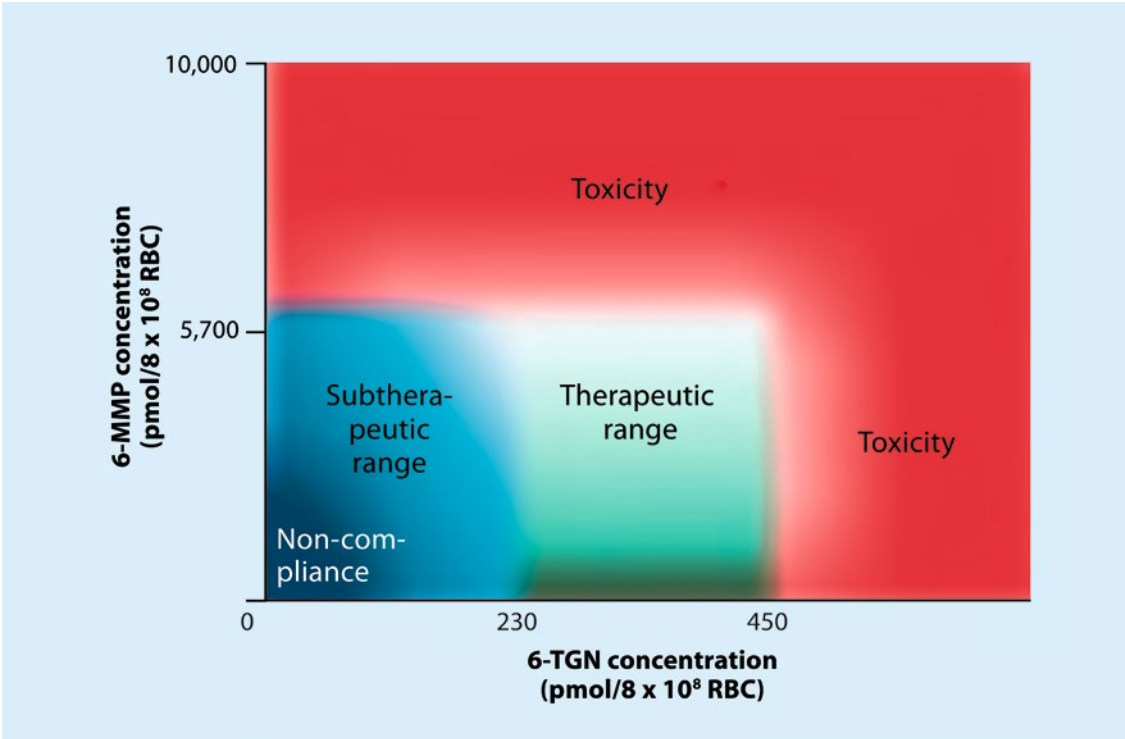


Fig. 12:
Therapeutic and toxic ranges of 6-thioguanine (6-TGN) and 6-methylmercaptopurine (6-MMP)
(source: 218 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5690305/); licensed under CC BY-NC-ND 4.0
(https://creativecommons.org/licenses/by-nc-nd/4.0/deed.de), Falk Foundation e.V. has no rights to this figure.

