The 12th EFLM Continuous Postgraduate Course in Clinical Chemistry

Under the Auspices of IFCC

NEW TRENDS IN CLASSIFICATION, MONITORING AND MANAGEMENT OF GASTROINTESTINAL DISEASES

Handbook

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Table of contents

Editorial

The 12th EFLM (former EFCC) Continuous Postgraduate Course in Clinical Chemistry: New Trends in Classification, Diagnosis and Management of Gastrointestinal Diseases

The Croatian Society of Medical Biochemists and Slovenian Association for Clinical Chemistry, together with the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM), organized in 2012 the 12th postgraduate weekend course entitled 'New Trends in Classification, Diagnosis and Management of Gastrointestinal diseases (GID)'. The Course is an advance study course in the frame of the Inter-University Centre (IUC) studies Dubrovnik promoting continuing postgraduate education of professionals also in clinical chemistry and laboratory medicine. The diploma of the IUC is recognized world wide. The Course is held under the auspices of Internal Federation of Clinical Chemistry and Laboratory Medicine (IFCC).

In this book the state-of-the-art on gastrointestinal classification and epidemiology as well as new approach to diagnosis and management of GID is presented by well-known experts. These renowned experts in different fields have tried to cover the clinical and laboratory aspects of gastrointestinal diseases with the accent to gastrointestinal nutrition-related disease, gastrointestinal disease in children, chronic gastrointestinal diseases and gastrointestinal oncology.

Integrated knowledge of the authors and the material prepared by these experts is intended to provide updated information of supreme quality to the reader interested in the field. The book contains two articles published in the Journal Biochemia Medica by generous permission of the Editor-in-Chief and Publisher of the journal.

Elizabeta Topić

Zagreb, November 2012

1. CLASSIFICATION AND EPIDEMIOLOGY OF GASTROINTESTINAL DISEASES

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1.1 INTRODUCTION

The gastrointestinal tract literally denotes stomach and intestine, but the term is usually used to describe the alimentary tract, which includes all organs from the mouth to the anus, with the main function of intake, processing and absorption of food. In an adult male, the gastrointestinal (GI) tract is 5 meters (20 ft) long, or up to 9 meters (30 ft) without the effect of muscle tone, and consists of the upper and lower GI tracts, with the border being set at the ligament of Treitz in the duodenum. The esophagus, stomach, small and large intestine are the main focus of gastroenterology. The digestive system is a broader term that includes other (accessory) organs involved in the digestion of food – the liver, the gallbladder, bile ducts and the pancreas.
The classification of GI diseases usually follows the anatomical distribution, so we usually consider every organ as an entity with a number of diseases of different etiology, i.e. inflammatory or autoimmune disorders, motility disorders, infectious diseases or tumors. Certain diseases are usually confined to and specific to an organ, such as peptic ulcer disease for the stomach and duodenum, or cirrhosis for the liver. The most common disorders of the digestive system will be reviewed here.

1.2 ESOPHAGUS

Esophageal hiatal hernia can be congenital, but is usually acquired. It is present when the stomach wall, at its junction with the esophagus, is dislocated 2 cm or more above the diaphragmatic hiatus. Radiologic surveys from Western countries found high prevalence of hiatal hernia (12%-69%, depending on age), as compared with the very low prevalence in Africa and Asia (1%-5%). Although the hiatal hernia itself rarely causes symptoms directly, it is the anatomic companion of all the major complications of gastroesophageal reflux.
Gastroesophageal reflux disease (GERD) is one of the major acid peptic diseases. According to the Montreal definition and classification, GERD is a condition which develops when the reflux of stomach contents causes troublesome symptoms and/or complications (1). Symptoms and signs of tissue injury can be present within the esophagus, oropharynx, larynx and respiratory tract, the later representing the extraesophageal manifestations of GERD. The prevalence of the disease differs depending on whether the analysis is based on symptoms or signs of disease. Approximately 20–40% of the adult population in Western countries suffers from heartburn and/or regurgitation, the characteristic symptoms of GERD (2), and over 45% of those who consult their physician in these regions because of reflux symptoms may have reflux esophagitis (3). Based on the presence of weekly reflux symptoms, the population prevalence of GERD has been reported to be 10–20% in Western countries and typically below 5% in Asia (4). The diagnosis is usually established by the presence of either recurrent symptoms (heartburn and/or regurgitation) or altered epithelial morphology, usually

visualized endoscopically or histologically. Although GERD is rarely a cause of death, it is associated with considerable morbidity as a result of its propensity to produce complications such as Barrett esophagus (8-20%), esophageal ulceration (5%), stricture formation (4-20%) and gastrointestinal hemorrhage (<1%). Barrett esophagus is defined as the presence of a simple columnar epithelium lining the lower esophagus and replacing the damaged stratified squamous epithelium. It was once considered an uncommon condition, but estimates of its frequency at autopsy (1/57 to 1/105 cases), on general endoscopic surveys (1/100 cases) and on endoscopic surveys in GERD patients (10/100 to 15/100 cases), indicate that it is not uncommon. It is principally a disorder of white men, with prevalence increasing with age, paralleling that of reflux esophagitis. The significance of Barrett esophagus lies in the 30- to 125-fold increased risk for the development of esophageal adenocarcinoma compared to the general population.

The two most common **esophageal malignancies** are squamous cell carcinoma and adenocarcinoma. Other epithelial tumors, benign (squamous papilloma, adenoma) and malignant (adenosquamous carcinoma, adenoid cystic carcinoma, neuroendocrine tumors, melanoma, etc.), and nonepithelial benign (leiomyoma, hemangioma, lymphangioma, fibroma, lipoma, etc.) and malignant (leiomyosarcoma, rhabdosarcoma, metastatic carcinoma, lymphoma, etc.) are rare. Representing the most common form of esophageal malignancy worldwide, esophageal squamous cell carcinoma is one of the leading causes of cancer mortality in men. Its incidence varies greatly according to geographic location, and globally ranges from 2.5 to 5.0 for men and 1.5 to 2.5 for women per 100 000 population. However, there are some high incidence regions, where rates may exceed 100 per 100 000 population, which include northern China, India, northern Iran, areas around Caspian Sea and the Transkei area of South Africa. The geographical variation in esophageal squamous cell carcinoma strongly hints at the contribution of environmental factors, although the genetic basis has also started to be elucidated. On the other hand, esophageal adenocarcinoma affects mostly Caucasians, with a male-female predominance of 3-5,5 : 1. The incidence typically begins to increase after the age of 40 years, and rises with each decade. The annual age-adjusted incidence rates of adenocarcinoma of the esophagus and gastric cardia in Caucasian men is 1.3 and 2.8 per 100.000, respectively. Once considered rare disease, the incidence of esophageal adenocarcinoma (and adenocarcinoma of the gastric cardia) has increased at a rate of 4% to 10% annually in different regions of the United States. This rapid rate of increase in the incidence is greater than that of any other cancer, but the reason for this increase is unknown. However, Barrett esophagus is recognized as an important precursor to esophageal adenocarcinoma. The incidence of adenocarcinoma arising in Barrett esophagus varies from 1 cancer per 55 patient years to 1 cancer per 441 patient years, or about 500 cases per 100.000, a figure greater than that for esophageal squamous cell carcinoma in high-risk areas of the world. Unfortunately, both esophageal cancers usually present themselves at advanced stages, when therapeutic options are limited. The overall 5-year survival rates are approximately 5% for squamous cell carcinoma and 7% for adenocarcinoma.

Achalasia is the most easily recognized motor disorder of the esophagus, characterized by failure of the lower esophageal sphincter to relax completely with swallowing, and aperistalsis in the smooth muscle esophagus. It is a rare disease in the Europe and the United States with an estimated incidence of 1/100 000 population per year and prevalence from 7.1 to 13.4/100 000, and is even rarer in Africa. Other peristaltic disorders include diffuse esophageal spasm, nutcracker esophagus, hypertensive lower esophageal sphincter and nonspecific esophageal motor disorders.

Primary esophageal infections are rare in an otherwise normal person in whom no permissive factor is present. In this setting, the most common pathogens are herpes simplex virus and

Candida species. Bacterial infections are rare and found almost exclusively in patients with hematologic malignancies and severe granulocytopenia. Mycobacterial involvement is uncommon, and protozoal infections occur almost exclusively in patients with AIDS. Unusual causes of esophagitis include corrosive agents, drugs and radiation. Esophagus can be affected by a number of systemic and dermatologic diseases, such as sarcoidosis, Crohn's disease, pemphigus vulgaris, and pehmphigoid and epidermolysis bullosa dystrophica.

1.3 STOMACH AND DUODENUM

Although the duodenum is anatomically part of the small intestine, it's pathology is closely related to the stomach, and they are sometimes referred to as the gastroduodenum.

Peptic ulcer disease (or acid peptic disorders) represents one of the most common pathologies in the GI tract with an enormous impact on the cost of health care. Data from the United States show that approximately 500 000 new cases and 4 million recurrences of gastric and duodenal ulcer occur every year, with the direct and indirect costs being estimated at 5-10 billion dollars each. The data for the incidence and prevalence are not very accurate, but some studies show the prevalence of active gastric and duodenal ulcer in the United States is approximately 1.8%, while the lifetime prevalence of peptic ulcer ranges from approximately 11-14% in men and 8-11% in women. Japanese male office workers older than 40 years of age have a 4.3% prevalence of duodenal ulcer, while in the Norwegian population between 20 and 49 years of age the annual incidence of duodenal ulcer was approximately 2 in 1000 men and 0.9 in 1000 women, with similar annual rates for gastric ulcer in both sexes. Two major epidemiologic observations have dramatically changed the manner in which we approach peptic ulcer disease. First is the landmark association of peptic ulcer disease with Helicobacter pylori infection, which leads us to consider the peptic ulcer as an infectious disease that needs to be treated with antibiotics. Second is the clear relationship between mucosal damage and ingestion of nonsteroid antiinflammatory drugs (NSAIDs) which instituted the need for preventive measures. Still, the mortality rates for duodenal and gastric ulcers decreased only modestly from 1976 to 1986, with gastric ulcer having higher mortality rate than duodenal ulcer. Surveys in the United States and Europe have documented a substantial increase in hospitalizations of elderly patients for ulcer-related complications (bleeding and perforation).The incidence of duodenal ulcer peaked between 1950 and 1970, decreased gradually until 1980, and stabilized thereafter. During the past 30 years, the death rates, surgical rates, and physician visits for ulcer has decreased by more than 50%, primarily because of decreased rates of ulcers among men.

Gastritis and gastropathy represent common problems in everyday practice. Gastritis, meaning inflammation of the gastric mucosa, can be caused by infectious agents (H. pylori being the principal causative agent), drugs, and autoimmune and hypersensitivity reactions. Gastropathy, as an epithelial/endothelial damage and regeneration, is caused by drugs (e.g. NSAIDs, alcohol), as well as by bile reflux, stress, hypovolemia, and chronic congestion. In western societies, gastritis is uncommon in childhood but increases in frequency with increasing age, reaching a prevalence of 50-60% by 50 years of age.

Although a dramatic decrease in the incidence of stomach cancer has been observed worldwide over the past 30-50 years, it is still considered the second most frequent cancer worldwide, accounting for approximately 9.9% of cancers. The distribution of the disease throughout the population is not uniform. It has predilection for men, occurring up to twice more frequently than in women. Its incidence increases with age, and shows great variation in the relative incidence through different geographical regions, with Japan, Costa Rica and San

Marino having the highest incidence (more than 8-10-fold higher compared to some other parts of the world). The stomach is a major site of cancer incidence in portions of Central and Eastern Europe, the former Soviet Union, Chile and China. Migrants from high- to low-incidence nations maintain ther susceptibility to gastric cancer, while the risk for their offspring approximates that of the new homeland. These finding suggest that an environmental exposure, probably beginning early in life, is related to the development of gastric cancer, with dietary carcinogens considered the most likely factors. Helicobacter pylori infection may also play a role.

Primary gastric lymphomas comprise less than 5% of all gastric malignancies, but make up the largest group after adenocarcinoma. Other tumors (carcinoid, lipomas, gastrointestinal stromal cell tumors) are rare. Gastric polyps are uncommon, occurring in less than 1% of autopsies or other surveys. They are almost always asymptomatic, but are of interest primarily because of their risk as potentially premalignant lesions.

Functional (or nonulcerous) dyspepsia is one of the most prevalent syndromes evaluated by gastroenterologists and is characterized by nonspecific upper abdominal symptoms in the absence of demonstrable organic disease. Several subtypes of functional dyspepsia have been defined, and disturbances of gastric and small intestinal motor activity are common in this entity (7). More serious disorders of gastric emptying include diabetic gastroparesis, postoperative, radiation-induced, and idiopathic and rarely other forms of gastroparesis.

1.4 INTESTINE

Since most of the intestinal disease are affecting simultaneously small and large bowel their classification and epidemiological characteristics will be reviewed as a one entity and ones who are organ specified will be elaborated separately. Absorption is a process that is carried on only in intestine therefore its impairment will be presented here.

Malabsorption refers to impaired absorption of nutrients (fat, protein, carbohydrate, vitamins and minerals) that are result of congenital defects in transport through small intestinal epithelium (primary) or in most of the cases loss of large viable part of a small intestine epithelium (secondary). It can be global when all the nutrients are deficient or isolated with impaired absorption of specific nutrient. Variety of disease can cause this syndrome and most common are gastric diseases (autoimmune gastritis, gastric resections), pancreatic insufficiency (chronic pancreatitis, cystic fibrosis, eg), obstructive biliary diseases, intestinal diseases (celiac and inflammatory bowel disease), short bowel syndrome and others. Clinical manifestations are diverse due to multifactorial etiology and consequent diverse nutrient malabsorption, but it is most commonly presented with diarrhea and weight loss. True prevalence is unknown because most of the patients are undiagnosed and the suspicion is made in late stage when patients need parenetral nutrition.

Inflammatory bowel diseases (IBDs), comprised of ulcerative colitis (UC) and Crohn's disease (CD), are characterized by chronic inflammation of the gastrointestinal tract in genetically susceptible individuals exposed to certain environmental risk factors. Crohn’s disease is characterized by transmural inflammation with skip lesions that can involve any segment of the GI tract from the mouth to the anus, and UC is determined by mucosal inflammation of colon. Ten to fifteen percent of patients cannot be classified into either type of disease and they are labeled as indeterminate colitis. Three mechanisms are involved in pathogenesis of IBD genetic predisposition, dysregulated immune response and an altered response to gut microorganisms. In favor of genetic predisposition says that first-degree relatives have a 5 to 20 fold increased risk of developing IBD and a child of a parent with

IBD has a 5% risk. In the United States, it is estimated that 2 million people have IBD, with an incidence of 70-150 cases per 100 000, prevalence of 396 cases per 100 000 annually (7). Although IBD can occur in any age two peaks exists, with the vast majority of new diagnoses made in people aged 15-40 years and smaller peak in patients aged 55-65 years. Ethnic and racial difference exists, so the incidence of IBD is lower in black and Hispanic populations compared to whites. Developed countries, colder-climate regions are areas of higher incidence of IBD. The highest prevalence of IBD worldwide was reported in Canada (20.2 per 100 000) and Europe, whereas Asia had the lowest prevalence. Nowadays incidence of IBD is increasing in Asian people, especially among first-generation children due to their emigration to western countries indicating a big role of environmental factors. Earlier, the incidence of Crohn's disease was several times lower than the one of UC but nowadays the difference is lower due to increasement of number of patients with CD and stable number of UC. The prevalence of CD in the United States is approximately 7 cases per 100 000 and of UC 35-100 cases per 100 000. Both have a bimodal distribution of age onset. In CD patients the first peak, in larger proportion of the patients, occurs between age 15 and 30 years, and the second peak occurs between age 60 and 80 years. In UC first peak is also in majority of the patients, at 15-25 years and a second one at 55-65 years. They are both more common in whites and in female gender. The lowest recorded rates of new cases of CD appear to be in South Africa and Latin America. One of the most adverse complications of IBD is toxic megacolon accounting approximately 1 to 5%.

Celiac sprue, also known as celiac disease or gluten-sensitive enteropathy, is intolerance to gliadin, the fraction of gluten, which is found in wheat, rye, and barley (9). Intrafamilial occurrence is frequent especially in first-degree relatives, approximately 10%. Now, estimations are that 1% of population, approximately 6 million people, is affected. The highest prevalence of celiac sprue 1 in 100 persons is in Ireland and Finland. The incidence of celiac sprue is increasing among certain populations in Africa, Asia (India), and the Middle East. Its incidence is slightly higher in females than in males and the age distribution has two peaks; first at 8-12 months, when gluten ingestion in infants begin, and the second in the third to fourth decade.

Infectious diarrheal diseases are increased in last two decades representing one of the five leading cause of death in general population, especially in developing countries where is the second cause of death in children. According to the duration of disease we can divide diarrhea into two groups: chronic and acute. Most cases of acute diarrhea are self-limited and are caused by bacteria (salmonella, campylobacter, shigella, enterotoxigenic E. coli, C. difficile, and others), viruses (rotavirus, adenoviruses, norovirus, and others), and protozoa (cryptosporidium, giardia, entamoeba, and others). Prevalence is impossible to establish because most of the patients do not seek medical help neither do physicians perform tests to affirm the pathogen. Prolonged bacterial infections of intestine usually occur in patients who recently traveled, ones with HIV infection, and after use of antibiotics. Bacterial usually responsible for chronic diarrhea are C. difficile, Aeromonas, Plesiomonas, Campylobacter, Giardia, Amebae, Cryptosporidium, Whipple's disease, and Cyclospora and others.

The diagnosis of small bowel tumors is often at a late state due to their variety in clinical manifestation and rarity. As of any origin, tumors of small bowel can be both malignant (adenocarcinoma, carcinoid, lymphoma, and sarcomas) and benign (adenoma, leiomyoma, lipoma). Small bowel tumors account for only 3 percent of all gastrointestinal tract neoplasm, but their age-adjusted incidence has risen from 1-3 per 100 000 to 14.8 per 100 000. Three major types of benign small bowel adenomas exist: villous, tubular, and Brunner's gland adenomas. Villous adenomas carry a significant potential for malignant transformation with the predilection in duodenum around the papilla (Ampulla of Vater). Patients with

adenocarcinoma of small intestine have an increased risk of large bowel adenocarcinomas and vice versa implying similar genetic and environmental factors involved in carcinogenesis. Males are somewhat more affected (male to female ratio of 1.5:1) and age at diagnosis depends on a type of tumor. Heritable cancer syndromes are associated with adenocarcinoma of both the large and small bowel, including hereditary non-polyposis colorectal cancer (HNPCC), familial adenomatous polyposis (FAP), and Peutz-Jeghers syndrome. Patients with adenocarcinoma and carcinoid are slightly older (67 to 68) compared to ones with sarcoma and lymphoma (60 to 62). Incidence of adenocarcinoma decreases throughout small intestine and carcinoid arises with predominant site in ileum (10). Patients with Crohn's disease are exception with predominant location of the adenocarcinoma in terminal ileum. Carcinoids represent approximately 40 percent of primary small intestinal malignancies. They have been reported in patients from 20 to 80 years old. The most common is a gastrinoma of the duodenum, which is defined by the associated clinical syndrome of excess gastrin secretion (Zollinger-Ellison syndrome). Gastrointestinal stromal tumors (GISTs) are most common in jejunum and ileum and account 10 percent of small bowel neoplasms.

Benign tumors of the large bowel can be divided on non-neoplastic (hyperplastic, mucosal, inflammatory psuedopolyps and submucosal) and neoplastic (serrated, hamartomatous and adenomatous) polyps. Most common non-neoplastic polyps are hyperplastic polyps that are usually small and their exact prevalence is not established because these are found usually accidental. In colonic examinations of asymptomatic patients over age 50 years, prevalence is 10%, but on sygmoidoscopy is 28%. Hyperplastic polyps predominate in rectum and sigmoid. Adenomatous polyps are divided into three groups based on histological characteristics (tubular, vilous and tubulovillosus), size and degree of dyplasia (mild, moderate, and severe). Tubular adenomas are small and exhibit mild dysplasia, and the most common of adenomatous polyps, account for 80 to 86%. Tubulovillous account for 8 to 16% and villous adenomas for 3 to 16% of adenomatous polyps. In countries with high prevalence of colon cancer adenomas tend to be larger with age. The prevalence of adenomatous polyps is determinate by four major factors; inherited risk, age, gender and positive family history. In population with high risk, such as Japan, prevalence is 30 to 40% and in low risk population account for 12%. Due to screening programs prevalence of adenomatous polyps have arisen, so in patients aged 50 years prevalence ranges from 24 to 47 % with predominance in males (1.5 is a relative risk). Race isn't likely to be independent factor.