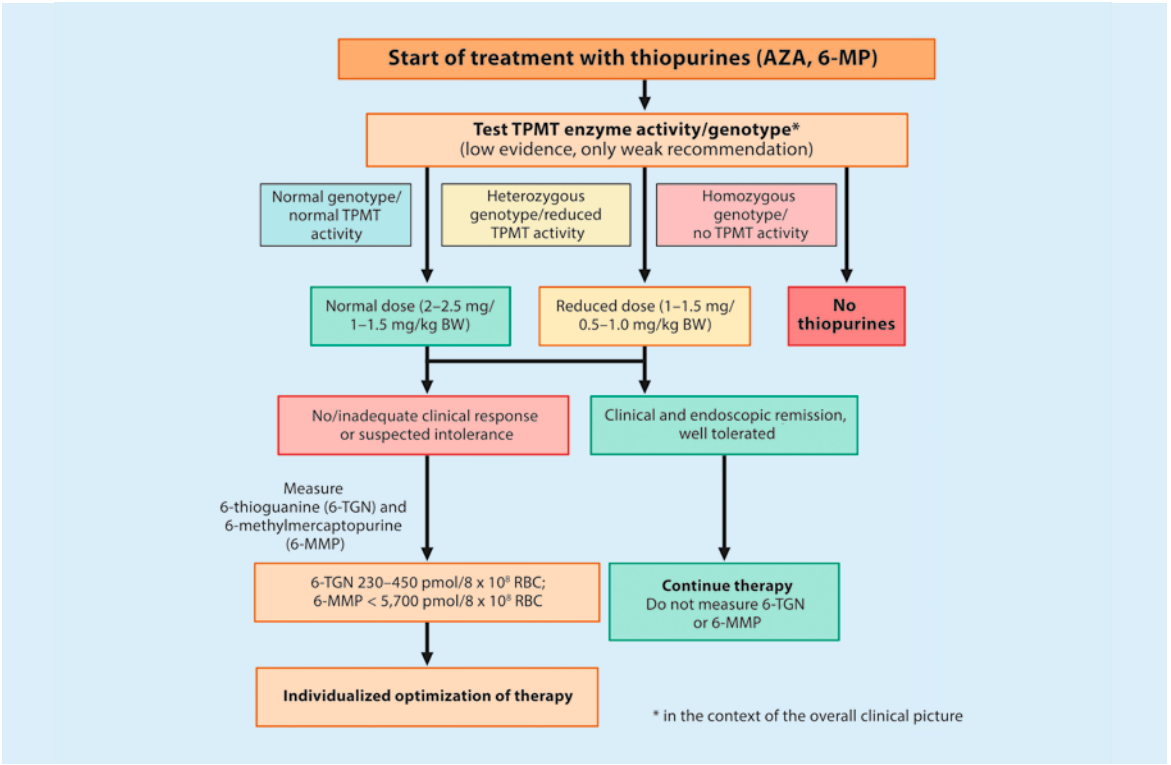


Fig. 13:
Monitoring of azathioprine (AZA) and 6-mercaptopurine (6-MP) metabolites (adapted from Vande Casteele et al.[223])

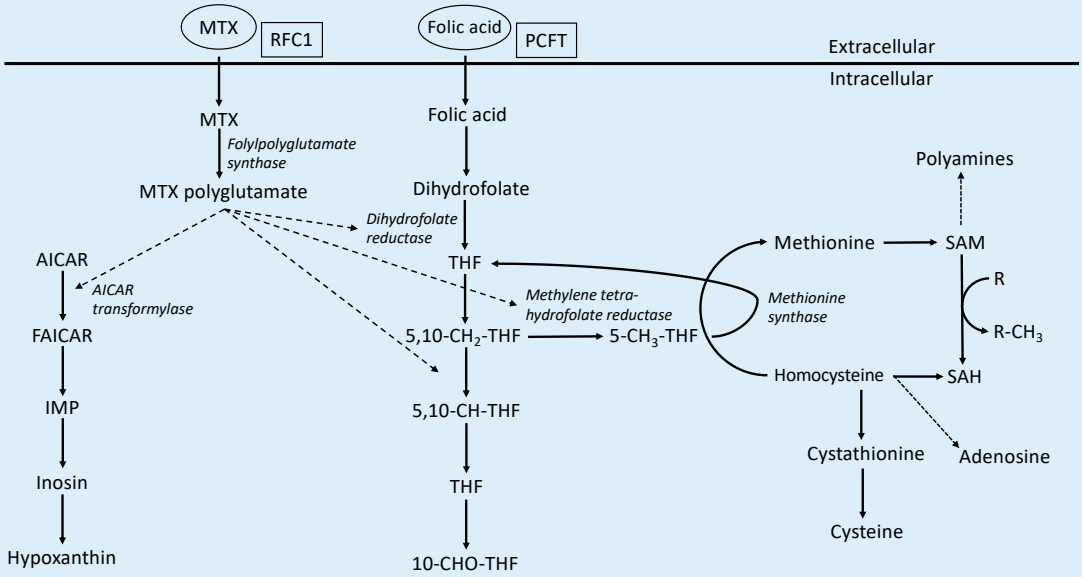
6-TGN level	6-MMP level	Interpretation	Strategy
Very low or absent	Very low or absent	Non-compliance	Talk with patient about compliance
Low	Low	Underdosage	Dose escalation
Therapeutic range	Normal or high (< 5,700)	Thiopurine-refractory	Consider alternative therapies
Low	High	Treatment-resistant/ "6-MMP shunters"	Different therapy or switch to allopurinol (100 mg) and reduce thiopurine dose to 25%
High	High	Overdosage	Dose reduction

6-TGN (6-thioguanine): low < 250 pmol/8 x 10^8 RBC; high > 450 pmol/8 x 10^8 RBC;
6-MMP (6-methylmercaptopurine): high > 5700 pmol/8 x 10^8 RBC (adapted from Leong et al.[222])

Table 19:
Tailored optimization of treatment for non-responders based on 6-TGN and 6-MMP levels

	Week 1	Week 2	Week 4	Week 8	Week 12	Every 3 months
Blood count	x	x	x	x	x	x
Bilirubin	x		x	x	x	x
Alkaline phosphatase	x		x	x	x	x
Transaminases	x		x	x	x	x
Lipase	x		x	x	x	x
Urinalysis	x					
Creatinine	x					x

Table 20:
Recommended laboratory tests in patients receiving azathioprine/6-mercaptopurine therapy



AICAR = 5-Aminoimidazole-4-carboxamide ribonucleotide; FAICAR = 5-Formamidoimidazole-4-carboxamide ribonucleotide; IMP = Inositol monophosphate; PCFT = Proton-coupled folate transporter; R = Residue; RFC 1 = Reduced folate carrier 1; SAH = S-Adenosyl homocysteine; SAM = S-Adenosyl methionine; THF = Tetrahydrofolate

Fig. 14:
Metabolism of methotrexate (MTX) (adapted from Bruns and Stallmach[224])

Calcineurin inhibitors

The calcineurin inhibitors cyclosporine (CsA) and tacrolimus (Tac) are lipophilic inhibitors whose bioavailability varies greatly within and between individuals and depends on the absorption, metabolism (cytochrome P450-3A, CYP3A) and biliary secretion of these molecules. There are also ethnic variations in CYP3A4 metabolism. For example, elevated CYP3A4 activity as a result of the CYP3A4*1B polymorphism is found in fewer than 4% of Caucasians but 46–66% of people of African origin. Similarly, patients with wild-type CYP3A5*1 require much higher doses of Tac. The frequently observed increase in Tac levels following discontinuation of steroids can be attributed to prior steroid-dependent induction of CYP3A and MDR1[224].

Decades of experience from transplantation medicine have demonstrated that measurement of trough levels is adequate for both calcineurin inhibitors to ensure sufficient monitoring of effectiveness and adverse effects.

Table 21 shows the therapeutic ranges for both calcineurin inhibitors. As in the postoperative phase after organ transplantation, the goal for IBD patients is to achieve trough levels within the upper half of the therapeutic range at the start of treatment; however, evidence-based data on this approach are not available. Toxicity is very likely at concentrations of > 400 ng/ml for CsA and > 20 µg/ml for Tac. Because cross-reactions by the corresponding metabolites of CsA and Tac are observed when drug concentrations are measured by immunoassays, more specific analytical methods like HPLC or liquid chromatography-mass spectrometry (LC-MS) should be used instead. Although 2-hour measurement (C2 monitoring) of CsA has been proposed as an alternative to trough levels in transplantation medicine, no data are available on this approach in IBD patients[225].

In addition to CYP3A4 and CYP3A5, the membrane-bound P-glycoprotein (ABCB1; P-gp) plays a key role in the pharmacokinetics of calcineurin inhibitors[226,227]. P-gp is a transport protein of the adenosine triphosphate (ATP)-binding group (ABC) that translocates substrates from the cell against their concentration gradient. P-gp was initially discovered in studies on the development of resistance to chemotherapy by cancer cells, but is also responsible for eliminating numerous xenobiotics,

Drug	Sample material*	Therapeutic trough level**		C2 level***
		At start	Maintenance phase	
6-Thioguanine	Heparin/ EDTA whole blood	250–450 pmol/ 8 x 10^8 RBC		–
Cyclosporine	EDTA whole blood	150–225 µg/l	100–150 µg/l	1,000/800/600 µg/l
Tacrolimus	EDTA whole blood	10–15 mg/l	5–10 mg/l	–

* Can be sent by mail at ambient temperature
** 12 hours after second dose
*** Preliminary cyclosporine reference values in blood 2 hours after administration of last dose in post-transplantation adults 0–6, 6–12, > 12 months post-transplantation