Colorectal cancer (CRC) is the third most common cancer in males and second in females. They evolve due to progression of premalignant lesion of colon (adenomas). In 2003, WHO reported an impressive number of 940 000 persons being diagnosed with CRC in general population and 492 000 died from it. Risk factor include heredity, environmental exposures (smoking, obesity and sedentary habits), and inflammatory syndromes (ulcerative colitis and Crohn's disease) affecting gastrointestinal tract. Seventy percent of cases arise in the colon. The highest incidence rates are in Australia and New Zealand, Europe and North America, and the lowest rates are found in in Africa and South-Central Asia (11). Incidence is territory depended therefore, in developed country is stable, in United States has declined and in Eastern Asia and Europe has risen. CRC incidence increases significantly after age of 40 years. Incidence is 25 % higher in men than in females and 20 % higher in blacks than in whites. Incidence has declined about 2 to 3 percent per year most likely as a consequence of good screening programs and also a shift toward right-sided cancers has been observed. The 5-year survival rate of CRC accounts 61 %.

Irritable bowel syndrome (IBS) is the most commonly diagnosed gastrointestinal characterized by chronic abdominal pain and altered bowel habits in the absence of any organic cause. Prevalence in North America and in Europe is similar around 11 %. Although

it can affect people of every age, gender and race it is more often diagnosed in young patients especially females (12). It is a functional disorder, commonly causing mild to moderate symptoms and therefore only 15 percent seek medical attention. Prevalence is hard to estimate because large number of patients is undiagnosed. The severity of a problem indicates the fact that 25 to 50% referrals to gastroenterologist are due to IBS symptoms is the second highest cause of work absenteeism.

Small intestine diverticula are multiple saclike mucosal herniation that bulge outward through weak spots of intestine and are five times more common in duodenum than in the rest of the small intestine. They are usually incidenatalomas so the true prevalence is unknown. Due to pathopathology logically their incidence arises with age therefore, 65 % patients are older than 85 years at the time of diagnosis. Although diverticula of colon are commonly left-sided in Asian people 75 % of cases are right-sided. Diverticulitis, an inflammation of the diverticula, is considered to be disease of elderly no meter of the gender. It is estimated that 65% older than 85 years have diverticula. Diverticulitis is characterized by recurrence (20-25% recurrence rate) and evolution of fistulas and adhesions. Studies has shown that patients with more diverticula have more often the inflammation; in 15-20 % of those. Since lifestyle and dietary modifications are one of the reasons for occurrence of diverticula and subsequent inflammation logically is more prevalent in Western countries.

Appendicitis is an inflammation of the inner lining of the appendix and is one of the most common causes of acute abdomen usually ending by urgent surgical procedure. It affects 7 % of the US population with incidence 233 per 100 000 (13). It is greater in Western countries and in men (male to female ratio 1.4:1). In pediatric population occurs more likely in 6-10 years because of lymphoid hyperplasia and later in the 10 to 19 year-old age group.

1.5 LIVER

There are many causes of liver diseases, from hereditary, infectious, autoimmune, malignant to drug induced that represent with a different pattern, but all can be categorized either as hepatocellular od cholestatic (obstructive). In cholestatic diseases (such as primary biliary cirrhosis, drug induced diseases, and malignant obstructions) predominate the features of inhibition of bile flow, and in hepatocellular (such as viral hepatits and alcoholic liver disease) predominate the features of liver injury, inflammation and necrosis. Disorders that are associated with hereditary defects in bilirubin metabolism are Crigler-Najjar syndrome, Gilbert's syndrome, Dubin-Johnson sydnrome, Rotor sydnrome, benign recurrent intrahepatic cholestasis and progressive familial intrahepatic cholestasis. Hepatitis, an inflammation of the liver parenchyma may result from various causes, both infectious (viral, bacterial, fungal, and parasitic organisms) and noninfectious (alcohol, drugs, autoimmune diseases, and metabolic diseases).

Acute viral hepatitis is a systemic infection that predominately affects the liver and is caused by many different viruses but in the majority of cases is caused by hepatitis virus A, B and C virus. Although all viruses have an acute form, in the clinical setting more important is the incidence and prevalence of the chronic hepatitis. Approximately 90-95% of neonates with acute hepatitis B (HBV) infection and 5% of adults with acute infection develop chronic HBV infection. According to the World Health Organization, HBV is the 10th leading cause of death worldwide (14). Around 350 million people have a chronic infection with hepatitis C virus (HCV), and about 20% of them will eventually develop HCV-related cirrhosis or hepatocellular carcinoma (HCC). HCV represents the most frequent cause of parenteral non-A, non-B (NANB) hepatitis worldwide. Its global prevalence in nations around the world is about 0.5-2%. The highest rates of prevalence are found in patients with hemophilia and in

intravenous drug users. Hepatitis D virus is found as a coinfeciton in 5% of the HBsAg positive, and hepatitis E is mostly present in nonurban settings and mostly in children. Prognosis of the viral hepatitis depends on the causative virus. Patients with chronic C and B infection have a risk of developing cirrhosis and eventually HCC.

More than 900 drugs and medications in general have been reported to cause liver injury, and drugs account for 20-40% of all instances of fulminate hepatic failure. Approximately 75% of the idiosyncratic drug reactions result in liver transplantation or death. The prognosis of the disease progression is highly variable depending on the patient's presentation and stage of liver damage.

Autoimmune hepatitis is a chronic disease of unknown cause, characterized by continuing hepatocellular inflammation and necrosis and tends to progress to cirrhosis. The diagnosis is based on present immune serum markers, liver specific and non liver specific autoantibodies and is often associated with other autoimmune diseases. The prevalence of autoimmune hepatitis is estimated to be 0.1-1.2 cases per 100,000 individuals in Western Europe. The reported prevalence of autoimmune hepatitis in Europe ranges from 11.6-16.9 cases per 100,000 persons. Autoimmune hepatitis accounts for about 3% of liver transplantations in Europe. Women are often more affected that men (in 80% of cases), and the disease has a bimodal age distribution with the first peak at 10-20 years of age and a second at 45-70. Approximately 50% of affected are younger than 20. Young age at presentation, type II autoimmune hepatitis, coagulopathy and severe initial histologic activity are factors associated with a worse prognosis, but in the end the prognosis depends primarily on the severity of liver inflammation.

Patients with alcoholic liver disease (ALD) can be divided into ones that have alcoholic fatty liver, alcoholic hepatitis, and alcohol related cirrhosis (15). Women develop more severe ALD more quickly and at lower doses of alcohol than men do. The prevalence of alcoholic hepatitis was found to be approximately 25-30%, although the true prevalence is unknown because patients with milder forms can be asymptomatic or never seek medical attention. The long-term prognosis of individuals with alcoholic hepatitis depends heavily on whether patients have established cirrhosis and whether they continue to drink, patients who have had a major complication of cirrhosis have a 5-year survival of less than 50%. Alcoholic hepatitis now represents a leading indication for liver transplantation (16).

Primary biliary cirrhosis (PBC) is a chronic and progressive cholestatic disease of the liver. The etiology is unknown, although it is presumed to be autoimmune in nature. Epidemiology has not been studied systematically, it is reported to be more prevalent in the Northern countries with a prevalence ranging from 2.2 - 24 cases per 100,000. About 70-90% of patients are women in their middle age with a mean of 39 years, but men are more likely to develop hepatocellular carcinoma.

Metabolic diseases that affect the liver are hepatic steatosis and nonalcoholic steatohepatitis, storage diseases such as Wilson’s disease, hemochromatosis, alpha-1 antitripsin deficiency and others. Fatty liver represents the accumulation of fat in the liver cells (17). In some patients, fatty liver may be accompanied by hepatic inflammation and liver cell death (steatohepatitis). Steatosis affects approximately 25-35% of the general population and NASH has been detected in 1.2-9% of patients undergoing routine liver biopsy (18). NAFLD is found in more than 80% of patients who are obese. Fatty liver occurs in all age groups and it has been found across all races, but NAFLD is most common in whites. Fibrosis or cirrhosis in the liver is present in 15-50% of patients with NASH. Approximately 30% of patients with fibrosis develop cirrhosis after 10 years.

Wilson disease is a rare autosomal recessive inherited disorder of copper metabolism. The condition is characterized by excessive deposition of copper in the liver, brain, and other tissues (19). The incidence of Wilson disease is 10-30 per million cases. The peak of the disease is in the age group from 5 to 40, although the disorder has been detected in children younger than 3 years and in adults older than 70 years (20). The major complications in patients with untreated Wilson disease are those associated with acute liver failure, chronic hepatic dysfunction with either portal hypertension or hepatocellular carcinoma.

Hemochromatosis is the most common genetic disorder of humans and it involves accumulation of abnormal amounts of iron due to inappropriate absorption from the intestine (21). Although genetic mutations for hemochromatosis are common, clinical manifestation are present in 40-50% of carriers.

Cirrhosis represents the final common histologic pathway for a wide variety of chronic liver diseases. Cirrhosis is defined histologically as a diffuse hepatic process characterized by fibrosis and the conversion of normal liver architecture into structurally abnormal nodules. The progression of liver injury to cirrhosis may occur over weeks to years. Cirrhosis is the ninth leading cause of death in the United States and is responsible for 1.2% of all US deaths. Many patients die from the disease in their fifth or sixth decade of life. Complications of liver cirrhosis include hepatic fibrosis and subsequently portal hypertension, ascites, hepatorenal syndrome, hepatic encophalopathy, manifestations related to the chronic disease that caused the cirrhosis, hematologic manifestation, hepatopulmonary syndrome and in the end hepatocelular and cholangiocarcinoma.

Simple cysts, multiple *cysts* arising in the setting of polycystic liver disease (PCLD), parasitic or hydatid (echinococcal) cysts, cystic tumors, and abscesses represent the cystic lesions of liver. The liver cysts have been estimated to occur in 5% of the population, and only 10-15% of these patients have symptoms that bring the cyst to clinical attention.

Tumors of the liver can be divided into benign and malignant carcinomas of the liver. Hepatocellular adenomas or hepatic adenomas are rare and benign tumors. They are mostly found in women in their third and fourth decade and are often associated with the use of contraceptive pills and estrogens in general (22). Focal nodular hyperplasia (FNH) is the second most common tumor of the liver (23). It occurs predominately in women, and it is often incidentally found in the ultrasound procedure (24). Hemangioma is the most common benign tumor affecting the liver; they are composed of atypical or irregular blood vessels (25). Women are affected more that men in a ratio of 4-6:1, they can occur at all ages, but are mostly diagnosed in the age between 30 and 50. The reported incidence rate of hepatic hemangiomas is approximately 2%, and the prevalence ranges from 0,5% to 7%.

Hepatocellular carcinoma is with 500 000 people affected at present the third leading cause of cancer deaths and it usually arises in the setting of a cirrhotic liver. The incidence of hepatocellular carcinoma is highest in eastern countries and Africa, where there is an endemic high prevalence of hepatitis B and hepatitis C. There has been noted worldwide that the incidence is twice as great in the developing nations in comparison to those developed, globally ranges of incidence is 7 to 17 per 100 000 in men and 2 to 6 per 100 000 in women. Over the past 20 years incidence has doubled, what can be associated with the epidemic of hepatitis C and alcoholic liver disease which subsequently results in more severe chronic liver disease. The mortality rate, following the incidence rate has similarly increased from 2.8 to 4.7 per 100,000 populations over the past 5 year (26). Metastatic tumors of the liver are common and are at least 20 times greater in incidence in comparison to hepatocellular carcinoma. They are found in 30-50% of autopsys of patients with malignant diseases. All

tumors except those primarily in the brain can metastasize in the liver. Most common are those of the gastrointestinal tract, lung, breast and melanomas.

1.6 THE GALLBLADDER AND BILE DUCTS

Cholelithiasis is the medical term for gallstone disease. Gallstones are concretions that form in the biliary tract, usually in the gallbladder. Migration of a a gallstone into the opening of the cystic duct may block the outflow of bile during gallbladder contraction and this can result in a characteristic type of pain known as a billiary colic. Cystic duct obstruction which persists for a couple of hours can lead to acute gallbladder inflammation, also known as acute cholecystitis. Choledocholithiasis is a term that refers to the presence of one or more gallstones in the common bile duct and this usually occurs when a gallstone passes from the gallbladder into the common bile duct and remains there. If the stagnation of the bile pertains the bile can get infected and bacteria can spread up the ductal system and cause inflammation of the bile duct called cholangitis. If gallstones are present for a longer period of time the irritation of the gallbladder wall can produce chronic cholecystitis and fibrosis that can be a trigger for formation of gallbladder cancer- cholangiocarcinoma.

In the United States, about 20 million people (10-20% of adults) have gallstones. In Europe prevalence of gallstones is highest in people in northern countries (27). It is approximated that every year 1-3% of people develop gallstones and about 1-3% become symptomatic. Each year, around 500,000 people develop symptoms or complications of gallstones that require surgical therapy. Women in their reproductive period are 2-3 times more likely to develop cholesterol gallstones than men, what is related to the estrogen levels (28). Risk of developing gallstones increases with age. Regarding mortality, gallstone disease is responsible for about 10,000 deaths per year in the United States and about 70% are attributable to acute gallstone complications.

Approximately 10-15% of patients will have an associated choledocholithiasis. Complications that are associated with this state are obstructive jaundice, acute pancreatitis and cholangitis. Cholangitis has a significant mortality and morbidity, especially if left untreated, with mortality rates that vary from 13-88%.

Acute cholecystitis is in 90% of cases associated with stones in the cystic duct (calculous cholecystitis), and the other 10% of cases represent acalculous cholecystitis (29). Risk factors for cholecystitis are similar to those for cholelithiasis and include increasing age, female sex, obesity or rapid weight loss, drugs, and pregnancy. Acalculous cholecystitis is related to biliary stasis, major surgery, severe trauma, sepsis, long-term total parenteral nutrition (TPN), and prolonged fasting. Aproximately one third of patients who have cholelithiasis will develop in some point of time acute cholecystitis. Complications of acute cholecystitis include emphysematous cholecystitis, empyema and hydrops of the gallbladder, gangrene, perforation, fistula formation and gallstone ileus.

Chronic cholecystitis, as mentioned, represents a chronic inflammation of the gallbladder usually in the setting of choleithiasis. Under the term porcelain gallbladder we presume calcium salt deposition within walls of a chronically inflamed gallbladder, a state which is connected with a high percentage of carcinoma of the gallbladder.

Primary sclerosing cholangitis is a chronic liver disease characterized by a progressive course of cholestasis with inflammation and fibrosis of the intrahepatic and extrahepatic bile ducts. The disease is believed to be caused by an autoimmune reaction and can lead to

cirrhosis of the liver. Disease is in most cases associated with inflammatory bowel disease, mainly ulcerose colitis, and is often complicated by the development of cholangiocarcinoma. Prevalence is estimated to be 6.3 cases per 100,000, although Scandinavian countries report a somewhat higher rate. Approximately 70% of patients with PSC are men, with a mean age of diagnosis around 40 years.

Cancers of the biliary tract include cholangiocarcinoma, cancer of ampulla of Vater and gallbladder cancer. All subtypes are rare and have an overall poor prognosis). About 20% arise from the extrahepatic biliary tract and 20% arise from the ampulla of Vater. Gallbladder cancer is the fifth most common gastrointestinal cancer in the United States and the most common hepatobiliary cancer. It accounts for 46% of the biliary tract (30). Gallbladder cancer incidence increases with age and is more common in women. Most patients have regional disease or distant metastases at presentation. Therefore, the prognosis of gallbladder disease is poor, with 5-year survival rates of 15-20% (31).

Cholangiocarcinomas originate in the liver and extrahepatic bile ducts (32). They are divided 3 regions: intrahepatic, extrahepatic (perihilar) also known as Klatskin tumors, which are the most common, and distal extrahepatic. More than 95% of these tumors are ductal adenocarcinomas; many patients present with unresectable or metastatic disease. Incidence in most Western countries ranges from 2- 6 cases per 100,000 people per year. Females are more affected that men, and the prevalence is the highest in the population of 60-70 years of age.

1.7 PANCREAS

Acute pancreatitis is an inflammatory condition of the pancreas characterized clinically by abdominal pain and elevated levels of pancreatic enzymes in the blood (33). The reported annual incidence of acute pancreatitis ranges from 4.9 to 35 per 100,000 populations. The incidence of acute pancreatitis is increasing in many European and Scandinavian countries due to increased alcohol consumption and better diagnostic capability (34). Although the pathogenesis of acute pancreatitis is not fully understood, a number of conditions predispose to acute pancreatitis such as gallstones, billiary sludge and microlithiasis as well as other causes of mechanical ampulary obstruction, alcohol, hypertriglyceridemia, hypercalcemia, drugs, infections and toxins, trauma, pregnancy, anatomical variation of pancreas, hereditary pancreatitis, post ERCP and postoperative pancreatitis and structural abnormalities. Gallstones and chronic alcohol abuse account for approximately 75 percent of all acute pancreatitis cases. In a systematic review of studies of acute pancreatitis, overall mortality was approximately 5 percent, 3 percent, and 17 percent in all cases of acute pancreatitis, interstitial, and necrotizing pancreatitis, respectively (35).

Chronic pancreatitis (CP) is a syndrome that contrasts with acute pancreatitis although the two conditions can overlap. It involves progressive and irreversible inflammatory changes in the pancreas that result in permanent structural damage, which leads to destruction of exocrine (acinar cell) and endocrine (islet of Langerhas) tissue (36). Recurrent episodes of acute pancreatitis may lead to chronic pancreatitis over time. Based on autopsy studies, the prevalence of chronic pancreatitis ranges from 0.04% to 5% but this can be misleading since not all patients have had symptoms during life (37). The incidence of CP ranges from 1.6 to 23 cases per 100,000 per year worldwide. The gradual rise in incidence observed in some countries may be attributed to an increasing alcohol consumption and earlier diagnosis. Observing the etiology of chronic pancreatitis the majority of cases are due to alcohol abuse, genetic causes such as mutations in the cystic fibrosis gene, hereditary pancreatitis, ductal obstruction, trauma, pseudocysts, stones, tumors, possibly pancreas divisum, tropical

pancreatitis, systemic disease such as systemic lupus erythematosus, hypertriglyceridemia, autoimmune pancreatitis and there is idiopathic pancreatitis. Although before it was estimated that in the Western countries alcohol is the cause of 70% to 90% of all cases of chronic pancreatitis new cohort studies have reported that these numbers were a bit overestimated and that it accounts for about 45 % of cases (38). Alcohol seems to be the most common etiology in men (about 59 percent of cases) and the least common etiology in women (28 percent). In women, both nonalcoholic and idiopathic etiologies were therefore more common (37 and 35 percent, respectively). There are a number of complications associated with CP that involve maldigestion, steatorrhea, diabetes mellitus, pancreatic pseudocys, uncomplicated and associated with inflammation, abscess, necrosis or bleeding, common bile duct obstruction, duodenal obstruction, pancreatic fistula, dysmotility and in the end cancer.

We can divide the **non endocrine pancreatic tumors** in three major groups: pancreatic cancer, cystic pancreatic neoplasms and other non endocrine pancreatic tumors. Pancreatic cancer has a high lethality, worldwide, pancreatic cancer is the eighth leading cause of cancer deaths in men and the ninth in women (39). The incidence of pancreatic cancer has been increasing since the 1930s, and since 1973 is more or less unchanged at 8,8 per 100,000 with a male to female ratio of 1,3:1. The disease is rare before the age of 45, but the incidence rises sharply thereafter (40). Factors that are associated with the increased incidence of pancreatic cancer are hereditary, other inherited factors that don't involve largely known inherited syndromes and environmental factors. Disease is associated with a bad prognosis (41). Cystic tumors of the pancreas are relatively uncommon, accounting for only 1% of pancreatic neoplasms. They include mucinous cystic neoplasms, serous cystadenoma, intraductal papillary mucinous tumors and rarely others (42).

The overall prevalence of **pancreatic endocrine tumors** is relatively low, with an overall annual incidence in the United States of 3-10 cases per million persons (43). Neoplasms of the endocrine pancreas can be divided into functional, which are more frequent and nonfunctional varieties. They can be associated with multiple endocrine neoplasia type 1 (MEN 1) or characterized as a sporadic form. Insulinoma, gastrinoma, Zollinger-Ellison syndrome, VIP-oma, Verner-Morrison syndrome, WDHA (watery diarrhea, hypokalemia, aklorhydria), glucagonoma, somatostatinoma, carcinoid tumors and other functional tumors represent this group of tumors, with insulinomas and gastrinomas accounting for the majority of cases. They are more frequent in women with an age mean of 30 to 50 years. Tumors that develop in the setting of MEN-1 can occur in the younger age, most commonly 10-30 years. Nonfunctional tumors account for 14-48% of all recognized neoplasms of the endocrine pancreas (44).

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2. THE ROLE AND IMPORTANCE OF SCREENING TESTS IN GASTROINTESTINAL DISEASES

Petr Kocna

2.1 INTRODUCTION

This paper 'has been prepared as proceeding for the 12th EFLM Continuous Postgraduate Course on Gastrointestinal Diseases. We summarize the general overview about screening, their benefits and rules (1-3) and main aspects for laboratory methods (Table 2.1) used as screening tests in gastrointestinal diseases, published in last years, as well as our experiences in the Czech Republic.