Table 21:
Pharmacokinetic data on immunosuppressants for IBD (adapted from Oellerich[225])

thereby protecting the body from the ingress of foreign substances (e.g. in the intestinal tract). It is expressed in many types of cells (brain, liver, gut) and is typically associated with CYP3A since most P-gp substrates are also metabolized by CYP3A4. In a retrospective analysis of 89 steroid-refractory ulcerative colitis patients treated with Tac, Herrlinger et al.[228] first showed that single-nucleotide polymorphisms (SNPs) in the P-gp gene (ABCB1 1236C>T) are significantly associated with response to Tac. In contrast, no association was found between treatment effects and Tac trough levels (> 10 ng/ml vs. < 10 ng/ml), confirming the results of an earlier study[229].

Anti-TNFα antibodies

About one-third of all patients treated with anti-tumor necrosis factor (TNF)α antibodies in clinical trials did not exhibit primary response (termed primary non-responders). The following parameters have been shown to be important clinical predictors of primary non-response: short disease duration, predominantly inflammatory disease, colonic involvement, non-smoker status, and moder-

ate to severe disease activity (reviewed by Yanai and Hanauer[230]).

A retrospective analysis of the ACT1 and ACT2 studies also demonstrated an inverse correlation between response and serum albumin concentration: Albumin levels < 3 g/dl correlated with a significantly worse initial response to treatment[231].

In contrast, some of the patients treated with anti-TNFα antibodies achieved primary response but experienced a decrease in effectiveness as their treatment continued (secondary non-responders). The primary cause of this phenomenon was initially thought to be the emergence of anti-drug antibodies (ADA)[232], as these were observed in 36–61% of patients with episodic doses of infliximab[233]. However, the hypothesis that ADA are the sole reason for failure of anti-TNFα therapy is increasingly being called into question. For example, several studies have found trough levels of infliximab to be low or undetectable even in the absence of ADA[234-236], suggesting other factors may be affecting the pharmacokinetics of this drug.

Although there is theoretically always a link between ADA and (secondary) non-response, studies from multiple groups have reported that measuring anti-TNFα levels is a significantly better predictor of non-response[230,234,237].

For example, high trough levels were found more frequently in patients with clinical and endoscopic remission. According to a recent consensus recommendation, infliximab trough levels of 3–8 µg/ml at week 14 or adalimumab trough levels of 5–12 µg/ml at week 4 are associated with a high probability of clinical remission. Trough levels of > 32 µg/ml for certolizumab pegol and > 2.5 µg/ml for golimumab were proposed, both at week 6[238].

Nonetheless, the specific method used (fig. 15) must always be kept in mind when interpreting the cut-offs for therapeutic antibody levels and ADA levels[239-241]. For example, enzyme-linked immunosorbent assays (ELISA) – which are the most frequently used and typically the easiest to perform – often have major limitations compared to the more complex RIA- or EMSA-based methods of detection (reviewed by Yanai and Hanauer[230], Steenholdt et al.[241], Bendtzen et al.[242]).

The phenotypical course of the disease must also be incorporated into any interpretation of drug concentrations. For example, higher infliximab

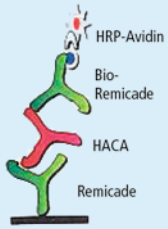
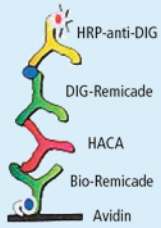
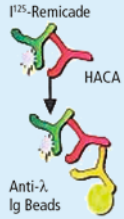
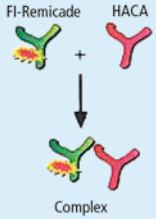
	"Sandwich" assay	Biotin-DIG-based "sandwich" assay	Radioimmuno-assay	Mobility shift assay
Construction				
Non-specific "background noise"	High	High	Low	Low
Sensitivity	Low	Moderate	High	High
False-positive/ false-negative rate	High	High	Low	Low
Detection of IgG4-HACAs	No	No	Yes	Yes
Detection of Ig isotypes	No	No	No	Yes
Tolerance to TNFα in the sample	Low	Low	High	High