Table 2.1 Recommended laboratory methods for screening in gastroenterology.

Gastrointestinal disease	**Recommended screening test**
Worldwide used screening	
Colorectal cancer	quantitative immunochemical Hb in stool
Celiac disease	IgG, tTGA and IgG DGP plasma antibodies
Evaluated new screening	
Chronic atrophic gastritis Helicobacter pylori infection	plasma pepsinogen I/II ratio Helicobacter pylori antigen in stool
Inflammatory bowel disease	calprotectin in stool

Legend: Hb - haemoglobin; IgG - imunoglobulin class G; tTGA - imunoglobulin A antibodies to tissue transglutaminase; IgG DGP - imunoglobulin G antibodies to deamidated gliadin peptide.

2.2 COLORECTAL CANCER

Colorectal cancer (CRCA) is the second most frequent malignant disease in Europe. Every year, 412 000 people are diagnosed with this condition, and 207 000 patients die of it (4) and estimated to cause 49 920 deaths in the U.S. in 2009 (5).

The pathogenesis of CRCA ordinarily occurs in a staged progression from normal mucosa, to adenoma, and finally carcinoma over a period of approximately 7–10 years (6). This sequenced progression over time provides an excellent opportunity for the utilization of screening tests for early detection of CRCA, with the goal of reducing cancer deaths by removal of pre-malignant adenomas and early localized cancer prior to onset of more advanced stages, and CRCA screening reduces mortality from colorectal cancer.

The introduction of national population-wide screening programs is a priority for the healthcare policy of individual states, and this is also being addressed at the highest level by European Union (EU) administrators. A screening program of one sort or another has been implemented in 19 of 27 European countries. The most frequently applied method is testing stool for occult bleeding (faecal occult blood test, FOBT). In the Czech Republic we started

CRCA screening programs in 1994 (7), and population-based, national screening with FOBT was started in 2002. The involvement of GPs has been found to improve patient compliance with bowel cancer screening (8).

The first level of FOBTs were guaiac based, gFOBT methods., which is still used in many countries as traditional methods of screening with high significance of Evidence-based-medicine, and recommended as one of many faecal screening methods by American College of Physicians (9). 20-years experiences with gFOBT were published recently in the UK (10). Guaiac methods are not specific for human haemoglobin. The gFOBT test is based on the oxidation of guaiac impregnated on the card) by hydrogen peroxide catalyzed by the peroxidase activity of haemoglobin. This oxidative reaction could as well occur with any peroxidase found in faeces (eg. plant peroxidases) or by certain chemicals (eg. vitamin C). The sensitivity of gFOBT for colorectal cancer is lower then 30% and these methods of FOBT will be changed in most of countries to immunological FIT.

The second level of FOBTs were immunochemical based, iFOBT (FIT) methods uses an antibody against human globin - the protein part of haemoglobin. The iFOBT are specific for human, haemoglobin, and are more sensitive than the gFOBT methods (11). Immunochemical-qualitative methods have very different accuracy and sensitivity in range 29-72% (12), use different sampling devices and different stability of haemoglobin extract in sampling buffer. Additional biochemical markers, haptoglobin and transferrin are used as a second detected analyte in these qualitative iFOBT tests, to increase screening accuracy (13,14).

The third level of FOBTs is now quantitative methods of faecal haemoglobin determination with automated analysers - qiFOBT. These modern methods increase accuracy to 90 - 95%, enabling setting to country-specific optimal cut-off and most important to be controlled by the External Quality Assurance Services (EQAS) programs. The European Group on Tumour Markers recommends use of a quantitative iFOBT with an adjustable cut-off point to all new centres undertaking FOBT for colorectal neoplasia (15), and organized faecal immunochemical test screening has been associated with an increase in annually detected CRC (16).

Three available analytical systems for quantitative FOBT test (Magstream HT, OC-Sensor/DIANA, FOB Gold/SENTiFOB) were compared for their accuracy, analytical sensitivity, and sample stability as well for sample mailing by tested subjects (17-20). Quantitative FOBT has found to be superior in compliance and colorectal cancer detection, compared with guaiac FOBTs as well with flexible sigmoidoscopy (21).

Diagnostic yield improves with collection of 2 samples of qiFOBT (22,23), increasing as well screening cost. Cost-effectiveness analysis of quantitative immunochemical test for colorectal cancer screening has been in high details described by Dutch working groups (24,25) publishing recently the optimal cut-off for qiFOBT to be 50 ug Hb/g. The pilot study with OC-Sensor qiFOBT recommends 50ng Hb/ml as optimal cut-off value for the screening in the Czech Republic (26).

Fecal immunochemical test results may be expressed as the haemoglobin concentration in the sampling device buffer and, sometimes, albeit rarely, as the haemoglobin concentration per mass of faeces. The current lack of consistency in units for reporting haemoglobin concentration is particularly problematic because apparently similar haemoglobin concentrations obtained with different devices can lead to very different clinical interpretations suggesting a proposal to standardize reporting units for qiFOBT (27,28).

Stool-based DNA testing for colorectal cancer is becoming a favored alternative to existing DNA screening tests. The basis for sDNA screening is the identification of genetic alterations in the initiation of a sequenced progression from adenoma to carcinoma, such as mutations in APC, K-ras, DCC, and p53 (29-32). Proteomics in combination with other techniques is rapidly being developed and there could be such a promising route for the diagnosis of early colorectal cancers (33). Hypermethylation of the plasma septin-9 gene shows promise as a nonstool-based screening tool (34,35). Blood-based biomarkers do not seem to be an alternative to FOBT-based CRC screening, but could be used in combination with iFOBT to increase colorectal screening accuracy (36-38). Tumour pyruvate kinase M2 (tumour M2-PK) is a key enzyme in the altered metabolism of tumor tissue. In cancer, it is known to be present in high concentrations in malignant tissue, plasma and other body fluids (39,40). Plasma and faecal levels of M2-PK could be used as other tumour markers better as prognostic marker, and it's not recommended for screening, its diagnostic efficiency was similar to that of gFOBT (41).

2.3 CELIAC DISEASE

Celiac disease (CD) is a common chronic small-bowel disorder of autoimmune origin occurring in both children and adults, and is one of the most commonly underdiagnosed diseases in general practice with incidence 1:100. This disease is genetically determined, has a strong HLA association with DQ2 (DQA1*0501/DQB1*02), and gliadin peptides derived from wheat gluten were identified as precipitating factors (42,43).

Screening strategies and diagnostic algorithms for the detection of CD, especially concerning serological markers, are included in research priorities of European Working Group on Serological Screening for Celiac Disease. The specificity and sensitivity of serological markers were reported in numerous studies for individual antibodies, ranging from 31% to 100%, and there no one marker could be neither 100% specific or 100% sensitive, and that a combination are able to detect all 100% celiac cases (44-7). The diagnostic accuracy of serology for CD has progressively increased in the last few years. IgA antibodies to tissue transglutaminase (tTGA) have been suggested as the first level test owing to their high sensitivity but accuracy could be confirmed by IgA endomysium antibodies (EmA), IgG tTGA should be performed in cases with a concomitant IgA deficiency, and IgA AGA is indicated for children under 2 year of age. A new antibody strategy designed for CD screening is therefore based on the combination of IgA tTG and IgG DGP (48). The new definition of celiac disease as well new ESPGHAN (European Society of Paediatric Gastroenterology, Hepatology and Nutrition) guidelines modifies the screening rules. TG2A levels exceeding 10 times the cut-off and confirmed in an independent blood sample by EmA testing are the first requirement for a celiac diagnosis without duodenal biopsy in symptomatic children (49,50).

Celiac disease has a prevalence of nearly 1% in the US and Europe, diagnosed cases of celiac disease only has a prevalence of about 0.27% or even less. The risk of celiac disease in various autoimmune diseases is approximately 5% - 10% (51,52). There is increased risk of complications in untreated celiac disease patients, which include malignancy and severe malabsorption. The early diagnosis of celiac disease and subsequent adherence to a gluten-free diet may prevent the development of other autoimmune diseases and decreases risk of mortality.

Mass screening for celiac disease (CD) as a public health intervention is controversial (53). The main argument against screening is adherence to the gluten-free diet, which might be low

in screen-detected patients, even in symptomatic patients (46). In contrast to the general population, screening in high-prevalence groups may prove to have a favorable cost–benefit ratio. The guidelines for targeted screening for celiac disease in the Czech Republic has been defined in the Bulletin of the Ministry of Health of Czech Republic in February 2011 indicating this high-prevalence subjects (Table 2.2). Recently new way in celiac screening started in 2011 in Italian primary schoolchildren with salivary anti-transglutaminase autoantibodies (54).

Table 2.2 Celiac disease associated disease, syndromes and signs indicating screening.

Associated symptoms and signs	Associated diseases and syndromes
Dermatitis herpetiformis	Type 1 diabetes
Osteoporosis, unexplained fractures	Autoimmune thyroiditis
Chronic diarrhoea with abdominal distension	Autoimmune liver disease
Anemia	Systemic lupus erythematosus
Chronic fatigue syndrome	Primary biliary cirrhosis
Polyneuropathy	Primary sclerosing cholangitis
Cerebellar ataxia, epilepsy	Sjögren syndrome
Spontaneous abortion and fetal growth retardation	Alopecia areata
Growth retardation, pubertal delay	IgA nephropathy
Involuntary weight loss	IgA deficiency
Unexplained anaemia (iron, folic acid)	
Dental enamel hypoplasia	
Recurrent aphthous stomatitis	
Hypertransaminasemia	

2.4 CHRONIC ATROPHIC GASTRITIS

Chronic atrophic gastritis (CAG) is an inflammatory condition characterized by the loss of gastric glandular structures, which are replaced by connective tissue (non-metaplastic atrophy) or by glandular structures inappropriate for location (metaplastic atrophy). Diagnosis and screening of CAG with stomach-specific plasma biomarkers could help as well with prevention of gastric carcinoma. Invasive gastric carcinoma are preceded by a cascade of precancerous lesions, multifocal atrophic gastritis (MAG) and intestinal metaplasia. It is accepted that a multistep process initiating from Helicobacter pylori-related chronic inflammation of the gastric mucosa progresses to CAG, intestinal metaplasia, dysplasia and, finally, leads to the development of gastric cancer (55,56). The diagnosis of Helicobacter pylori infection are now simple, easy with high sensitivity and specificity 92-98%, using both UBT (Urea Breath Test) and stool antigen tests (57,58).

Parallel assays of PGI, of the PGI/II ratio, and of amidated gastrin-17 comprise an exact and validated set or panel of biomarkers that reflect the degree of mucosal inflammation, the extent and grade of atrophic gastritis in the stomach, and the capacity of the existing mucosa to secrete acid and gastrin-17 (59,60). The sensitivity and specificity of these biomarker test panel (commercial test panel (GastroPanel, Finland) were 71-83% and 95-98%, respectively (61). High prevalence, more then 3%, of advanced atrophic corpus gastritis (ACG) among Finnish adult volunteers without specific complaints was diagnosed last year (62).

2.5 INFLAMMATORY BOWEL DISEASE

Differentiating patients with inflammatory bowel disease (IBD) from patients without intestinal pathology, in particular those with irritable bowel syndrome (IBS), poses a diagnostic challenge. Current guidelines suggest performing invasive endoscopy with histological sampling for further diagnosis. There is, consequently, a need for a reliable, non-invasive, simple, and cheap test that could provide objective evidence of whether the underlying disease is organic or functional. Measuring calprotectin, a neutrophilic protein, in faeces has been proposed as a surrogate marker of intestinal inflammation. Calprotectin values have been shown to reliably differentiate between IBD and non-organic disease in symptomatic patients and, when elevated, warrant early endoscopic investigation to rule out IBD and other organic pathologies (63-5). On the other hand, the use of faecal calprotectin as a screening test substantially could reduce the number of invasive measurements necessary in the diagnostic work-up of patients with suspected IBD (63), in adults, using faecal calprotectin as a screening test in suspected IBD to decide upon the need for endoscopy would result in a 67% reduction of patients requiring endoscopy (66). Calprotectin, lactoferrin, M2-pyruvate kinase, and other faecal markers could help us in differential diagnostics as well in screening of large bowel disease, colorectal cancer and IBD.

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3. GUIDELINES ON COLORECTAL CANCER SCREENING

Michael Neumaier

3.1 INTRODUCTION

Epidemiologically, colorectal cancer (CRC) is a leading cause in cancer mortality worldwide and, with 608.000 annual deaths, accounts for approximately 10% of all worldwide cancer casualties (1). Approximately 3.000 new CRC cases are diagnosed in Croatia every year. Of those, around 1.900 patients will die in the course of progression of the disease (2). A comprehensive national screening effort has been undertaken between 2007 and 2011. This has shown the potential to optimize prevention and early detection for the reduction of morbidity and mortality of CRC.

Guidelines are consensus instruments written by expert panels to optimize disease management based on clinical and scientific evidence. As colon tumours (and CRC) are considered to be highly curable diseases if diagnosed early, practice guidelines emphasize screening matters. There are a number of updated guidelines from various national medical societies used for decision making in screening (3-11): National Academy of Clinical Biochemistry (NACB) Laboratory Practice Guidelines on the use of tumour markers (biomarkers) in CRC based on published reports (3). In these, the use of screening markers is recommended in accordance with guidelines of other organisations. The German S3 Guideline is an extensive and current document (featuring 795 references), and its updated version has been published in 2010 (4). Very recently, the international colorectal cancer screening network, established in 2003 has performed an extensive survey on 43 organized CRC screening programs. The data presented by Benson et al (5) cover 35 eligible programs from 24 countries and present extensive information on the status of CRC screening on an international basis.

3.2 LABORATORY SCREENING FOR CRC

The screening aspects that are addressed here do not refer to recommended clinical procedures (colonoscopy, CAT scan etc.), but the laboratory methods only. There are three methodologies for laboratory screening for CRC dealt with in various guidelines: i) stool tests: fecal occult blood tests (FOBT) using different routine methods and stool DNA test for tumour mutations (sDNA), ii) concentrations of tumor-associated antigens (tumour markers) in the serum and iii) genetic predisposition testing for predictive medicine.

3.2.1 The fecal occult blood test (FOBT) in stool is the oldest method to screen for CRC. The classical guaiac (also referred to as gFOBT) colour test measuring pseudoperoxidase activity of heme has been shown to reduce mortality. There are several variants to the method, brands and protocols, in part differing significantly in their diagnostic sensitivities (see 5 and 6). While it is accepted that immunochemical variants (iFOBT) detecting human globin are more sensitive than gFOBT, this better performance is still a matter of some debate requiring studies and comparing test procedures. The National Comprehensive Cancer Network (NCCN) recommends taking three successive stool testings during a prescribed diet (7).

In recent years, nucleic acids testing has been proposed for CRC screening in stool. Specifically, targets often mutated in CRC (*kras, APC, p53, BAT-26*) have been evaluated

using sensitive PCR methods. Amplification methods yield significantly higher sensitivities and specificities, but are more complex to perform and more expensive. Direct comparison between FOBT and sDNA has been carried out some years ago (8). Most guidelines recommend using sDNA testing as an option. Finally, epigenetic markers designed to detect tumour methylation signatures in peripheral blood have been proposed recently, but have not found their way into recommendations so far.

All guidelines recommend being aware of harmful side effects as result of positive screening tests like psychological anxiety, complications during colonoscopy or the possibility of over-diagnosis. However, in the light of the natural course and progression of CRC, over-diagnosis is far less problematic as compared to other very slowly growing cancers with very high prevalence like prostate cancer. Guideline specifically warn of false negative results, while it must be said that due to low sensitivities and specificities and low prevalence, the negative predictive value can be calculated as being very high indicating that a healthy (i.e. asymptomatic and unsuspicious of disease) individual with a negative result has a greater than 95% chance of indeed being healthy.

Table 3.1 Guidelines on colorectal cancer screening.

	ASCO * (9)	**EGTM (10)**	**NACB (3)**	**ESMO (11)**	**NCCN (7)**	**ACS (12)**
FOBT		yes	yes		yes	yes
CEA screening	no	no	no			
CEA prognosis	yes	yes	yes	yes	yes	
CEA follow-up	yes	yes	yes	yes	yes	
CEA monitoring	yes	yes	yes	no	no	
FAP	yes			yes	yes	
MSI			yes		yes	
HNPCC	yes			yes	yes	

Legend: *ASCO - American Society of Clinical Oncology; EGTM - European Group on Tumor Markers; NACB - National Academy of Clinical Biochemistry; ESMO - European Society of Medical Oncology; NCCN - National Comprehensive Cancer Network; ACS - American Cancer Society; FOBT - fecal occult blood tests; CEA - Carcinoembryonic Antigen; FAP - Familial adenomatous polyposis; MSI - microsatellite instability; HNPCC - hereditary nonpolyposis colon cancer.

3.2.2 The Carcinoembryonic Antigen (CEA) is the most prominent tumour marker used for diagnosis of CRC. It cannot be used for the screening of healthy individuals, as low sensitivity and low specificity leads to a high number of false positive results due to very low positive predictive values. Among other factors, CEA may be used for planning of surgical treatment. Patients with increased CEA serum concentrations should be evaluated for progressed disease or metastasis. Preoperative CEA concentrations are no decision criterion for adjuvant chemotherapy. Postoperative measurements of CEA should be done quarterly for at least 3 years. In patients with advanced CRC, CEA should be determined regularly. Increases indicate progressive disease or distant metastasis, particularly to the liver, depending on the amplitudes of the rising concentrations. Other serum markers possess an even lower performance and should not be used for routine diagnosis let alone for screening.

3.3.3 Aside from **screening for somatic mutations, the genetic testing for CRC predisposition**, i.e. monogenic traits or familial CRC background is recommended in the

context of human genetic counselling and should only be done in context with careful assessment of the family history. Mainly, defects from the *wnt*-signalling pathway (*APC, CTNNB*) for FAP or the DNA mismatch repair pathway (microsatellite instability; MSI) for HNPCC are suitable targets. Taken together, only a small number of markers can be used, although many candidates have been proposed over the years.

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4. NEW MOLECULAR DIAGNOSTIC TESTS FOR SCREENING AND MONITORING OF COLORECTAL CANCER

Michael Neumaier

4.1 INTRODUCTION

Malignant tumours regularly show a multitude of genetic and epigenetic defects and vary considerably even within given tumour entities. While they can be clinically and phenotypically very similar, they often show considerable differences at the cell or cell cluster level. This heterogeneity is believed to be the result of constant selective pressure acting on the malignant cells in context with therapeutic response or resistance. The resulting divergence of the genetic and epigenetic make-up in a tumour is the basis of biomarker-driven diagnostics either using markers with high prevalence or individual defects in the sense of "personalized medicine". Importantly, biological differences are now increasingly characterized as to their pathobiochemical importance and form the basis of molecular classifications that impact on the choice of therapeutic regimens.

Epidemiologically, colorectal cancer (CRC) is a leading cause in cancer mortality worldwide and, with 608,000 annual deaths, accounts for approximately 10% of all worldwide cancer casualties (1). In Croatia, approximately 3,000 new CRC cases are diagnosed every year with around 1,900 patients dying of the disease (2). A comprehensive national screening effort has been undertaken between 2007 and 2011. This revealed a relatively low return rate for fecal occult blood tests (17.1%) with some significant differences between the 20 Croatian counties. 6.9% of the FOB tests (n=12477) were returned positive, and subsequent colonoscopies were performed on 66% of the participants (n=8541). These revealed CRC in 5.5% (n=472) and adenomatous polyps in 39% (n=3329). Within Europe, Croatian citizens have a relatively high incidence to contract and die of the disease placing emphasis on better compliance with screening programs and the need to improve early diagnosis.

4.2 MOLECULAR TESTING FOR COLORECTAL CANCER (CRC)

It is firmly established that CRC develops from normal colon epithelium through a distinct accumulation of successive, ordered genetic and epigenetic alterations, a process termed "multistep carcinogenesis". Today these alterations can easily be identified in tumour material and also peripheral blood specimens of patients.

Most CRC are spontaneous in their occurrence, but their genetics closely resembles the defects found in rare hereditary CRC syndromes suggesting that these defects are critical for tumour development and progress in general. A major question for today´s diagnostics and ist future development is, whether or not we will be able to identify genetic tumour predisposition reliably enough for prediction and prevention of full-fledged CRC.

Genetic predisposition factors for CRC show different modes of inheritance and penetrance. Firstly, monogenic defects act with high penetrance in the carriers i.e. between 70-100% will develop the cancer during early lifetimes. For example, in 2-5% and <1% of the cases, monogenic defects cause hereditary CRC termed **H**ereditary **N**on-**P**olypous **C**olorectal **C**ancer (HNPCC) or „**F**amilial **A**denomatous **P**olyposis coli" (FAP) syndromes, respectively.

HNPCC is caused by defects in the DNA mismatch repair system and will lead to microsatellite instable (MSI) cancer. In contrast, the FAP is caused by defects in the *wnt*-signalling pathway (most often due to mutations in the *APC* gene) and will lead to chromosomally instable (CIN) polypous cancers. Furthermore, biallelic loss of the *MYH* gene function leads to *MHY*-associated polyposis and CRC with 100% of the carriers bearing a malignant tumour by the age of 60. Conversely, one third of the polypous CRC patients without APC defects will carry biallelic *MHY* defects. Finally, there are inherited colon tumor syndromes like the Peutz-Jeghers´ disease (*LBK1* defects), juvenile polyposis (*SMAD4, BMPR1A* defects) or Cowden´s disease (potentially *PTEN* defects) that show a CRC risk between 10-60%.

Next to these high penetrance gene defects of monogenic hereditary disease, there is a less defined bigger number of CRC genes with intermediate or low penetrance. Altogether, it is assumed that up to 35% of all CRC possess a familial genetic component identified by linkage and association studies. Many of these candidate genes still require confirmation, as their functional relevance is not understood in the disease process. Also, there are clear indications that risk alleles of intermediate or low penetrance will substantially increase the cancer risk, when accumulating in an individual. For example, recent studies independently confirm that the increasing numbers of low penetrance genes raise the CRC risk in these families significantly (3,4). While possessing 10 risk alleles was found to carry the risk of the general population, additional alleles led to substantiate risk increase by 2-5 folds. It was judged that the 10 strongest loci accounted for approximately 10% of the familial CRC risks.

Approximately 20 years ago, epigenetic silencing of gene expression by DNA hypermethylation has first been implicated in carcinogenesis of the colon and has since then been increasingly appreciated as a mechanism for very early tumour lesions. Today, it is very clear how epigenetic silencing of gene lead to the failure of tumour suppressor functions like DNA repair, leading to genetic defects in their wake that eventually cause malignant transition of the colon mucosa cells. Hypermethylation of CpG dinucleotides is a robust and stable DNA modification that can easily be tested for in DNA previously treated with high concentration of sodium-bisulfite.

COMMENT: Genetic data clearly show that different routes must be taken for the molecular diagnostics of CRC depending on individual risk. Inherited CRC requires the analysis of very few, but usually large tumour suppressor genes. In contrast, low penetrance genes are important for the polygenic risk and require analysis by larger numbers. Since a functional role often cannot be deduced, interpretation of the results will relay on bioinformatics classification algorithms to identify their significance. It can be expected that technologies like next-generation sequencing (NGS) will increase the number of potential defects reported. Epigenetics testing will become an increasingly important area of CRC diagnostics, as it allows identifying early tumour forms of not yet malignant phenotype. Epigenetic markers are comparably stable and can be tested for in the peripheral blood and other biomaterials.

4.3 MOLECULAR SCREENING AND EARLY DIAGNOSIS OF CRC

There have been numerous attempts at using molecular techniques for an advanced diagnosis in screening programs. They have met with mixed success so far. The detection of *k-ras* mutated DNA in stool samples has been performed with different techniques including Digital Melting Analysis, single base extension or allele-specific PCR and results in an increase in diagnostic sensitivity over the classical Guajak-Test for fecal occult blood (FOB) (6,7). However, these tests are expensive, require sophisticated protocols and equipment and thus

have not been adapted widely.

A first test has been introduced recently allowing the analysis of *SEPT9* methylation in the peripheral blood as a means of early diagnosis of CRC. While this marker shows some sensitivity and specificity, it has all the limitations of classical serum tumour markers like CEA or CA19-9. However, it proved the point that tumour-associated methylation patterns can be revealed from circulating DNA. More recently, Lind and colleagues have screened methylation markers in order to establish a multiparametric assay for colorectal tumour development. They finally resorted to 6 markers that, when simultaneously determined, showed a combined AUC of 0.976 in the detection of adenomas and carcinomas from peripheral blood specimens (5).

COMMENT: epigenetic screening is an interesting new test modality for the detection of early tumour forms. It does not differentiate well between adenomas and CRC. However, since adenomas are precancerous lesions and discussing the low compliance (of age groups at risk) with colonoscopy programs, their early determination may help to identify colon tumours in a not yet malignant stage. Multiparametric analysis appears to be more robust to inter-individual tumour behaviour than a single marker like SEPT9.

4.4 STRATIFICATION FOR THERAPY AND DETECTION OF RELAPSE

EGFR is an important receptor conveying growth and differentiation signals to the tumour cells. Blocking this signal by using monoclonal antibodies like Cetuximab and Panitumumab is a therapy recommended for advanced CRC. *Kras* and *braf* are signal transduction molecules involved in forwarding the EFGR signal to the nucleus. Mutations of these molecules lead to an EGFR-independent downstream activation of tumour-associated growth. Antibody treatment will therefore have no therapeutic effect (8). Due to the high costs of these biologicals and their unwanted side effects, genetic testing of the *kras* and *braf* status is now mandatory to stratify patients. As 65% of the CRC with *kras* wildtype are resistant to EGFR-blockade, the search for additional biomarkers for stratification continues (9,10,11). Detection of CRC progression and metastasis should be an important actionable health information. Accordingly, the molecular detection of circulating tumour cells, circulating tumour DNAs or microRNAs have been evaluated starting in the early 1990s. In a recent extensive metaanalysis starting with 1864 papers, the clinical utility of various test formats including also a large body of immunochemical methods has been evaluated (12,13). Finally using 36 appropriate studies, the authors come to the conclusion that circulating tumour cells are valid targets. In an attempt to use NGS for tumour profiling and the detection of minimal residual disease, Forshew et al. have reported a special method allowing a highly sensitive targeted resequencing of a gene panel from patient plasma (14).

4.5 PERSONALIZED MOLECULAR TESTING

A promising area of molecular testing in CRC relates to grading of the tumour. The Onctype Dx or the Coloprint panels test mRNA-expression in array formats. A number of studies are on their way, but preliminary reports suggest that molecular risks for progression-free survival and metastasis can be assessed with these tests (15,16). Presently, these approaches can be seen more as means of stratification than personalized diagnostics. However, for the future we may extrapolate individual therapeutic options based on molecular profiling.

Recent additions to the diagnostic arsenal of personalized diagnostics are reports that use

NGS as an even more powerful technology to characterize individual tumour biomarkers for personalized diagnostic use. Specifically, Leary et al. have used NGS to identify the position of tumour-specific chromosomal translocation from CRC tissue. Using this information, the authors have devised patient-specific PCR tests to monitor free circulating DNA in the plasma (17).

4.6 SHORT CATALOGUE OF MOLECULAR DIAGNOSTIC TARGETS

APC: Adenomatus Polyposis Coli. Downstream Regulator of Wnt signalling pathway; gate keeper defects lead to familial adenomatous polyposis (FAP, <1% of all CRC). Chromosomally instable phenotype.

MUTYH: Mutation of the Y Homologue; MYH-associated attenuated polyposis; the MUTY protein is involved in DNA excision base repair

hMLH1, hMSH2, hMSH6, PMS2; human Mutation L Homologue 1, human Mutation S Homologue 2 & 6. Central genes of the DNA mismatch repair system. Highly penetrant genetic defects; responsible for the hereditary non-polypous colorectal cancer (HNPCC, Lynch-Syndrom; 2-5% of all CRC). Microsatellite-instable phenotype.

SEPT9; early methylation marker in the plasma; positive predictive value is low in screening situations. Being evaliuated as stratification tool for colonoscopy programs.

KRAS: mutations of the kirsten-ras Protooncogen lead to activation of the ras/raf/MAP kinase pathway; important stratification marker for CRC antibody therapy.

BRAF: v-Raf murine sarcoma viral oncogene homologue B1; a Serin/Threonin protein kinase influencing cell division and differentiation as part of the *kras* pathway. *Braf* mutations exclude *kras* mutations within the same tumour.

Epigenetic risk panel: **CNRIP1, INA, BEX1, FBN1** und **SNCA** are genes of different functions, whose acitivity is diminished or lost in >90% of the CRC, but in <5% of the normal mucosat.

Expression arrays: OncoType Dx Colon Cancer™ measures mRNA expression levels in 7 CRC-associated genes ***(BGN, INHBA, FAP, MK167, MYBL2, MYC, GADD45B)*** and 5 internal control genes. Expression profiling is used for grading and to the risk for relapse and prognosis during follow-up. The test is exclusively available at Genomic Health Inc., Redwood, CA, USA

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5. WHAT IS THE ROLE OF EGFR AND *RAS* PATHWAY IN COLON CANCER?

Sanja Kapitanović, Tamara Čačev

5.1 INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers and one of the leading causes of cancer related death in the Western world (1). Of all human cancers, the molecular genetic alterations in colorectal cancer are best understood. The disease arises from the accumulation of mutations in oncogenes, tumor supressor genes and MMR genes during progression from normal colon epithelium to adenoma and metastatic carcinoma (2).

Colorectal cancer represents the third most diagnosed cancer in both men and women. The majority of colon cancers (75%) are sporadic and only 25% of the patients have a family history of CRC. However, only 5-6% of CRC arise from inherited mutation high penetrant colorectal genes while the remaining of the familial form are the result from interactions between less penetrant genes and enviroment. (3,4).

The development of colorectal cancer is a multistep process caused by progressive accumulation of genetic and epigenetic changes that cause activation of oncogenes and/or inactivation of tumor supressor genes. The earliest trigger is the mutation of the *APC* (adenomatous polyposis coli) gene. Mutations in oncogenes (*KRAS*) and other tumor suppressor genes (*SMAD4*, *TP53*) drive tumor towards malignancy and metastasis. Alongside with gene mutations, deregulated expression of oncogenes and/or tumor suppressor genes can also occur as a consequence of epigenetic modifications of their promoters (3-7).

Improved understanding of genetic events that underline tumor development, progression and metastasis may contribute to new strategies for prevention, screening, diagnosis as well as for therapy. Survival of patients with metastatic colorectal cancer has improved over the past several decades, due to the development of new combinations of standard chemotherapy such as 5-fluorouracil, irinotecan, and oxaliplatin, as well as to the introduction of new targeted therapies. Among the available targeted therapies are two monoclonal antibodies that target the epidermal growth gactor receptor (EGFR), cetuximab and panitumumab, which have clearly demonstrated efficacy in the treatment of metastatic CRC (8,9).

Individual patient response to therapy could be very diverse, even if their disease seems similar when evaluating clinicopathological parameters. Many drugs commonly used in clinical practice show interindividual variations in efficacy, dose requirements as well as the presence of side-effects. There is increasing evidence that treatment response is dependent on the genetic background of the individuals as well as on the molecular-genetic changes in the tumor itself. The study of the genetic determinants influencing interindividual differences in drug response is known as pharmacogenetics. Knowledge about the influence of polymorphisms and mutations on drug response can be used to identify, through pretreatment genetic screening, the patients with the best chance of responding to a specific drug and those at greater risk to develope an adverse drug reaction (10,11).

In recent years the focus in this field has shifted towards the development of targeted therapies and the use of molecular analyses to identify patients most likely to respond to these therapies. The epidermal growth factor receptor (EGFR) has become an important target of cancer therapy. For instance, the anti-epidermal growth factor receptor (EGFR) monoclonal

antibodies panitumumab and cetuximab have been shown to be effective therapies for metastatic colorectal cancer, but, as with most cancer therapies, not all patients derive clinical benefit from these treatments (12). Cetuximab (Erbitux®, Merck KgaA, Darmstadt, Germany) ia a chimeric mouse/human antibody targeted against the extracellular domain of the EGFR. Binding of cetuximab to the receptor prevents ligand binding, induces receptor internalization and causes a direct inhibition of the receptor tyrosine kinase activity (13,14). This in turn blocks downstream signal transduction via the PI3K/AKT and RASRAF/MAPK pathways inducing pro-apoptotic mechanisms and inhibiting cellular proliferation, angiogenesis and metastasis (15,16). As an IgG1 antibody cetuximab may also induce antibody-dependent cell-mediated cytotoxicity (ADCC). However, the clinical relevance of ADCC with regard to antitumor efficacy is likely to be rather low (14). Panitumumab (Vectibix, Amgen Thousand Oaks, CA, USA) is a fully human antibody which is also directed against the EGFR but being an IgG2 monoclonal antibody lacks ADCC activity (13). There has recently been an increase of interest in the relevance of several biomarkers for the selection of patients who will benefit from EGFR-targeted therapies for the treatment of colorectal cancer. Genetic analyses showed that the presence of mutations in the gene *KRAS* can predict lack of response and resistence to panitumumab and cetuximab in patients with metastatic CRC (13).

5.2 EGFR PATHWAY

The epidermal growth factor receptor (EGFR) is a 170 kDa transmembrane tyrosine kinase receptor that is present in most epithelial tissues. It plays an important role in cell growth and function controlling several signaling pathways. EGFR also represents an important target for cancer treatment because its activation stimulates key processes involved in tumor growth and progression, including proliferation, angiogenesis, invasion, metastasis, and drug sensitivity. It is a member of the human epidermal growth factor receptor erbB/HER family of cell surface receptors tyrosine kinases, which consists of four structurally related proteins: EGFR (also called HER1/erbB1), erbB2 (HER2/neu), erbB3 (HER3) and erbB4 (HER4)(17,18). The EGFR transmembrane protein is composed of three domains: an extracellular ligand binding domain, a lipophilic transmembrane domain, and an intracellular tyrosine kinase domain. Apart from erbB2, specific ligands have been identified for each of the erbB receptors. Among these, the epidermal growth factor (EGF) and the transforming growth factor alfa (TGF α) selectively bind to the EGFR (8,12,13).

In normal cells, the EGFR signaling cascade begins with ligand activation of EGFR. Ligand binding induces conformational change and dimerization of the receptor with formation of homodimers and heterodimers, which leads to the activation of tyrosine kinase. The intracellular tyrosine kinase residues then become autophosphorylated, inducing activation of multiple signal transduction pathways including the RAS-RAF mitogen activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), Akt and phospholipase C pathways. All together, these EGFR induced signaling pathways control gene transcription, cell cycle progression, cell proliferation and survival, adhesion, angiogenesis, migration, and invasion (19).

Signaling through the EGFR pathway is a complex process that requires tight regulation. The first level of complexity is encountered at the receptor level, where are shared and lateral signaling occurs between members of the erbB family. Then there are positive and negative feedback loops built into the pathways and differential activation of transcription factors, depending upon the cell type. When this system, due to molecular genetic changes becomes unregulated it can contribute to malignant transformation and tumor progression through

increased cell proliferation, prolonged survival, angiogenesis, anti-apoptosis, invasion and metastasis (20-22).

Based on importance of the EGFR axis in colorectal cancer, drugs that interfere with various functional domains of the receptor have been developed as it is already mentioned before. Currently, two anti-EGFR monoclonal antibodies have been approved in several countries for the treatment of colorectal cancer, cetuximab and panitumumab. Both antibodies have been shown to reduce the risk of tumor progression and to improve overall survival, progression-free survival and quality of life in patients with colorectal cancer refractory to standard chemotherapy (19,23,24).

Overexpression as well as mutations of *EGFR* are found in a range of solid tumor types and have been linked to poorer outcomes. Since the EGFR is primary target of monoclonal antibodies such as cetuximab or panitumumab, it is reasonable to evaluate the predictive potential of EGFR protein expression with regard to sensitivity towards these drugs. Analyses performed by immunohistochemistry (IHC) indicate and EGFR protein expression in 60-80% of colorectal tumors. However only a small proportion of IHC positive tumors also show *EGFR* gene amplification (25) Clinical analyses have also consistently reported no correlation between the EGFR overexpression detected by IHC and response to anti-EGFR therapy (13).

Mutations of the *EGFR* gene have been reported in non small cell lung cancer (NSCLC) and were linked to the clinical efficacy of tyrosine kinase inhibitors such as gefinitib. It appears, however, that *EGFR* gene mutations are rare events in colorectal cancer and have no clinical relevance with regard to the activity of anti-EGFR therapy (20,26).

5.3 KRAS AND THE EGFR PATHWAY

The *KRAS* gene encodes a 21 kDa guanosine 5'-triphosphate (GTP) binding protein at the begining of the MAPK signaling pathway, downstream of tyrosine kinase receptors including EGFR, in a complex signaling cascade involved in the development and progression of cancer. Somatic *KRAS* mutations are found in many cancers, including 40-60% of colorectal cancers, as well as in tubular and villous adenomas. The identification of *KRAS* mutations in almost 50% of adenomas suggests that this is an early event in CRC carcinogenesis. Up to 90% of activating mutations of the *KRAS* gene are detected in codons 12 and 13, but less frequently also in codon 61 and 146. With regard to codon 12/13 mutations only, 70% of mutations occur in codon 12 and 30% in codon 13 (8,27,28).

KRAS mutations, most commonly codon 12/13 missense mutations, lead to constitutive activation of the KRAS protein by abrogating GTPase activity. Because KRAS is the downstream effector of EGFR, mutations in the *KRAS* gene lead to an independent activation of the downstream signal transduction system, and that tumors with activating *KRAS* mutations do not benefit from anti-EGFR therapies. This unreguated downstream signaling as a result of *KRAS* mutation will not be blocked by antibodies that target the EGFR receptor. Not predicitve for outcome with standard chemotherapy, *KRAS* mutation status is a strong predicitive marker of resistance to EGFR targeted therapy in patients with metastatic colorectal cancer. *KRAS* mutations strongly predict a lack of response to anti-EGFR monoclonal antibodies cetuximab and panitumumab (29). Unfortunately at least 60% of patients with wild type KRAS gene will still not respond to anti-EGFR therapies, highlighting the need for additional biomarkers to help separate responders from nonresponders (29,30).

The *BRAF* gene encodes a serine-threonine protein kinase that is downstream of KRAS in the MAPK signaling pathway and mutations in these two genes are mutually exclusive. *BRAF* mutations occur in 5-22% of all colorectal cancers, and in 40-52% of CRC with microsatellite

instability (31,32). The most frequently reported *BRAF* mutation ia a valine-to-glutamine amino acid (V600E) substitution. Unlike *KRAS* mutations, *BRAF* mutations have an impact on prognosis and survival. Patients with a *BRAF* mutation in a microsatellite-stable CRC have significantly poorer survival than those without the mutation, but the *BRAF* status does not affect survival of patients with microsatellite-unstable tumors. *BRAF* status also predicts response to anti-EGFR tharapy. Of metastatic CRC that are found to be *KRAS* wild type at codon 12/13, 5-15% can harbour *BRAF* mutations and show resistence to anti-EGFR tharapy (20).

Analysis for mutations in both genes could identify as many as 40% of patients who have no chance of responding to this class of drugs. Retrospective studies suggest that concomitant detection of *KRAS* and *BRAF* mutations, combined with detection of mutations in two other genes (*PIK3CA* and *PTEN*), can identify up to 70% of patients who are unlikely to respond to anti-EGFR therapies (30).

The *PIK3CA* gene encodes phosphatidylinositol 3-kinase (PI3K), a key signal transducer in the PI3K-AKT pathway. Mutations in *PIK3CA* occur in 14-18% of CRC, and most mutations involve hotspots in exon 9 and 20 (7,20). There is a strong association between *PIK3CA* exon 9 mutations and *KRAS* mutations. As a prognostic marker, *PIK3CA* mutations are associated with shorter survival, but this effect may be limited to patients with *KRAS* wild-type tumors. As a predicitve marker, only *PIK3CA* exon 20 mutations appear to be associated with worse outcome after cetuximab therapy (33).

The *PTEN* gene encodes a protein tyrosine phosphatase enzyme (PTEN) that acts as a tumor supressor protein, dephosphorylates phosphatidylinositol-3,4,5 triphosphate (PIP3) and thereby inhibits PI3K function. Loss of PTEN results in constitutive activation of the PI3K-AKT pathway. *PTEN* mutations and loss of heterozygosity (LOH) at the *PTEN* locus have been reported in 13-18% and 17-19% of colon cancers, respectively. PTEN protein inactivation may also be a negative predictor of response to anti-EGFR therapy (20,34).

Mutations involving the EGFR axis (KRAS, BRAF, PIK3CA, PTEN) can cause its constant activation and result in continous activation of the downstream RAS, MAPK or PI3K pathways leading to uncontrolled cell proliferation, regardless of wheather the EGFR is activated or pharmacologically blocked. Such activation enhances transcription of various oncogenes, including *MYC*, *CREB*, and the gene for nuclear factor kappa B (*NFκB*) (12,18,35) (Figure 5.1).

5.4 CONCLUSION

The EGFR signaling pathway is a complex and tightly regulated signaling network that is involved in growth, proliferation, and survival of normal cells. When this network becomes unregulated it can lead to growth, proliferation, survival and metastasis of neoplastic cells. Alterations within the EGFR signaling cascade, such as gene mutations in its downstream regulators, have been shown to contribute to colorectal carcinogenesis, but are also predictve biomarkers of anti-EGFR therapy (12,20,36).