Fig. 15:
Methods used to detect HACAs (human anti-chimeric antibodies) (from Wang et al.[247])

trough levels (> 10 µg/ml) appear to be necessary to heal perianal fistulas in Crohn's patients[243].
In patients with detectable ADA and concurrently low trough levels, continuation of treatment only results in successful outcomes in 17% of patients. In contrast, treatment response was observed in approx. 92% of patients who were switched to a second anti-TNFα antibody.
Moreover, a study by Yanai et al. with nearly 250 IBD patients reported that drug monitoring led to therapeutic consequences in > 70% of patients[244].
On the other hand, monitoring should initially be repeated after 2 months in patients with ADA and adequate therapeutic drug levels, since ADA may be transient and become undetectable again in about two-thirds of patients[245,246].
Figures 16a + b summarize the current recommendations for therapeutic drug monitoring of anti-TNFα antibody treatment of IBD.

Vedolizumab and ustekinumab

Although clinical experience with vedolizumab and ustekinumab is still less extensive than with anti-TNFα antibodies, the first non-interventional studies suggest that drug monitoring may be useful for both antibodies in order to optimize treatment.
The first meta-analysis for vedolizumab was published in 2019 based on data from over 500 IBD patients. This analysis revealed that patients with ulcerative colitis who achieved endoscopic and clinical remission had significantly higher vedolizumab trough levels during maintenance therapy. Trough levels > 20 µg/ml at week 6 and > 12 µg/ml during maintenance therapy were associated with improved outcomes in the analysis. ADA against vedolizumab were observed in 1.7–3% of patients on maintenance therapy[248].
The cut-offs reported to be associated with a high probability of treatment response in Crohn's disease were peak concentrations of > 105 µg/ml within 2 weeks after induction for ustekinumab[249] and trough levels of 2 µg/ml at week 8 and 4.5 µg/ml at week 26[250,251]. The corresponding data are not yet available for ulcerative colitis.

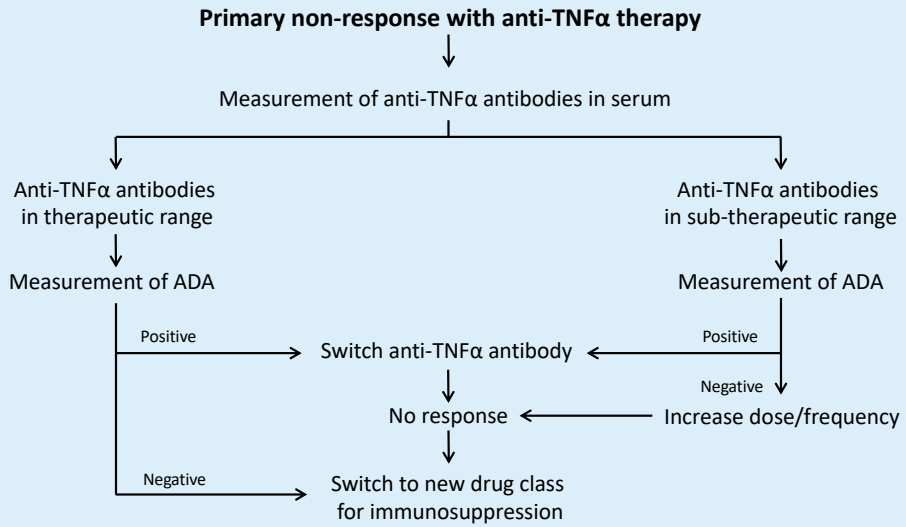


Fig. 16a:
Drug monitoring of anti-TNFα therapy using trough levels and ADA measurement: primary non-response