As more biomarkers will be identified and validated predicting testing will be used more extensively in clinical decision making. Optimization of tools to predict the risk for developing cancer, to diagnose a disease at an early stage, to give a prognosis, and predict treatment response in patients, is of huge importance to the patient itself, as well as to the health professionals.

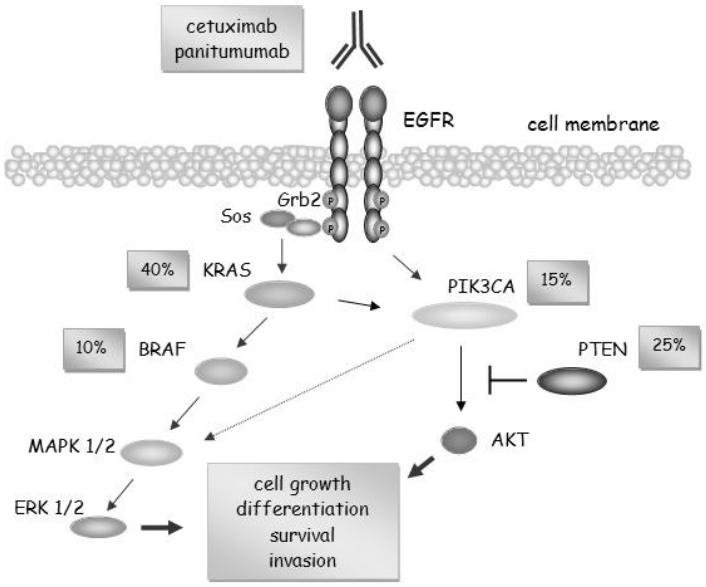

Figure 5.1 Mediator of epidermal growth factor receptor (EGFR) signaling and anti-EGFR antibodies. EGFR forms a homodimer after ligand activation, which results in autophosphorylation/activation of the intracellular kinase domain and a cascade of downstream signaling. Monoclonal antibodies used to treat patients with metastatic colorectal cancer including cetuximab and panitumumab bind to the extracellular domain of EGFR and inhibit signaling in some patients. Activating mutation that confer resistance to these drugs occur in *KRAS* (40%), *BRAF* (10%), *PIK3CA* (15%) and *PTEN* (25%) gene.

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6. SCREENING AND CONFIRMATION OF MALABSORPTION

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6.1 INTRODUCTION

6.1.1 DEFINITION OF MALABSORPTION

Malabsorption is a failure of normal absorption of nutrients and micronutrients regarding transport, digestion or absorption of nutrients in the gut. It differs from malnutrition, which is an inadequate food intake (1,2). Malabsorption can be generalized, affecting absorption of a range of nutrients, or specific, where the absorption of only a single nutrient is impaired. Principially three mechanisms are involved in pathophysiology of malabsorption: premucosal (luminal), mucosal and postmucosal (postabsorptive). Premucosal mechanisms lead to maldigestion, mucosal and postmucosal mechanism lead to real malabsorption (1,3,4).

6.1.2 CLINICAL SYMPTOMS

Main clinical symptoms are weight loss, abdominal pain, chronic diarrhea, bloating, steatorrhea, anemia, osteopathies and neuropathies. If the process affecting absorption is mild, malabsorption may stay clinically silent, as it is compensated by bigger food intake. In more severe cases it presents by one or more signs and symptoms of the malabsorptive state. Fat malabsorption usually presents by diarrhea, steatorrhea, weight loss, reduced triceps skin-fold

Table 6.1 Basic laboratory tests for investigation of malabsorption

Tests to seek evidence of malabsorption	Tests for course of malabsorption
Global tests Blood count Mean erythrocyte corpuscular volume Mean erythrocyte corpuscular hemoglobin Serum iron Serum ferritin Serum cobalamin Serum and red blood cell folate Prothrombin time Plasma proteins Serum electrolytes Liver enzymes Thyroid stimulating hormone Occult bleeding quantitative test in stool	**Infection markers** C-reactive protein Erythrocyte sedimentation rate Immunoglobulins Stool bacteriology/parazitology tests **Celiac disease** anti-tissue transglutaminase anti-deamidated-gliadin antibodies Beta carotene **Carbohydrate malabsorption** Hydrogen breath tests ^{13}C substrate labeled breath tests Xylose test **Fat malabsorption** 72-hour faecal fat collection Pancreatic elastase I in stool ^{13}C substrate labeled breath tests **Protein malabsorption** Alpha1-antitrypsin clearance in stool Bowel permeability tests

thickness, fat soluble vitamin deficiences (night blindness, dry eyes, osteomalacia and prolonged prothrombin time). Protein loss brings edema, reduced mid-arm muscle circumference, reduced creatin and creatinine: height ratio. Iron deficiency presents by glossitis, angular stomatitis and pallor; group B vitamins deficiency shows glossitis, magenta tongue, pellagra and peripheral neuropathy (1,3-7).
Major diseases involved in malabsorption are: celiac disease, Crohn's disease, lactose intolerance, small bowel lymphomas and intestinal infections, gastric and liver diseases, chronic pancreatitis, systemic diseases and neuroendocrine tumors.
Diagnosis is based on physical examination, physical history, imaging methods; routine and special laboratory tests (Table 6.1). The use of scoring systems like MUST (malnutrition universal screening tool) is advisable (4,8,9). MUST scoring system uses patient's height, weight, recent unplanned weight loss, considering effect of acute disease, to determine malabsorption risk and to manage it if present.

6.2 LABORATORY TESTS FOR MALABSORPTION

Laboratory tests can be divided according to biological material from which they are performed: blood (serum, plasma), stool and breath samples, while latter two can be assessed non-invasively and are suitable even for children and good for screening. From those pancreatic elastase I in stool is widely used to assess severity of exocrine pancreatic dysfunction and hydrogen breath tests to show suspected monosacharides and disacharides intolerance. Breath tests are summarized in Table 6.2 (10-13). In general hydrogen breath tests and ^{13}C-labeled substrate breath tests can be performed. For carbohydrate malassimilation both types can be used employing lactose, fructose, sorbitol, saccharose, D-xylose and starch. Small intestinal bacterial overgrowth can be objectified by glucose, lactulose, glycocholate and D-xylose breath tests. For orocaecal transit time assessment lactulose, inulin and lactoseureide as substrates are used. ^{13}C-labeled substrates are of great importance in exocrine pancreatic function assessment: ^{13}C-mixed triglycerides, ^{13}C-triolein and other substrates are widely used. Permeability tests are sometimes performed (14,15).

Table 6.2 Breath tests used in malabsorption screening and diagnosis.

Hydrogen breath tests substrates	**^{13}C-breath tests substrates**
Carbohydrate malabsorption	
Lactose	^{13}C-lactose
Fructose	^{13}C-fructose
Sorbitol	^{13}C-starch
Saccharose	^{13}C-saccharose
D-xylose	
Fat malabsorption	
	^{13}C-mixed triglycerides
	^{13}C-triolein
Small intestinal bacterial overgrowth	
Glucose	^{13}C-D-xylose
Lactulose	^{13}C-glycocholate
Orocaecal transit time measurement	
Lactulose	^{13}C-lactoseureide ^{13}
Inulin	

6.3 NORMAL BOWEL PHYSIOLOGY

Normal absorption relies on multiple processes, some starting as soon as food is seen, smelt and tasted. Chewing starts the physical transformation of food and secretions from the salivary glands, stomach, pancreas, liver and intestine dissolve components of the meal and lubricate its passage. Coordinated muscle function is needed to swallow the bolus, gastric motility is crucial for mixing food in the stomach and emptying the semi-liquid chyme into duodenum. Intestinal peristalsis propels and mixes nutrients during digestion and absorption. These processes are controlled by nerves and hormones (16,17). Reabsorption of secreted water, electrolytes and bile acids takes place in the distal intestine. Bacterial action releases some further nutrients that can be absorbed in the colon (4).
For adequate digestion exocrine secretion of enzymes is essential. Digestive enzymes are also present on brush-border apical membrane and cytoplasm of the enterocyte. Main enzymes involved in digestion of macronutrients are shown in Table 6.3 (4,18,19).
Proteolytic enzymes are produced as inactive precursors and intestinal brush-border enterokinase activates trypsinogen to trypsin, which then activates other pancreatic proteases. Important non-enzymatic secretions are hydrochloric acid from the stomach (stomach enzymes better work at lower pH) and bicarbonate present in pancreatic juice and bile (intestinal enzymes prefer alkali pH). Bile acids and phospholipids from the liver form micelles with ingested lipids and improve their absorption.

Table 6.3 Enzymatic digestion of macronutrients.

Site	Carbohydrates	Proteins	Lipids
Salivary glands	Salivary amylase		
Stomach		Pepsins	Gastric lipase
Pancreas	Pancreatic amylase	Trypsin Chymotrypsin Elastase Carboxypeptidases	Lipase Colipase Phospholipase Cholesterol esterase
Intestine	Orocaecal transit Sucrase Lactase Maltase	Enterokinase Aminopeptidases Endopeptidases Oligopeptidases Dipeptidylpeptidase	

Water absorption is regulated by absorption of major electrolytes (sodium, chloride). Sodium is co-transported with many other nutrients (glucose, amino acids), in ileum and colon specific mechanisms of sodium and chloride ions absorption exist.
Carbohydrates are relatively easily digested. Monosacharides are absorbed in duodenum and upper jejunum rapidly. Sucrose is split by sucrase to glucose and fructose. Lactose, the milk sugar, is broken down by lactase to galactose and glucose. Starches are longer to digest and absorb, dietary fiber precedes through small intestine almost unchanged (4,20).
For protein digestion numerous enzymes are present in brush-border membrane; after full or partial digestion amino acids, dipeptides, tripeptides and oligopeptides are absorbed and further digestion proceeds in the enterocyte cytoplasm (4,21).
Triglycerides and phospholipids are not completely digested but absorbed as monoglycerides and lysophospholipids together with free fatty acids. Cholesterol esters are digested to

cholesterol and fatty acids. Inside the enterocyte are re-esterified and apolipoproteins synthesis takes place. These are incorporated into chylomicrons and very low-density lipoproteins and secreted at the basolateral enterocyte membrane (4,22).
Minerals, vitamins and other micronutrients usually have specific absorptive transport mechanisms. Iron and calcium are more soluble in acidic conditions and are absorbed in proximal intestine. Cobalamin has to be bond with stomach intrinsic factor and has limited region of absorption in the terminal ileum (23-25). Conjugated bile acids are reabsorbed (26).

6.4 GENERALIZED MALABSORPTION VERSUS SPECIFIC MALABSORPTION STATES

6.4.1 GENERALIZED MALABSORPTION

Generalized malabsorption commonly results from small intestine diseases, but pancreatic diseases and other organ diseases can be involved. Major intestinal findings are summarized in Table 6.4.

Table 6.4 Mechanisms of intestinal malabsorption.

Condition	Mechanisms
Short bowel syndrome	Loss of absorptive area Loss of digestive enzymes Rapid transit
Celiac disease	Reduced absorptive area due to villous atrophy Reduced enterocyte digestive enzymes Impaired intestinal hormonal secretion
Crohn's disease	Loss of functioning intestinal area Bypassed gut Small intestinal bacterial overgrowth
Lactose intolerance	Normal genetic non-persistence of lactase Secondary forms from mucosal injury
Bile acid malabsorption	Secondary to impaired bile acids reabsorption Overproduction in primary bile acids diarrhea
Infections	Reduced brush border enzymes Metabolic effects on enzyme and nutrients Villous atrophy, lymphangiectasia

Short bowel syndrome

Short bowel syndrome occurs when there is insufficient functioning bowel to meet individual's needs for macronutrients, salt and water from an ordinary diet, without artificial supplementation. This usually occurs when less than 200 cm of functioning bowel remains. The most common causes in children are congenital disorders and volvulus resulting in small bowel resection, in adults these are Crohn's disease and mesenteric vascular occlusion (1,4).

Coeliac disease

Celiac disease is chronic autoimmune condition in genetically susceptible individuals triggered by dietary gluten. There is a wide scale of disease severity: from clinically silent to severe malabsorption. Concomitant anemia and osteoporosis are usual, small intestine lymphomas and adenocarcinomas are rare. Association with other autoimmune diseases like

type 1 diabetes mellitus (27) and autoimmune thyreopathies is described (28). Diagnostically anti-tissue transglutaminase (IgG tTGA) and anti deamidated-gliadin (IgG DGP) plasma antibodies are recommended, and small bowel biopsy is performed.

Crohn's disease

Crohn's disease is a chronic inflammatory bowel disease that can affect any part of digestive tract; small bowel is involved in 70% of cases. Genetic susceptibility plays an important role. Major features in pathogenesis are gut microflora, mucosal permeability and host immunity. Malnutrition in Crohn's disease is multifactorial: anorexia, increased energy expenditure and reduced intestinal absorption. In patients with extensive small bowel involvement, picture similar to short bowel syndrome occurs, both macro- and micronutrients being malabsorbed together with imbalances in water and electrolyte metabolism. Terminal ileum is involved often; therefore cobalamin and bile acids are malabsorbed.

Laboratory tests include routine tests and special antibodies determination: pANCA (perinuclear anti-neutrophil antipody) and ASCA (anti-Saccharomyces cervisiae antipody). Colonoscopy and imaging methods are being involved (4,6,29).

Whipple's disease

Whipple's disease is a systemic disease caused by Gram-positive bacterium Tropheryma whipplei. Except of bowel disease affects joints, nervous and cardiovascular systems. Due to blocked lymphatics mostly postabsorptive malabsorption is present. Diagnosis is based on typical histological findings in small bowel biopsy: lamina propria populated by periodic acid-Schiff-positive foamy macrofages, PCR analysis is available (1).

Other infectious causes

Acute or chronic infection underlies many cases of malabsorption in developing world; similar conditions may affect travelers to such countries.

Acute tropical sprue follows acute (mostly viral) infections; chronic tropical sprue is probably associated with chronic bacterial infection of the upper gastrointestinal tract. Generalized malabsorption is often combined with specific folate malabsorption; diagnosis is made upon histology in small bowel biopsy.

Giardiasis is caused by upper small intestine colonization by trophozoite Giardia lamblia. Once established infestation by these leaf shaped microorganisms covering intestinal mucosa causes malabsorption by barrier effect, down regulation of brush-border enzymes is also present. Diagnosis is made from histological and microbiological observation in small bowel biopsy sample (1).

6.4.2 CARBOHYDRATE MALABSORPTION

Lactose intolerance

Lactose intolerance is most common type of malabsorption in Europe. Mucosal lactase concentrations are highest at birth and are essential for the absorption of energy from milk monosacharides. Lactase levels decrease following weaning during childhood in most populations (lactase non-persistance) resulting in intolerance of dairy products. Congenital defects are rare, secondary deficiency accompanies mucosal injury – like in celiac disease, giardiasis and radiation therapy. Undigested lactose reaches the colon where it is metabolized by bacteria, producing hydrogen, methane, and short chain fatty acids – this leads to symptoms of bloating, cramping and diarrhea. Diagnosis is based upon clinical findings,

exposition tests, hydrogen breath test, rapid test from biopsy and histology of small bowel sample (20,30-38).

Fructose intolerance

In general, fructose is less well absorbed than glucose and galactose. Malabsorption can be explained by low-capacity of carrier mediated facilitated diffusion, consumption of fructose without glucose and amino acids present in the same meal. In diagnosis, exposition and hydrogen breath tests are generally used (13,20,39).

6.4.3 FAT MALABSORPTION

Fat digestion and absorption is complex process with many organs involved, but chronic exocrine pancreatic insufficiency and lack of bile acids together with short bowel syndrome are the major causes of fat malabsorption. Tests available to uncover fat malabsorption are 72-hour fecal fat collection and fecal fat assessment (in Europe almost not used due to low compliance of patients and laboratories) and pancreatic elastase I in stool determination.

6.4.4 PROTEIN MALABSORPTION AND PROTEIN LOSING ENTEROPATHY

Protein losing enteropathies leading to protein malabsorption are rather the syndrome than single disease itself. Patient presents by hypoproteinemia and edemas in the absence of proteinuria, defects in protein synthesis or protein malnutrition. According to the type of mucosal alteration protein loosing enteropathy can be classified into three groups:

Mucosal ulceration occurs in ulcerative colitis, Crohn's disease, gastrointestinal carcinomas or peptic ulcer. *Non-ulcerated mucosa with evidence of altered permeability* is represented by celiac and Whipple's diseases and colagenous colitis. *Lymphatic dysfunction* presents either primary lymphatic disease or secondary to partial lymphatic obstruction.

Laboratory diagnosis is performed using alph1-antitrypsin clearance test from stool or tests using radio-labeled proteins (Gordon's test) (40).

6.4.5 MALABSORPTION OF SPECIFIC NUTRIENTS

Iron

Malabsorption is just one of the causes of iron deficiency, others are dietary inadequacy and low bioavailability, increased demands (childhood) and increased looses (cow's milk enteropathy, menstruation, hook worm infection, bleeding from polyps and tumors, Crohn's diesease). Iron intestinal malabsorption itself usually accompanies protein energy malnutrition where mostly mucosal mechanism is involved (24).

Vitamins

Thiamine (vitamin B_1) malabsorption occurs in states with diarrhea, dysentery, tropical sprue, ulcerative colitis. In some food are present thermo-labile thiaminases (raw fish) and thermo-stabile polyhydroxyphenols (coffee, tea, red cabbage, blueberries, blackcurrants etc.) that can impede thiamine absorption.

Riboflavine (vitamin B_2) absorption is decreased by deficiency of bile salts in patients with cirrhosis or hepatitis; biliary absorption increases when vitamin is taken together with food. Alcohol ingestion interferes with the absorption of dietary riboflavin by inhibiting the hysrolysis of food flavins to riboflavins as well as inhibiting its active transport mechanisms.

Vitamin B_6 (pyridoxol, pyridoxine, pyridoxamin vitaminers) in food is present as vitaminers phosphate. Prior absorption it has to be dephosphorylated by means of a membrane-bound alkaline phosphatase present in intestinal brush border (23).

Cobalamin (vitamin B_{12}) in nature is synthetsized exclusively by microorganisms and is present in animal but not plant products. Food cobalamin is attached to proteins and heating, gastric pepsin and hydrochloric acid action makes is available for binding to R-protein, from which it is released in the jejunum by pancreatic proteases, cobalamin binds to intrinsinic factor and proceeds to ileum where the complex has specific receptor. Main causes leading to malabsorption are pancreatic insufficiency and disease of the ileal region (Crohn's disease, Whipple's disease) (23,25). Diagnostically Schilling's test or novel ^{13}C labeled breath test can be performed (41).

Vitamin C is found almost exclusively in foods of plant origin. Gastrointestinal absorption of vitamin is fast and efficient, active carrier-mediated system has been described. With adequate intake malabsorptive states are extremely rare.

Fat soluble vitamins (A,D,E,K) are hydrophobic substances dissolved in the upper portion of gastrointestinal tract in the lipid phases and emulsions. Pancreatic lipase binds to the surface of fat droplets and catalyzes fat digestion; mixed micelles are formed, with the lipid core containing fat soluble vitamins. These micelles are small enough to gain close proximity to the microvillar surfaces of the intestinal mucosa and facilitating diffusion of fat soluble vitamins across enterocyte membrane. All fat soluble vitamins absorption can be affected by cholestasis, cystic fibrosis, chronic diarrhea, pancreatic insufficiency, Crohn's disease, small bowel resection, jejunal bypass and celiac disease (23).

Bile acids

Bile acids circulate in the neterohepatic circulation 4-6 times per day. Secondary malabsorption occurs when resected or diseased segment of terminal ileum reduces the proportion of reabsorbed bile acids, these then pass into the colon where they produce water and electrolyte secretion leading to diarrhea. When the pool of bile acids is significantly depleted, emulsification of fat in the small bowel is reduced, leading to fat malabsorption (4,40). Bile acids malabsorption can be investigated by selenium-75-homocholic acid taurine scans (26).

6.5 CONCLUSION

There are many diverse causes of malabsorption. Treatment depends on understanding mechanisms of malabsorption involved, identifying the cause by means of laboratory and imaging methods based tests and procedures and giving specific therapy where possible and available.

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7. NEW TRENDS IN THE CLASSIFICATION AND DIAGNOSIS OF EATING DISORDERS

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7.1 INTRODUCTION

7.1.1 INTRODUCTION TO THE CONTROVERSIES IN SYSTEMS OF CATEGORISATION

The taxonomy of psychiatric disorders is under scrutiny as both the World Health Organisation International Classification of Diseases (WHO ICD) and the American Psychiatric Association Diagnostic and Statistical Manual of Mental Disorders (DSM) are being revised. There are questions as to whether this form of categorical approach remains appropriate for psychiatry in the twenty first century. Ideally a system of categorisation links causes and potential treatments. It is uncertain whether the current or proposed eating disorder categorisation fulfils this aim and captures scientifically valid and clinically distinct syndromes using the developing neuroscience information base (1,2).

7.1.2 LIMITATIONS OF THE CATEGORICAL APPROACH TO EATING DISORDERS

7.1.2.1 Eating Disorder Symptoms: fluctuations and migration between syndromes

There are also particular problems within the classification of eating disorders. Cultural changes in eating, exercise behaviours and food production, technology and distribution have led changes in body weight and meal patterns as evidenced by the increasing prevalence of obesity and abnormal eating behaviours (1-7). Both dietary restriction and binge eating (with or without compensatory strategies such as vomiting or excess exercise) can cause large fluctuations in weight and impact on social and emotional functioning. Symptoms such as these now present across a broader diagnostic spectrum than the classical presentation of eating disorders as anorexia nervosa (AN) or bulimia nervosa (BN). For example, abnormal eating patterns or attitudes are common in bipolar disease (8) or Aspergers (9).

Another problem with the categorical approach for eating disorders is that some of the symptoms and behaviours change within an individual over time. For example, it is common for AN to evolve into BN or binge eating disorder (BED)(2,3). Moreover over time people with BN develop addictions and people with BED develop obesity. Therefore, it is possible that an approach which includes underlying quantitative traits may be of more value for categorisation.

7.2 COMORBIDITY AND TRANSDIAGNOSTIC APPROACHES

In the period leading up to the changes in the DSM, a variety of suggestions were made relating to associations between eating disorders and other forms of psychopathology. For example, one idea was to consider AN within the obsessive compulsive disorders spectrum (10-12). An allied idea was that AN could be considered within the autistic spectrum of disorders (13,14). This concept has been revisited and it was concluded that a subgroup of patients may have autistic spectrum disorder neurodevelopmental traits, but that for the majority these occur as a secondary consequence of starvation (15). In addition it has been argued that eating disorders should be categorised within the anxiety set of disorders (16). Yet another proposition was to include obesity as an eating disorder. The idea for this was based

upon similarities in the neural circuits in obesity to those found in the addictions (17). However, a literature review was not supportive of this latter proposition (18). Nevertheless, there is increasing evidence to support the idea that binge eating may be a form of addictive behaviour (19-21). These ideas for a broader transdiagnostic categorisation would fit within a system that would use a dimensional approach to categorisation of eating disorders by registering shared underlying traits that are common to other categories of mental illness.

7.3 THE NOT OTHERWISE SPECIFIED CATEGORY

The fundamental premise of the validity of the categorical approach is clinical utility. Therefore the system has to be usable, reliable and predictive of the course, complications and treatment response. In the DSM IV there were three categories of eating disorders, AN, BN, and eating disorder not otherwise specified (EDNOS) (the latter included, binge eating disorder, purging disorder and night eating syndrome) and also a section of feeding disorders in children. A major problem with this system was that over 50% of the eating disordered population presenting for treatment fell into the EDNOS category. This problem with a lack of specificity meant that one of the aims of the next phase of categorisation in DSM 5 was to reduce the size of the non-specified category.

7.4 THE PROPOSED CHANGES INTO DSM5

The process of revising the DSM 5 categories involved commissioning 13 literature reviews published and freely available in a 2009 edition of the International Journal of Psychiatry. The preliminary recommendations were published in 2010 on the DSM 5 web site (www.dsm5.org). In order to reduce the size of the EDNOS category several changes have been proposed for eating disorders in DSM 5.

7.4.1 ANOREXIA NERVOSA (AN)

The criteria used to define AN have been changed so that they are clearer and more broadly applicable.

Criteria A: restriction of energy intake relative to requirements leading to a significantly low body weight in the context of age, sex, developmental trajectory, and physical health. Significantly low weight is a weight that is less than minimally normal, or, for children and adolescents, less than that minimally expected.

Criteria B: intense fear of gaining weight or becoming fat, or persistent behavior that interferes with weight gain, even though at a significantly low weight

Criteria C: disturbance in the way in which one's body weight or shape is experienced, undue influence of body shape or weight on self-evaluation, or persistent lack of recognition of the seriousness of current low body weight"

Criteria D: this criteria (the need for amenorrhoea) has been dropped.

7.4.2 BULIMIA NERVOSA (BN)

There has been very little change in the criteria for BN. The key change has been broadening criteria by reducing the requirement of two binges per week for diagnosis, to only one per week: "The binge eating and inappropriate behavior both occur, on average, at least once a

week for three months". The other change is that sub-typing into purging and non purging categories has been dropped.

7.4.3 BINGE EATING DISORDER

This category has been moved from the appendix into the main section. The duration and frequency of binges has been changed in order to produce parity with BN: "The binge eating occurs, on average, at least once a week for 3 months"

7.4.4 CHILDHOOD FEEDING DISORDERS

The childhood feeding disorders, such as pica and rumination disorders, are subsumed into the DSM5 feeding and eating disorders category. Avoidant/Restrictive Food Intake Disorder (ARFID) replaces feeding disorder of infancy and early childhood in order to provide more specificity.

7.4.5 FEEDING AND EATING CONDITIONS NOT ELSEWHERE CLASSIFIED (FEC-NEC)

Feeding and Eating Conditions Not Elsewhere Classified (FEC-NEC) will replace the term eating disorders not otherwise specified (EDNOS) and will contain the following subcategories.

- Atypical Anorexia Nervosa
- Sub threshold Bulimia Nervosa
- Sub threshold Binge Eating Disorder
- Purging Disorder
- Night Eating Syndrome

In summary the provisional recommendations for the DSM 5 Feeding and Eating disorders category will include the following categories:

- Pica
- Rumination Disorder
- Avoidant/Restrictive Food Intake Disorder
- Anorexia Nervosa
- Bulimia Nervosa
- Binge Eating Disorder
- FEC-NEC

7.4.6 ICD 11

The task force for updating the ICD form of classification is currently meeting. One aim is dissolve divisions between childhood and adult types of disorder and other aims include to ensure that different cultural presentations of illness are recognised. For example fear of obesity (laparophobia) is not a universal sign of AN and in other cultures the individual may present with subjective complaints of epigastric pain, gastritis, and bloating.

7.5 DIMENSIONAL APPROACHES TO CATEGORISATION

It has been argued that quantitative traits may be more useful than categorical taxons in psychiatry. One argument to support this approach is the high level of comorbidity for most disorders with personality and developmental disorders and other axis one disorders. Within the US the NIMH has launched the research domain RDoC project

(http://www.nimh.nih.gov/research-funding/rdoc/nimh-research-domain-criteria-rdoc.shtml). This aims to move psychiatric diagnosis from the use of categories onto a system that reflects the organisation of neural circuits and their associated behaviours. An additional aim of this project is to improve translation of basic science into new treatments. This is an integrative approach with a dimensional approach to genetic, neural and behavioural features. It consists of a matrix of 5 domains of functioning (positive valence, negative, cognitive, social and arousal systems) with a variety of constructs within them and seven units of analysis: genes, molecules, cells, circuits, physiology, behavior and self report. This system at the moment is "work in progress" and expert workshops are being held to consolidate the ideas (Table 7.1).

Table 7.1 A grid summarising Research Domain Criteria (NIMH) Eating Disorders.

Domains	Gene	Mol	Cell	Circuit	Physiology	Behaviour	Self Report	Comment
Positive Valence				+		+	+	Differences between binge & restriction
Negative Valence						+	+	State effects
Social				+		+	+	State effects
Cognition				+		+	+	Differences between binge & restriction
Arousal					+			

7.5.1 APPLYING RESEARCH DOMAIN CRITERIA TO EATING DISORDERS.

In the following section we summarise the evidence for abnormalities within each construct citing systematic reviews wherever possible. The neuroscience literature in eating disorders is an area of rapid growth and the aim is not to synthesis all of the available literature but rather to explore what a dimensional framework might have to offer in terms of structuring research and clinical work.

7.5.2 POSITIVE VALENCE

A variety of constructs have been delineated within the positive valence domain. These include approach motivation in which there are sub categories such as reward, valuation, effort valuation/willingness to work, expectancy, action selection (preference based decision making). The following constructs describe the processes after reward consummation and include the initial response to reward attainment, the longer term response to reward attainments, followed by reward learning and habit formation.

Many of these constructs have been studied in eating disorders using self report, behaviour and neural circuit paradigms. A systematic review of this construct concluded mainly based on the self report literature that people with restrictive AN had reduced approach to reward whereas the opposite was the case for people with BN (22). Scanning paradigms suggest that people with BN may have greater activation to anticipation (approach motivation) to reward but less activation to consummation (taste) of the reward (23). However the response to food anticipation has been variable even in studies in the same group (24). The differentiation between the subsets of constructs with the positive valence domain may help synthesise the literature. It is probable that biological underpinning either over or loss of control of eating can be delineated.

7.5.3 NEGATIVE VALENCE

The constructs defined within the negative valence system include, acute threat (fear), potential harm (anxiety), sustained threat, frustrative non reward and loss. A systematic review of sensitivity to punishment and anxiety concluded mainly from self report data that this construct was relevant to all form of eating disorder (22). Indeed a shared conceptualisation of anxiety and eating disorders has been developed (16). Studies using behavioural paradigms suggest that there is vigilance to social threat (25-27) and less modulation by positive cues (28). Scanning paradigms have found anomalies only in cerebellar activation to standard images that elicit fear and disgust in the acute state (29) whereas no difference in the response to happy and sad faces was found after recovery from AN (30). Some of the anomalies within this system may be state rather than trait effects, and anxiety may be general or limited to weight, shape, food and interpersonal meanings.

7.5.4 SOCIAL PROCESSES IN EATING DISORDERS

The five fundamental elements of social processing as defined by the National Institute of Mental health NIMH Research Domain Criteria (RDoC) include theory of mind, social dominance, facial expression recognition, attachment and self regulation. A systematic review of the eating disorder literature suggests that there are both self-report and behavioural abnormalities in theory of mind and emotional recognition (31). Scanning studies also find anomalies (reduced amygdala and parietal activation) in the response to faces depicting social emotions such as anger in BN (32) and to a theory of mind task in people with a past history of AN (33). More work to understand this aspect of the psychopathology of eating disorders is needed.

7.5.5 COGNITIVE PROCESSING

The six cognitive constructs in the RDoC include attention, perception, working memory, declarative memory, language behaviour and effortful control. Not all of these are constructs have been studied within the eating disorders field whereas other constructs which are not in the system such as set shifting have been studied in detail. For example, self report, behavioural and neural activation to set shifting paradigms are impaired in people with eating disorders (34-36).

There may be differences in these constructs across the eating disorder spectrum. For example, adolescents with AN have demonstrated superior working memory performance (37) whereas people with obese binge eating have demonstrated limited performance (38). Moreover state effects occur as working memory is reduced in the presence of food cues (39,40). People who binge eat have less effortful control on both self-report and behavioural measures (41).

Weak central coherence (a tendency to prioritise detail over the bigger picture) might be considered to be a form of perceptual anomaly and is found particularly in AN (42).
Anomalies in attentional processes towards food and body image have been found in people with eating disorders (43-45). The pattern of cognitive anomalies varies across the eating disorders spectrum and within the illness state.

7.5.6 AROUSAL AND REGULATORY STATE

These constructs include both arousal and resting state regulation. Increased stress reactivity is found in eating disorders (46) and one conceptual model summarises the evidence for eating disorders to arise as a form of chronic stress reaction (47). The literature in this area has not been very active over the last decade but scanning studies of the resting state are in progress.

7.5.7 CONCLUSION TO DIMENSIONAL APPROACH

In table 1 the form of evidence across the constructs framed the proposed research domain criteria project (RDoC) in eating disorders is summarised. Many cells in this matrix are empty and studies which combine a variety of technologies such as genes/molecular paradigms with circuits are needed.

7.6 CONCLUSION IN SUMMERY

The standard categorical method for making a diagnosis for eating disorders is undergoing change. However, it is possible that cultural changes in food content and eating behaviour may foster a wider problematic relationship to food weight and shape, which may not be adequately captured by this type of approach. The dimensional approach holds promise. Over time it may be possible to populate the matrix proposed by the RDoC with more data. This dimensional approach may clarify the different forms that eating problems take, and explain how the disorder changes over time. This framework can structure research and may suggest ways to frame the clinical assessment and formulation and possibly tailor treatment.

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8. REFEEDING SYNDROME: WHAT DOES IT REALLY MEAN?

Jasna Leniček Krleža

8.1 INTRODUCTION

Refeeding Syndrome (RFS) is a set of clinical symptoms (heart, lung, liver, and kidney, neurological, metabolic and haematological) that occur as a result of fluid and electrolyte shifts in malnourished patients in response to an inappropriately high-calorie diet. This applies to any form of complementary foods after several hours of fasting. It includes enteral or parenteral nutrition, as well as uncontrolled and unlimited oral intake. Changes in fluids and electrolytes are caused by hormonal and metabolic changes that can cause severe complications and death. The main biochemical feature of refeeding syndrome is hypophosphataemia, although the syndrome is complex and may include a disruption in the balance of water and nitrate content, alterations in the metabolism of glucose, fat and protein, thiamine deficiency, hypokalaemia and hypomagnesaemia. These deficiencies vary from case to case. They depend on the severity of the health status of the organism (type and duration of primary disease), on the nutritional status or on the severity of the malnutrition of the organism which suffers from the amount of the retained energy, vitamins and minerals "reserve" as an effect. Their deficiency is visible by measuring their concentrations in the blood. In doing so, a deficiency in all minerals is not necessarily pronounced; all combinations are possible with various severity levels for each. Thus, the symptoms that occur as a consequence of this deficiency are unpredictable and can range from asymptomatic cases to the dysfunction of any of the vital organs; since is not treated (correction of electrolyte imbalances) they can cause a coma and death (1-5).

Over the last sixty years, since RFS was first clinically described and published, what we in fact call RFS has still not been clearly defined.

A clear international consensus has not been set on when we can truly say that RFS has been developed: is RFS just a laboratory diagnosis or a moment when clinical symptoms are presented. A laboratory diagnosis of RFS refers to moment when during the feeding of malnourished patients, the serum concentration of one or more major electrolytes (particularly phosphates) declined without any clinical symptoms (or without any clearly expressed symptoms), while the appearance of symptoms (disturbance in vital function), in laboratory findings implies electrolytes disturbance (primarily low phosphates)(6,7).

In a review of the published literature, we can find individual case reports or reports on series of cases, cohort studies and expert opinions. The level of evidence (LOE) of the published studies is III and IV. On this basis, in 2006 the National Institute for Health and Clinical Excellence (NICE) claimed that the recognition and timely inclusion of compensatory mechanisms is essential to reduce, if not completely eliminate the morbidity and mortality associated with this phenomenon. Guidelines for the management of RFS in adults have been published. These guidelines define the criteria for risk and high risk patients (Table 8.1), as well as instructions for the compensation mechanisms if electrolyte deficits are presented. However, in medical practice, RFS still frequently occurs (2,4).

Table 8.1 High risk criteria for development refeeding syndrom.

One or more of the following symptoms:	**Two or more of following symptoms:**
• BMI less than 16 kg/m^2 • unintentional weight loss greater than 15% within the last 3–6 months • little or no nutritional intake for more than 10 days • low levels of potassium, phosphate or magnesium prior feeding.	• BMI less than 18.5 kg/m^2 • unintentional weight loss greater than 10% within the last 3–6 months • little or no nutritional intake for more than 5 days • a history of alcohol abuse or drugs including insulin, chemotherapy, antacids or diuretics.

Modifed from National Institute for Health and Clinical Excellence (NICE) guideline − Nutrition support in adults (2).

Legend: BMI - Body Mass Index

RFS is generally a term associated with marked malnutrition, primarily with anorexia. However, many clinical conditions represent a risk for RFS. There are three basic groups of patients that are at risk of developing RFS: (1) individuals with a low intake of nutrients, (2) individuals with an increased loss of nutrients, and (3) individuals with a reduced absorption. These groups include a wide range of individuals for whom various medical conditions as well as social, economic and psychological statuses that affect all age groups are responsible for malnutrition and risk (6,7).

8.2 THE INCIDENCE OF REFEEDING SYNDROME

The lack of a clear definition of RFS entails the lack of randomized controlled trials and published data on its frequency. Moreover, as was already mentioned, often mild symptoms frequently remain unrecognized, and low electrolytes are often associated with secondary diseases. For these reasons, the frequency of RFS remains unknown. According to a study by Camp and Allon, in 10197 hospitalized patients, the incidence of severe hypophosphatemia was 0.43% (8). The published results of studies on patients who were on total parenteral nutrition (TPP) revealed that 30% to 38% of patients who received phosphate had hypophosphataemia, as did 100% of patients who received no phosphates in the parenteral nutrition (9), while the frequency RFS in oncological patients was observed in more than 25% of patients (10).

8.3 THE PATHOPHYSIOLOGY AND CHARACTERISTICS OF REFEEDING SYNDROME

Under normal circumstances, the body uses glucose as a primary source of energy. This requires a continuous intake of carbohydrates. Two to three hours after the intake of carbohydrates, blood glucose becomes available and is retained as glycogen. The amount of glycogen is limited and provides a short-term source of energy to the body in situations where food intake fails. The body thus retains proteins that would not be used for energy purposes. Excessive food intake, that is, excessive energy intake, is stored by the body as fat, which represents the most important energy reserve in the human body (11).

In a short period of fasting (24 hours), glycogenolysis takes place in the liver and in muscles to compensate for glucose. After the glycogen is expended, the gluconeogenesis process begins. Amino acids from muscle protein and fatty acids from fatty tissues provide the organism glucose as an energy source through metabolic reconstruction. With gluconeogenesis pyruvate and lactate may also provide glucose. This initial fasting period marks increased protein degradation (11).

If fasting continues, the body will slow down and reduce its basal metabolism rate by about 20 to 25%. Most organs and tissues in these conditions switch to fatty acids as an energy source. The brain is the organ that primarily uses glucose, and can only partially switch to ketones as an energy source. By switching to fat as an energy source in the body, protein and muscle mass are maintained. The reduction of the proteolysis rate, an increased migration of fatty acids and formation of ketone is characteristic of this period. In addition, at this stage there is also a decrease in intracellular concentrations of minerals (electrolytes) and vitamins. Serum electrolyte concentrations usually remain normal because of intracellular space contraction, decreased renal excretion and withdrawal from storage in the body. Under these conditions, the body slowly begins to consume itself and this is its mode of survival. Bearing these metabolic changes in mind, they are changes that mostly occur in thin or emaciated and malnourished people. However, they can also occur in overweight people who resort to starvation as a means to decrease their body weight (11).

During the supplemental feeding process, the increase in blood glucose leads to increased insulin secretion and decreased glucagon secretion. This hormonal shift results in the stimulation of the synthesis of glycogen, protein and fat. Minerals such as phosphor and magnesium, as well as cofactors such as thiamine, are necessary for these processes. Insulin stimulates the absorption of potassium and glucose into the cell, and magnesium and phosphates enter the cell. This leads to a reduction in serum concentrations of phosphates, potassium and magnesium which are already in deficit in the body. Clinical symptoms of refeeding syndrome occur as the result of a lack of these minerals and rapid changes in the basal metabolism rate (1,11).

Phosphor is the primary intracellular mineral. It is found in blood in the form of free and protein-binding inorganic phosphate. It is essential for many intracellular processes: it activates enzymes and messengers, stores energy in the form of adenosine triphosphate (ATP), regulates the affinity of haemoglobin for oxygen and thus regulates the supply of oxygen to the tissues, participates in the regulation of acid-base balance, is an integral part of DNA, RNA and cellular membranes and is responsible for its integrity. The depletion of phosphor in the whole body occurs in the "refeeding" syndrome and insulin secretion leads to increased absorption and utilization of phosphate in the cell. This leads to a deficit of intracellular and extracellular phosphor. In these conditions, even slight decrease in serum phosphate levels can lead to a significant dysfunction of cellular processes that affects virtually every physiological system. Hypophosphatemia is a surrogate marker for RFS, but may be present before the feeding of undernourished patients with malnutrition, in diabetes, alcoholism, or in prolonged severe respiratory alkalosis and in vitally endangered patients. Moreover, antacids can also be a cause of hypophosphatemia. (12,13,14).

Potassium, the main intracellular cation, is also deficient in malnutrition, although its serum concentrations may remain normal. Starting anabolic processes leading to the absorption of potassium into the cell stimulated with insulin, which results in severe hypokaliemia, changes in electrochemical membrane potential, abnormal heart rhythm and heart failure. Hypokaliemia is probably the most common electrolyte deficit in clinical practice. The causes of hypokaliemia, besides in refeeding, are an increased loss of stool (diarrhea), an increased

loss of urine, metabolic alkalosis, or secondarily as a result of taking many drugs (diuretics, b-adrenergic agents, high-dose glucocorticoids, insulin)(12,14,15).