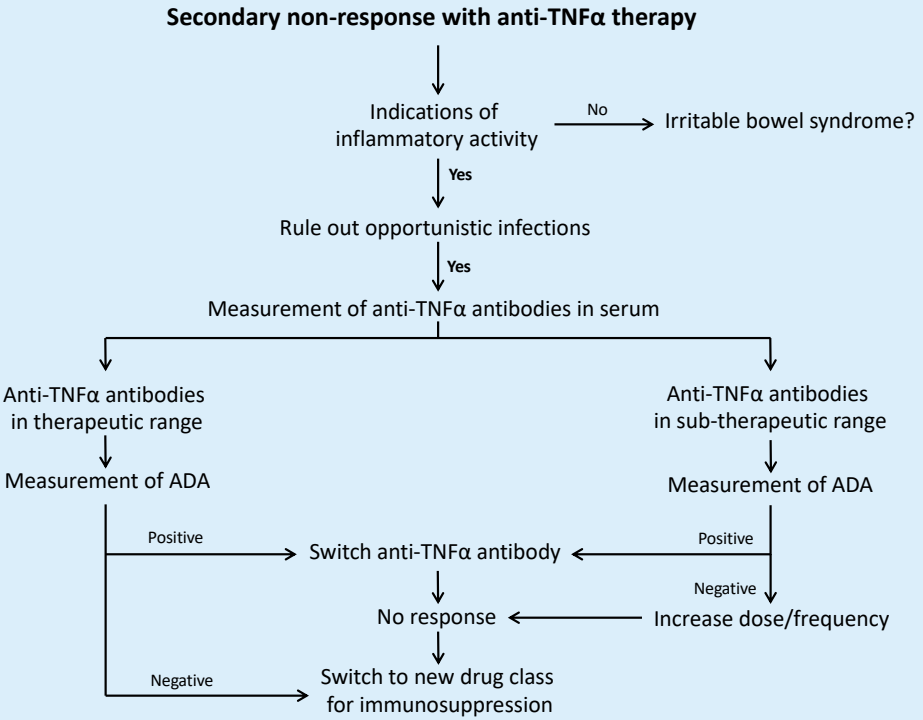


Fig. 16b:
Drug monitoring of anti-TNFα therapy using trough levels and ADA measurement: secondary non-response

VIII Genetics

Epidemiological studies have consistently demonstrated familial clustering among IBD patients. In various publications, the prevalence of IBD in first-degree relatives has been reported at 0.35–4.5% for patients with Crohn's disease and 0.3–2.7% for patients with ulcerative colitis.[252].

These prevalence rates point to the likely existence of genetic susceptibility factors. Indeed, a 2011 meta-analysis of six twin studies reported Crohn's disease concordance rates of 30.3% for monozygotic and 3.6% for dizygotic twins[253].

In 2001, three different groups independently identified mutations in the NOD2/CARD15 gene among Crohn's patients using classical genetic linkage analyses[254-256]. In 2004, DLG5 was identified as a second gene linked to the disease[257].

Two years later, AGR2 was the first susceptibility gene to be linked specifically to ulcerative colitis[258]. Since then, over 240 genetic risk loci have been identified for IBD using genome-wide association studies, next-generation sequencing, and other methods. About 30 of these loci are linked to both ulcerative colitis and Crohn's disease[259] (fig. 17). Of these, the Th17 cytokine IL-23 and other components of this signal transduction pathway (IL-23R, IL-12B, STAT3, JAK2, TYK2) represent typical susceptibility genes for Crohn's disease and ulcerative colitis, suggesting a key role for maintenance of immune homeostasis in the GI tract[260].

Single-nucleotide polymorphisms (SNPs) in the autophagy gene ATG16L1[261] and in the NOD2/CARD15 gene are primarily linked with classical Crohn's terminal ileitis. In contrast, IL-10 and IL-17REL are thought to be susceptibility genes for ulcerative colitis[262]. Mutations in the genes encoding for epithelial barrier proteins (ECM1, CDH1, HNF4A, LAMB1, GNA12) also appear to be primarily associated with ulcerative colitis[260].

Nonetheless, studies on the genotype-phenotype associations for these genes have been unsuccessful to date, save for a few exceptions. For example, mutations in the NOD2/CARD15 gene are associated with terminal ileal involvement in Crohn's disease and more than two mutations are associated with a much higher risk of complications[263]. Meanwhile, mutations in HLA DRB1*0103 are associated with extensive spread, and usually with severe cases, of ulcerative colitis[264].