Magnesium, predominantly an intracellular cation, is an important cofactor in most enzyme systems, such as in those involved in oxidative phosphorylation and ATP production. It is essential to the structural integrity of DNA, RNA and ribosomes, it affects the membrane potential of cells and its deficiency can lead to changes in neuromuscular excitability and heart disorders. Besides in refeeding, where we find acute hypomagnesemia, the cause of reduced magnesium levels may be diarrhea, pancreatitis, malnutrition, alcoholism, metabolic acidosis, or secondarily as a result of therapy (amphotericin B, furosemide, aminoglycosides, cisplatin, cyclosporin). Hypomagnesemia can cause hypocalcemia and hypokalemia (K activator, the ATP-ase pump, and impairs the release of parathyroid hormones). It can also be falsely lowered in cases of significant hypoalbuminemia. The correction of a potassium deficiency along with magnesium values (the lower reference range or lower) is not possible without correcting the magnesium. (12,14,16).

Hypocalcemia is a sign of lower total serum calcium levels and is present relatively often in different states (sepsis, pancreatitis, trauma). Serum calcium concentrations return to normal values 5 to 6 days after recovery from an acute illness. The etiology of hypocalcemia is multi-factorial and not fully understood. However, the interpretation of findings of total serum calcium may be incorrect in situations where there is markedly reduced albumin, which is often found in malnourished patients. Under conditions in which the concentration of serum albumin is low, it is necessary to determine the ionized calcium level (12,17).

Thiamine deficiency is the most common vitamin deficiency that occurs as a re-alimentation complication. Thiamine is an essential cofactor of key enzymes in the metabolism of carbohydrates and synthesis of ATP. Its deficiency leads to Wernicke's encephalopathy (ataxia, ophthalmoplegia, confusion, hypothermia, coma) or Korsakoff's syndrome (amnesia, confabulation)(4,18).

Changes in carbohydrate metabolism lead to changes in **sodium and water balance**. The introduction of carbohydrates in the diet leads to a decrease in the renal excretion of water and sodium. If fluid intake is increased to maintain dieresis, this can lead to fluid overload, congestive heart failure and pulmonary edema (1,3).

An excessive intake of glucose can lead to **hyperglycaemia, osmotic diuresis and dehydration**. There can also be a stimulation of lipogenesis, which can lead to fatty changes in the liver, increased production of carbon dioxide, hypercapnia and respiratory failure (1).

Mutual hormonal and biochemical relations and the consequences that arise during feeding after starvation can be seen on Figure 8.1 (1).

During fasting, due to the reduced food intake and the minerals and vitamins in the body, there is a lack of them in the body. Nevertheless, through control mechanisms and their redistribution between compartments, concentrations of electrolytes in the blood are mainly normal. Baseline concentrations of electrolytes were measured before refeeding; this is why they are not a true reflection of the state of the organism. Reduced basal electrolyte concentration values can be found when due to other disturbances compensatory mechanisms are no longer sufficient. The principle of complementary foods in such cases requires a specific approach, but is basically clear.

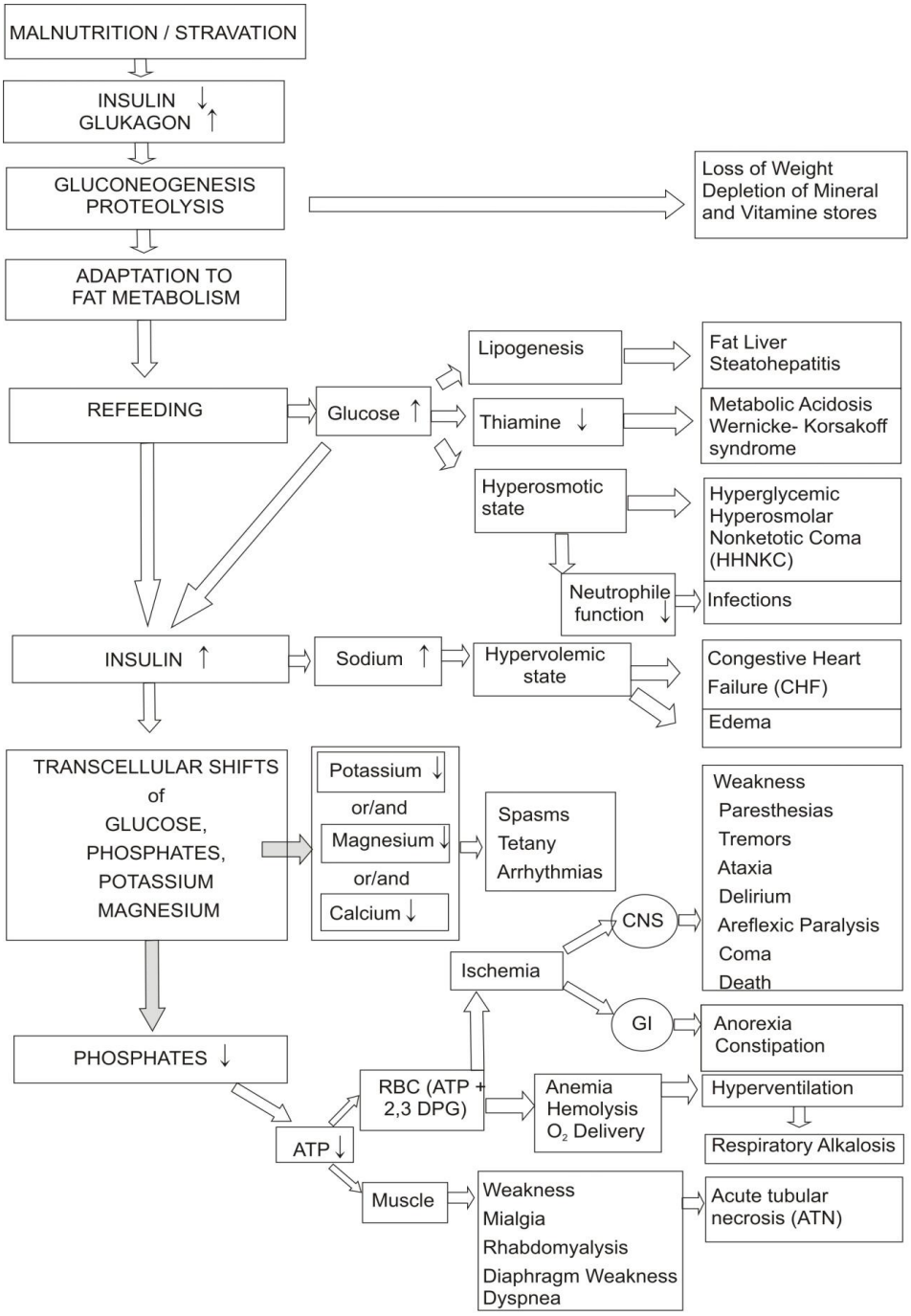

Figure 8.1 Consequences of the major metabolic and biochemical changes in the refeeding syndrome. Modified from Boateng (1).

8.4 LABORATORY MONITORING

During the supplemental feeding of malnourished patients, laboratory monitoring are an integral part of NICE guidelines and come in the form of protocols. The protocols include biochemical tests along with the dynamics of definition and also note explanations and interpretations. These protocols were developed by a group of professionals (Guideline Development Group, GDP) on the basis of good practice point (GPP) and as a recommendation for the best practice based on experience (LOE 3 or 4) and/or formal consensus (2,3). According to this protocol, laboratory monitoring includes:

Sodium, potassium, urea and creatinine measurement for the assessment of renal functions as well as the status of water, Na and K. The interpretation of results includes insight on therapies and fluid balance.
After the initial values, the dynamics of determination include monitoring once a day until the results are stable, and then 1 to 2 times a week. The determined of K and Na in the urine can be of use when tracking and replenishing electrolytes that are lost gastrointestinally is necessary.

Glucose measurement for monitoring glycaemia and due to possible resistance to glucose, which is not a rare occurrence.
After the initial value, values need to be determined twice a day or more if necessary, until the results are stable; then they are to be determined once a week.

Magnesium and phosphates in the body are often depleted despite initial values that are often within the reference range.
After the initial values are determined, once a day during the first week (there is risk of developing refeeding syndrome), then 2 to 3 times a week until the values are stable, then once a week.

Liver function tests including **International Normalised Ratio (INR)**: deviation of normal values is common, although the reasons can be complex and should be interpreted taking this into consideration. Deviation in laboratory tests could be a result of parenteral nutrition, sepsis or other disorders.
The initial values are determined, then again twice a week until values are stable, then once a week.

Calcium and albumin: a possible presence of hypo and hyperkalemia. Albumins are not a measure of the status of protein, rather a measure of malnutrition or disease. It is necessary to correct serum calcium levels because of albumin.
The initial values are determined, then again once a week.

C-reactive protein (CRP) is necessary to assess the presence of acute phase reaction. The CRP value is important in interpreting, but can also be helpful in assessing the results of other parameters (proteins, trace elements, vitamins).
The initial values are determined; then again 2 to 3 a week until results are stable.

Zinc (Zn) and copper (Cu), trace elements, are often lacking in malnutrition, even when the loss is increased. When interpreting the results it should be borne in mind that the acute phase (an elevated CRP level) can be reflected in a reduced Zn level and an increased Cu level.
The initial values are determined, then, depending on the results, again every 2 to 4 weeks.

A selenium (Se) deficiency is found in severe disease and sepsis, but also in long-term nutrition support. The acute phase lowers Se values.
Is determined only when a deficiency is suspected, and further determinations depend on the initial one.

Complete blood count (CBC) and MCV: Anaemia due to iron deficiency or folic acid is common in malnourished patients. Sepsis significantly affects the results of the CBC.

The initial values are determined; then again 1 to 2 times a week until results are stable, then once a week.

Iron (Fe) and ferritin. Iron deficiency is common in long-term parenteral nutrition. When interpreting the results, the acute phase reaction should be paid close attention to, when the Fe is reduced and ferritin elevated.
The initial values are determined, then again every 3 to 6 months.

Folate, B12: Serum folate/B12 sufficient, with full blood count.
The initial values are determined, then again every 2 to 4 weeks.

Manganese and 25-OH vitamin D. These tests are rarely needed, unless there is cause for concern (2,3).

8.5 REFEEDING SYNDROME FROM A LABORATORY PERSPECTIVE

RFS is a complication that arises during the supplemental feeding of malnourished patients. The dynamics of laboratory monitoring of these patients according to the protocols of published guidelines (NICE, 2006) (2) allows the identification of the development RFS before the onset of symptoms and with the correction of the deficiency symptoms are prevented from being developed. In this respect, RFS is a laboratory diagnosis. This understanding includes laboratory professionals in and commits them to the team that treats and cares for such patients. What can a laboratory expert do in this regard? What is important for good laboratory monitoring? How can laboratory specialist help a physician in interpreting the laboratory test results?

From a laboratory perspective, the most important feature of RFS is the rapid and uncertain change in the concentration of glucose and electrolytes in serum, which depends on the applied therapeutic procedure. These treatments are generally not known to the laboratory professional, and neither is the weight of the underlying disease, which may or may not be present.

Standardized methods of determination, standardized complete pre-analytical procedures and analytical phases of laboratory work, as well as the implementation of quality control are the basic requirement for good laboratory practices (according to ISO 15189). Laboratory errors were thus reduced to a minimum. The interpretation of the laboratory test results is part of the post-analytical phase, and it is common to both biochemists and clinicians.

In medical practice, most often the obtained laboratory tests results are compared with the reference values that are determined by the age and sex of patients. This is the so-called longitudinal evaluation of laboratory test results. The usefulness of such assessments in laboratory monitoring may be calculated through an index of individuality (II) for each analyte. It represents the ratio of inter-individual (CVI) and intra-individual variability (CVintra). The smaller the ratio (II), the more pronounced the individuality of each analyte is, and hence the usefulness of the reference interval lessens (19,20).

Laboratory monitoring is used to monitor the effects of therapeutic procedures on the health status of patients. This means that the assessment of laboratory test results is relevant to the comparison of the obtained values to the preceding results. The slightest differences between two obtained concentrations in the required medical reports that could be linked to actual changes in the patient’s medical status are referred to as critical differences, or "reference change values" (RCV). The usefulness of this estimate is far greater because it involves biological (intra-individual) and analytical variation. The formula and method of calculating the RCV according to Ricos and colleagues can be found in literature (19). The database containing biological and analytical variations is continually updated and is available on the website (21). Ricos and colleagues calculated the RCV values for 216 analytes (22), while Table 8.2 shows the RCV values for laboratory tests that are used in the daily laboratory monitoring of RFS. The RCV values were calculated from changes in healthy subjects, and were present at a 95% probability level, provided that the CVA < CVI (approximately CVA CVI x = 0.5). RCV values in diseased states in individual analytes were significantly higher, although studies have shown that RVC in diseased and healthy states do not significantly

change (20,22,23). However, in clinical terms, this is not significant because it is better to register the change, and then to decide that this is clinically unimportant, rather than to ignore the change in the patient's condition. Furthermore, the analytical imprecision of the (CVA) is replaced by the desired analytical quality specifications that is based on biological variation (CVI 0.5). Of course, a prerequisite for this is the daily implementation of internal quality control on the laboratory's analytical phase to achieve the desirable specifications for imprecision, inaccuracy and allowable total error (21). For the proper interpretation of the laboratory test results, knowledge of the possible interferences is also necessary. It is particularly important to reduce the impact of all the factors from the preanalytical phase on the results (potential errors in blood tests, transport conditions, separation, sample storage)(20,23). This is done by introducing clear and precise protocols in and outside of the laboratory, and is based on guidelines published by the Clinical and Laboratory Standards Institute (CLSI) and the World Health Organization (WHO) (24,25). Furthermore, the effects of the most common interferences (haemolysis, icterus, lipemia) should be clearly defined, as well as other known interferences (drugs), or states in which the analytes can be altered.

The determination of the initial or basic values of all laboratory parameters provided by the working diagnosis is of great importance. These are values that are determined prior to any therapeutic or diagnostic procedure.

The longitudinal assessment of the initial laboratory tests allows a series of therapeutic procedures. It also presents a comparative value along with the value of the next scheduled laboratory monitoring point.

Table 8.2 Biological (intra- and interindividual) variation, analytical variation, index of individuality and reference change values for analytes in daily monitoring of refeeding syndrome.

Analyte	CV_I (%)	CV_{INTRA} (%)	Index of individuality (II) (CV_I/CV_{intra})	CV_A Desirable (%)	$RCV_{95\%}$ Desirable specification ($CV_A \sim 0.5CV_I$)(%)
Phosphate	8.5	9.4	0.90	4.3	26.3
Potassium	4.8	5.6	0.86	2.4	14.9
Magnesium	3.6	6.4	0.56	1.8	11.2
Calcium	1.9	2.8	0.68	1.0	5.9
Calcium ionized	1.7	1.9	0.89	0.9	5.3
Sodium	0.7	1.0	0.7	0.4	2.2
Glucose	4.9	6.1	0.80	2.5	15.2

Legend: CV_I intra-individual coefficient of variation; CV_{INTRA}: inter-individual coefficient of variation; CV_A: desirable coefficient of analytical variation; $RCV_{95\%}$: reference change values at a 95% probability level.

8.6 CONCLUSION

Key points:

1. RFS is a pathophysiologically well-described condition, and with respects of the protocols of laboratory monitoring in published guidelines (NICE, 2006) it has become a laboratory diagnosis.
2. The RCV value in laboratory monitoring should be an aid in interpreting differences in serial laboratory test results from individuals.

3. The introduction of the RVC in the laboratory reports the value of the reference range would most certainly help the clinician in making medical decisions.
4. This requires good and organized policies implementation to control the quality of the laboratory work.

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Review

Bowels control brain: gut hormones and obesity

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Abstract

Peptide hormones are released from the gastrointestinal tract in response to nutrients and communicate information regarding the current state of energy balance to the brain. These hormones regulate appetite, energy expenditure and glucose homeostasis. They can act either *via* the circulation at target peripheral tissues, by activation of the vagus nerve or by acting on key brain regions implicated in energy homeostasis such as the hypothalamus and brainstem. This review gives an overview of the main gut hormones implicated in the regulation of food intake and how some of these are being targeted to develop anti obesity treatments.

Key words: peptide YY (PYY); glucagon-like peptide-1 (GLP-1); glucagon; ghrelin; bariatric surgery; obesity

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Introduction

Obesity is one of the greatest health care challenges of our time. Currently there are estimated to be 2 billion adults who are overweight worldwide (body mass index 25-30 kg/m^2) and a further 500 million are obese (BMI greater than 30 kg/m^2). Being overweight or obese carries an increased risk type 2 diabetes, ischaemic heart disease, stroke and cancer, and carries an increased risk of both all cause, and cause-specific mortality (1,2). This has prompted a concerted effort to identify effective novel treatments for obesity. The role of peripheral hormones and the gut/brain axis in the regulation of appetite has become a hot topic in recent years, owing to the growing global obesity crisis. Of particular interest has been the potential of these peripheral signals to provide novel targets for developing anti-obesity therapies. The focus of this review is to provide a synopsis of the gut-brain cross talk involved in the regulation of food intake.

Neuroendocrine control of appetite

The hypothalamus and the brainstem are the main central nervous system regions responsible for the regulation of energy homeostasis. Although it is important to remember these regions are not solely responsible. Both the hypothalamus and the brainstem receive peripheral neural and hormonal signals that relay information regarding energy availability, both acutely i.e. nutritional state and long term availability i.e. adiposity (3). Neural afferents and hormonal signals from the periphery are integrated with higher brain centre signals (e.g. relaying reward drive and mood) to regulate appetite and control energy expenditure (4) (Figure 1).

The arcuate nucleus (ARC) of the hypothalamus is believed to play a crucial role in the regulation of food intake and energy homeostasis. The ARC contains two populations of neurons with opposing effects on food intake (5). Orexigenic neurons (i.e. those stimulating appetite) express neuropeptide Y (NPY) and Agouti-related protein (AgRP) (6-8). Whilst anorexigenic neurons (i.e. those inhibiting appetite) in the ARC express alpha-melanocyte-stimulating hormone (alpha-MSH) derived from pro-opiomelanocortin (POMC), and cocaine- and amphetamine-regulated transcript (CART) (9).

The ARC is adjacent to the median eminence, a 'circumventricular organ' with fenestrated capil-

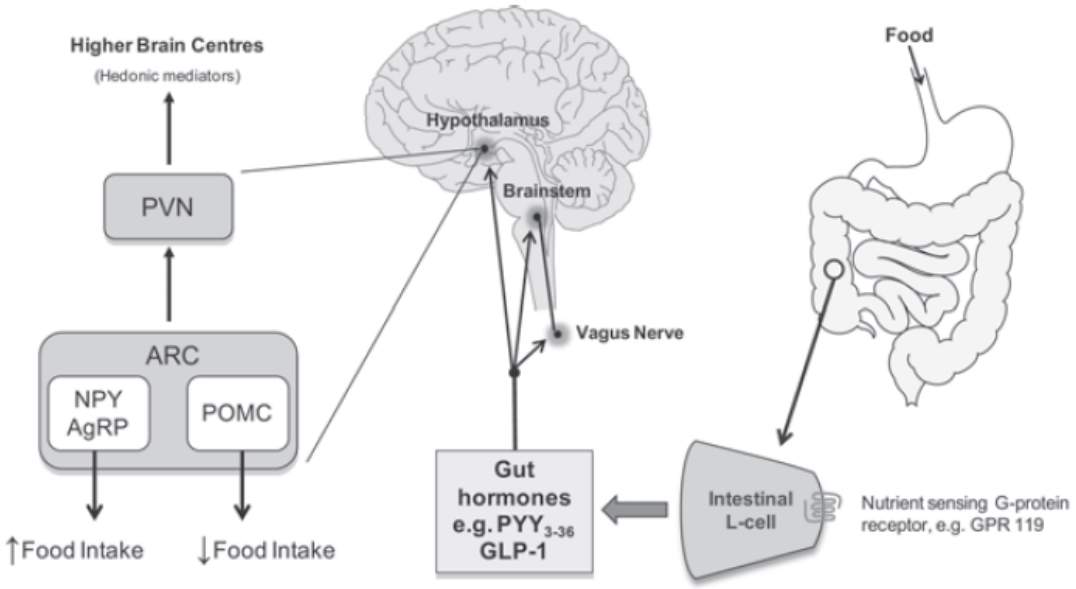

FIGURE 1. Gut-brain axis: regulation of food intake.
Nutrients created by the digestion of food are proposed to activate G-protein coupled receptors on the luminal side of enetroendocrine cells e.g. the L-cell. This stimulates the release of gut hormones which may influence food intake at three sites: the vagus nerve, brainstem and hypothalamus. Within the arcuate nucleus of the hypothalamus two neuronal populations are thought to be critical conduits through which peripheral signals are integrated to alter the drive to eat, the orexigenic NPY/AgRP neurons and the anorexigenic POMC neurons. Further connections between hypothalamic nuclei and higher brain centres may exist which control the hedonic aspects of food ingestion.
ARC - arcuate nucleus; AgRP - agouti related peptide; GLP-1 - glucagon like peptide-1; NPY - neuropeptide Y; POMC – propiomelanocortin; PVN - paraventricular nucleus; PYY - peptide YY.

laries and hence an incomplete blood-brain barrier (10). Circulating hormones are able to pass across the median eminence and influence the activity of the ARC neurons directly. Gut hormones are released from the gastrointestinal tract on a meal to meal basis and signal short term nutrient availability to the ARC. Other circulating factors such as insulin and leptin (a circulating peptide released from adipose tissue) relay information about long-term energy stores and adiposity (11). Thus the ARC has been described as a conduit through which the body can balance its energy requirements to maintain weight.