It remains unclear whether genetic testing will allow prediction of treatment response in IBD – as

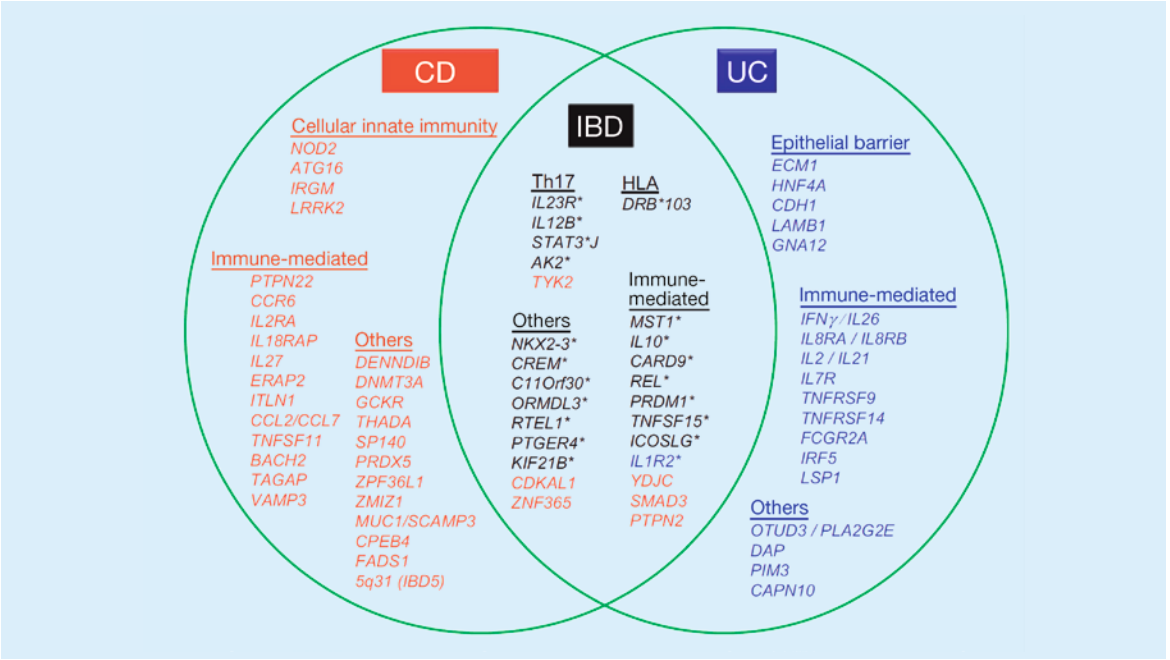


Fig. 17:
Susceptibility loci for Crohn's disease (CD) and ulcerative colitis (UC) (from Lees et al.[260])

is the case for hepatitis C (genotype 1), for which a polymorphism in the IL28B gene correlates with durable viral clearance[265].

A genome-wide association study on IBD subphenotypes published in 2016 that incorporated data from 16,902 ulcerative colitis and 12,597 Crohn's disease patients from 16 countries around the world found no significant links with disease outcome or complications. However, a strong correlation was observed between early age of initial onset and three loci (3p21 [MST1], NOD2, and HLA). Moreover, there was high concordance between NOD2 and ileal involvement (but not colonic involvement or high severity/complications, as had been previously assumed). The most important clinical insight from this study was the fact that it was no longer possible to sub-classify IBD in a binary fashion between Crohn's disease and ulcerative colitis, but rather that the disease represents a continuous spectrum with ulcerative colitis and ileal Crohn's disease at opposite poles[266].

IX Markers of potential malignant transformation

The risk of cancer is only slightly elevated in IBD patients. When malignant transformations do occur, they are most common in the gut itself or in the hepatobiliary system, although they can also arise in other organ systems such as the lymphatic system (lymphoma). Nonetheless, cancer does contribute to the mortality associated with IBD.

Is it possible to detect malignant transformation by laboratory tests?

Since conventional tumor markers such as CEA are often elevated in a non-specific manner due to the mucosal inflammation in IBD, they cannot be used to detect dysplastic lesions in the mucosa.

Hence, assessing this risk will continue to require monitoring by chromoendoscopy and biopsy in order to rule out low-grade or high-grade dysplasia by conventional histology.

Nonetheless, more modern techniques allow specific biomarkers such as the $p14^{ARF}$ gene[267] to be detected by molecular biology in bioptic or surgical specimens. This raises hopes that dysplastic changes may be recognized relatively early even in areas of the mucosa that appear endoscopically unremarkable.

Qualitative analysis of the mucin composition of goblet cells may also gain importance in the detection of malignant transformations. In this situation, the content of molecules called sulfomucins and the percentage of O-acetylated sialomucin is altered in cells adjacent to dysplasias[268]. To date, qualitative measurement of these molecules has been accomplished by immunohistochemistry on paraffin sections of biopsies. No laboratory test is currently available.

X References

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