Additionally short term availability of nutrients is signalled by gastrointestinal vagal afferents. Following a meal the vagus is activated by both mechanoreceptors and chemoreceptors. The resultant neural signals converge in the nucleus of the tractus solitarius (NTS) within the brainstem. These signal are then fed forward Neuronal from the NTS to the hypothalamus. Circulating factors such as gut hormones are also thought to act at the NTS, which like the ARC is adjacent to a circumventricular organ, the 'area postrema' (AP). For example, ablation of both the AP and another circumventricular organ, the subfornical organ (SFO), has been shown to delay the anorectic action of the gut hormone peptide tyrosine tyrosine (PYY) (12). Gut hormones also alter the activity of the ascending vagal pathways from the gut to the brainstem (13).

Hence, the hypothalamic ARC orexigenic and anorexigenic neurons are influenced by numerous neural and hormonal inputs. These ARC neurons in turn project to a number of extra-hypothalamic and intra-hypothalamic regions, including in particular the hypothalamic paraventricular nucleus (PVN), where some of the important efferent pathways regulating energy expenditure arise.

Enteroendocrine cells of the gastrointestinal tract

There are at least 15 different types of enteroendocrine cells diffusely distributed throughout the gastrointestinal epithelium (14). These cells produce and release a variety of hormones and signalling molecules, which together constitute the largest endocrine organ in the body (14,15). Chemosensing of gut luminal contents by the enteroendocrine GI cells plays a critical role in the control of functions such as digestion, pancreatic secretion, food intake, and metabolic regulation. Evidence that endocrine cells can directly sense luminal contents has been demonstrated in PYY and glucagon like peptide -1 (GLP-1) expressing L cells. Both human and rodent L cells express receptors previously identified in the oral epithelium for detecting sweet (T1R2 and T1R3) and bitter sensation (T2R), as well as amino acids (T1R1 and T1R3) (16,17). Additionally, the gustducin G protein which is associated with these taste receptors has been identified in L cells (16,17). Activation of these receptors is thought to lead to increased intracellular calcium and release of gastrointestinal peptides from enteroendocrine cells (17). The presence of the sweet taste receptor subunit T1R3 and gustducin may also underlie a luminal glucose sensing mechanism, since activation of these receptors mediates the postprandial release of GLP-1 from intestinal L cells (16).

Fatty acids derived from digestion of dietary fats appear to be sensed *via* separate mechanisms. The short-chain fatty acid receptors GPR43 and GPR41 are expressed in PYY-containing enteroendocrine L cells (18,19). Short chain fatty acids have been shown to increase both PYY and GLP-1 secretion in rats when delivered directly into the colon (20,21). GPR119 is another G protein coupled receptor found in intestinal endocrine cells as well as pancreatic beta cells (22). Administration of oleoylethanolamide (OEA), an endogenous long chain fatty acid derivative, and other GPR119 agonists increases GLP-1 secretion, both *in vitro* and *in vivo* in rodents (22,23).

The enteroendocrine L cells therefore have the capacity to integrate complex nutrient sensing in the gut and to respond appropriately by releasing gut hormones. In addition to chemical stimulation, the endocrine cells of the gut also respond to neural and physical stimulation of the cell by releasing peptide containing granules at the basolateral side of the cell. These peptides can have an endocrine role, a local paracrine role, and/or activate receptors present on nerves innervating the GI mucosa (24).

Gut hormones regulating food intake

The gastrointestinal tract releases more than 20 different regulatory peptide hormones that influence a number of physiological processes (25). The release of gut hormones such as PYY, GLP-1, and oxyntomodulin (OXM) is stimulated by distension of the stomach, production of nutrients from the digestion of food, and by neuronal signals (26,27).

Gut hormones are believed to contribute to the short-term feelings of satiety and hunger (28). These peptides are thought to reduce food intake by decreasing hypothalamic orexigenic signalling and increasing anorectic signalling (13,29). These peptides also mediate inhibitory feedback mechanisms on intestinal transit, contributing to prolonged gastric distension, and increased satiety between meals (30,31). These combined CNS effects and 'intestinal brake' mechanisms facilitate the control of food intake and postprandial transit through the gastrointestinal tract and thereby the immediate availability of energy. Below the focus of the review will concentrate on 5 of the most studied gut hormones which have been shown to control food intake and body weight and which are being actively pursued as anti-obesity targets.

Peptide tyrosine tyrosine (PYY)

PYY belongs to the 'PP-fold' family of proteins like NPY and pancreatic polypeptide (PP). These peptides are 36 amino acids in length and share a common tertiary structural motif known as the PP-fold. C-terminal amidation of these proteins is a necessary requirement for biological activity. PYY exists endogenously in two forms: PYY_{1-36} and PYY_{3-36} (32). The enzymatic cleavage of secreted

PYY_{1-36} at the amino terminal by the cell surface enzyme dipeptidyl peptidase IV (DPP-IV) gives rise to PYY_{3-36} (33), which is the predominant form of circulating PYY immunoreactivity. PYY_{1-36} and PYY_{3-36} exert their effects through the neuropeptide Y family of receptors (34). PYY_{1-36} binds with similar affinity to all of the Y receptors, however PYY_{3-36} is a selective high affinity agonist at the Y_2 receptor subtype (Y_2R) (35). The Y_2R is thought to be the receptor responsible for mediating the reduction of food intake by PYY. It is an auto-inhibitory pre-synaptic receptor found on NPY neurons within the ARC (36), and deficiency of the Y_2R abolishes the anorectic effects of PYY (29). Furthermore, the anorectic effects of PYY_{3-36} are attenuated by Y_2R antagonists (37). PYY_{3-36} is therefore thought to reduce food intake through activation of the Y_2R.

Low levels of PYY are detected in enteroendocrine cells in the stomach, and levels increase distally along the small and large intestine, reaching their highest levels in cells in the colon and rectum (26). Endogenous circulating concentrations of PYY are lowest in the fasting state, and rise post-prandially in proportion to caloric intake (26). Plasma levels of PYY rise within 30 minutes of a meal, and in humans, circulating levels plateau at 1-2 hours post-prandially, remaining elevated for up to 6 hours (38). Protein rich meals cause the greatest increase in PYY levels compared to other macronutrients (39,40). Peripheral administration of PYY_{3-36} reduces food intake and weight gain in rodents (29,41-43). Intravenous administration of PYY inhibits food intake in humans and unlike leptin is equally effective in normal and obese subjects (44).

The anorectic effects of PYY_{3-36} appear to be mediated centrally via the ARC, as peripheral administration of PYY_{3-36} increases c-fos expression in this hypothalamic nucleus (29). Peripheral administration has been reported to decrease expression and release of NPY whilst activating POMC neurons (29). However, others have reported PYY_{3-36} inhibits POMC neurons via postsynaptic Y_2R (45). Moreover, POMC knockout mice maintain their acute anorectic response to peripherally administered PYY_{3-36}, suggesting that POMC is not critical to the inhibitory effects of PYY_{3-36} on feeding (46).

A vagal brainstem mediated pathway may also be involved since PYY is expressed by the neurones of the myenteric plexus and the Y_2R receptor is expressed by the vagus nerve (47). Furthermore the anorectic effect of PYY_{3-36} on both food intake (47,48), and ARC activation of feeding neurons, are abolished following bilateral sub-diaphragmatic total truncal vagotomy or following transection of the brainstem–hypothalamic pathway in rodents (48).

Interestingly, it has recently been shown that acute effects of gastrointestinal bypass surgery on body weight are lost in *Pyy*KO mice (49), and that wild-type mice losing weight after gastrointestinal bypass surgery exhibit increased colonic *Pyy* expression and circulating fasting PYY levels (49). Suggesting PYY plays a key role in mediating the early weight loss that occurs following gastrointestinal bypass surgery.

The effects of PYY_{3-36} on satiety and central control of appetite are clear. Most are mediated via anorectic neuronal populations in the ARC, but vagal/brainstem-mediated pathways and peripheral effects of PYY on gastric emptying and intestinal motility may also play a part. High plasma concentrations of PYY result in nausea, but the importance of PYY_{3-36} at physiological levels in the regulation of energy intake make it a prime focus for new obesity therapies, targeted either at PYY itself, or against the Y_2 receptor.

Glucagon-like peptide-1 (GLP-1)

GLP-1 is a 30 amino acid peptide produced from the cleavage of preproglucagon (50). The two bioactive forms of GLP-1, $GLP\text{-}1_{7-37}$ and $GLP\text{-}1_{7-36}$ amide, are released into the circulation from L cells of the gastrointestinal tract in response to an oral glucose load (51). Physiologically, GLP-1 is an important incretin, augmenting glucose-dependent insulin release (52). In addition, GLP-1 inhibits the secretion of glucagon, thereby inhibiting endogenous glucose production (53). The net effect is to reduce blood glucose following a meal. GLP-1 also delays gastric emptying (54), and increases satiety (55,56).

Like PYY, GLP-1 has been shown to act centrally at hypothalamic nuclei known to be implicated in

the control of appetite including the ARC, PVN and supraoptic nucleus (57). Both acute peripheral and central administration of GLP-1 reduce food intake in rats (58,59) and chronic administration of GLP-1 reduces weight gain (55). The intravenous administration of GLP-1 to normal and obese humans decreases food intake in a dose dependent manner (60) as well as reducing gastric emptying (61,62). These effects are thought to be mediated through vagal and brainstem pathways since peripheral administration of GLP-1 activates neurons within the brainstem in rats (63). Furthermore, this increase in neuronal activity, and the anorectic effects of GLP-1, are abolished following vagotomy in rodents (48,63). More recently, functional magnetic resonance imaging (fMRI) has confirmed the activation of the VMH and PVN following peripheral administration of GLP-1 (64).

GLP-1 is rapidly degraded in the circulation by DPP-IV, making native GLP-1 unsuitable for therapeutic use. Longer acting GLP-1 mimetics have been developed (65). Exendin-4 is a naturally occurring GLP-1 mimetic isolated from the venom of *Heloderma suspectum*, a lizard native to several southwestern American states (66). A truncated form of this peptide, exendin 9–39, acts as a competitive antagonist at the GLP-1 receptor. Acute intracerebroventricular administration of exendin 9–39 increases food intake and chronic administration increases body weight in rats (55,59). Suggesting endogenous peripheral GLP-1 may physiologically reduce appetite and food intake. However, GLP-1 receptor knockout mice do not have altered food intake or body weight (67). This may be because developmental changes compensate for the lack of GLP-1 signalling, or may reflect that GLP-1 has a more important physiological role in controlling blood glucose than in regulating food intake.

The discovery of exendin-4 has led to the development of a synthetic version, exenatide. Exenatide has a much longer *in vivo* half-life than native GLP-1, stimulates insulin release, suppresses glucagon and lowers blood glucose. It is the first incretin mimetic approved for the treatment of type 2 diabetes (68). Exenatide has also been shown to reduce body weight in treated diabetics in phase III clinical trials (69-71). The weight loss associated with exenatide is considered a significant advantage as many anti-diabetic treatments are commonly associated with weight gain. Nausea is a relatively common side effect of Exenatide treatment. However, it does not seem to be intrinsically linked to the effects on appetite (3). Whilst GLP-1 has been developed as a treatment for diabetes due to its incretin properties, the observed effects of GLP-1 on satiety and weight loss are a valuable secondary effect. Indeed recent data suggests liraglutide may be useful for the treatment of obesity, causing sustained weight loss over 2 years but with a 50% rate of nausea and vomiting in the 3.0 mg/day group in the first year (72). The newest long acting analogues of GLP-1, exenatide-LAR (Amylin Pharmaceuticals, FDA approved January 2012), taspoglutide (Ipsen and Roche) and Albiglutide (GlaxoSmithKline), have been shown to effectively control glucose and to reduce weight. These agents allow for less-frequent dosing schedules, improved glycemic control throughout the day, and improved treatment satisfaction compared to some available agents (73). It remains to be seen whether these drugs perform well enough in specific weight loss paradigms such that they could be used as anti-obesity agents.

Oxyntomodulin (OXM)

OXM, like GLP-1, is also a product of the preproglucagon precursor molecule. It is a 37 amino acid peptide released post-prandially from L cells in proportion to caloric intake (27). OXM delays gastric emptying and reduces gastric acid secretion (74), and has been shown acutely to decrease food intake and in the longer term to decrease weight gain in rodents (75,76). In addition, chronic administration of OXM produces greater weight loss compared to pair-fed controls, suggesting an increase in energy expenditure may also help to reduce body weight (77). OXM has been shown to reduce food intake in normal weight human volunteers when administered intravenously or subcutaneously (78). Given preprandially to obese subjects it reduces both food intake and body weight (79). As in rats, there is evidence that OXM may also increase energy expenditure in humans (80).

Although OXM has some agonist activity at the glucagon receptor, there is evidence that its anorectic effect is predominantly mediated via the GLP-1 receptor (75,81). The anorectic effects of OXM are abolished in GLP-1 receptor knockout mice (81) and in the presence of the GLP-1 receptor antagonist exendin 9-39 (76). OXM has a 50-fold lower affinity for the GLP-1 receptor than GLP-1 itself, but despite this, it reduces food intake with similar potency (75). Furthermore, although the administration of exendin 9-39 directly into the ARC blocks the anorectic effects of OXM, it does not block those of GLP-1 (76). Therefore, it is possible that OXM may act via an as yet unidentified receptor. Studies using manganese-enhanced magnetic resonance imaging MRI (MEMRI) has shown that intraperitoneal administration of OXM produces a distinct pattern of neuronal activation compared to GLP-1 (82), implying that these two hormones act via different hypothalamic pathways.

Glucagon

Glucagon is a 29 amino acid peptide secreted from the α-cells of the pancreatic islets of Langerhans. It is a further product of preproglucagon cleavage alongside OXM and GLP-1. Glucagon is released into the portal vein in fasted states and also in response to exercise, and acts on the liver to promote hepatic glycogenolysis and gluconeogenesis and maintain glycaemic balance (83-86).

Glucagon mediates its effects via the glucagon receptor, a 7-transmembrane G-protein coupled receptor which has a wide tissue distribution. It is expressed in the gut, adrenal glands, brain, heart, pancreas, spleen and in adipocytes, but is predominantly found in the liver and kidney (87).

As a potential treatment for obesity, glucagon has been shown to increase energy expenditure in rats, and also in humans during insulin deficiency (88). It also significantly reduces food intake, with a subjective reduction of appetite in man (89). Infusion of glucagon into the portal vein but not the inferior vena cava causes a reduction in meal size in rats (90).

Glucagon presents an interesting prospect in the treatment of obesity due to its effect on increasing energy expenditure, and increasing satiety. It has been demonstrated that the potentially unfavourable effect on glucose tolerance due to glucagon's actions on hepatic glycogenolysis and gluconeogenesis is effectively counteracted by dual agonism at the glucagon and GLP-1 receptors (91,92). The data from these studies demonstrated highly effective weight loss in diet-induced obese mice whilst avoiding the hyperglycaemia that might be expected from agonism at the glucagon receptor.

Ghrelin

Ghrelin is a 28-amino acid acylated peptide secreted from the stomach. It was originally identified as an endogenous ligand for the 'growth hormone secretagogue' receptor (GHS-R) and is a growth-hormone-releasing peptide (93).

Ghrelin is the only orexigenic gut hormone (94), causing an increase in food intake and weight gain in rodents following both peripheral and central administration (95-97). Intravenous administration of ghrelin has also been shown to stimulate gastric acid secretion and motility in rats (98). In normal subjects, ghrelin levels are highest in the fasted state (99), and levels are chronically higher in people with weight loss due to anorexia nervosa or dietary reduction (100-102). In contrast to other gut hormones, plasma ghrelin levels decrease after meals (100,103) and are low in obese subjects (102). Ghrelin concentrations are also reduced after gastric bypass surgery, and this may contribute to weight loss in such patients (101).

Ghrelin receptors are found in the ARC of the hypothalamus suggesting a central mode of action. Consistent with this *c-fos* expression is increased in the ARC after peripheral administration of ghrelin (104) and ablation of the ARC blocks ghrelin induced food intake (105). When given centrally, ghrelin also stimulates *c-fos* expression in other nuclei known to be involved in appetite control including the PVN, dorsomedial nucleus, and lateral hypothalamus as well as in the AP and NTS in the brainstem (95). Ghrelin and its receptor are both

expressed in vagal afferents in mice (106), and blockade of the gastric vagal afferent has been shown to abolish ghrelin-induced feeding, growth hormone secretion, and activation of NPY-producing and growth hormone-releasing hormone producing neurons in rats suggesting an additional mode of action (107).

Diet induced obesity is associated with a blunting of ghrelin's orexigenic effect. There has therefore been recent interest in the interaction between the ghrelin system and macronutrients. High fat feeding has been shown to render NPY/AgRP neurones relatively ghrelin resistant (108), and diets high in fat have been shown to directly inhibit the hyperphagic effect of ghrelin (109,110). These data have significant implications for developing anti-obesity treatments targeting the ghrelin system and suggest success of these approaches could depend on the fat content of the diet the patient consumes. More recently, ghrelin has been shown to engage neurons in the ventral tegmental area of the brain and may provide a link between the gut and neuronal control of stress-induced eating of 'comfort foods' (111).

Other gut peptides

A number of other gut-derived peptides have been shown to reduce food intake. However, the physiological role of these peptides in the regulation of food intake and energy homeostasis remains unclear.

CCK is released post-prandially from the small intestine (3), and has also been shown to co-localise with PYY in L cells (112) Two types of CCK receptor have been identified in the CNS and peripheral tissues CCK1 and 2 (113). CCK is released post-prandially in response to saturated fat, long-chain fatty acids, amino acids and small peptides that would normally result from protein digestion (114,115). CCK release and signalling via the CCK-1 receptors in response to these long chain fatty acids mediates stimulation of PYY release and inhibition of ghrelin (an orexigenic gut hormone) in human subjects (116).

The effects of CCK on appetite are well documented. Peripheral administration of CCK in rodents results in a dose dependant reduction in food intake, decreasing both meal size and duration (117). CCK administration is also associated with an increase in postprandial satiety behaviours such as increased grooming and decreased locomotor activity (117). In humans, intravenous administration of physiological doses of CCK reduces food intake and increases the perception of fullness (118). Unfortunately, the therapeutic potential of CCK as a treatment for obesity is limited by nausea and tachyphylaxis of the anorectic effects associated with chronic administration (119).

PP is an amidated 36-amino acid peptide and belongs to the 'PP fold' family of peptides. It is released post-prandially under vagal control by pancreatic islet PP cells (120-122). PP binds to all the members of the Y receptor family, but has the highest affinity for the Y_4 receptor subtype (123). The effects of PP are likely to be mediated by both the hypothalamus and brainstem (124). PP is comparable to other anorectic intestinal peptides such as PYY, being secreted in proportion to caloric intake. Circulating levels rise after meals and remain elevated for up to 6 hours post-prandially (120). Intraperitoneal injection of PP acutely reduces food intake in fasted mice (124), an effect that remains apparent for 24 hours after injection. Furthermore, chronic administration of PP over 6 days in *ob/ob* mice significantly reduces body weight gain and improves glucose profile (124). Intravenous infusion of PP at doses that achieve normal postprandial plasma concentrations reduces appetite in lean humans and inhibition of food intake persists for 24 hours after infusion (125). PP has also been shown to reduce food intake at lower infusion rates (126). Furthermore, pancreatic polypeptide has been shown to reduce food intake in patients with obesity secondary to Prader-Willi syndrome (127). Additionally, PP has also been implicated in energy homeostasis, with exogenous administration of PP causing an increase in oxygen consumption (124), thus implying that part of the effect of PP on body weight may be due to increased energy expenditure. It has also been shown to increase spontaneous locomotor activity in mice (128). These data have lead to a concerted effort to develop long acting PP analogues, which have completed Phase I trials (129).

NT was first isolated from hypothalamic tissue, but is widely distributed throughout the central nervous system. However, the majority of NT is found within enteroendocrine cells of the GI tract (130). NT regulates a number of digestive processes, including gastrointestinal motility, and pancreatic and biliary secretion (131). It also has trophic effects on the pancreas and small intestine (132,133). Plasma levels of NT increase after a meal, with intraluminal fat being the most potent stimulus (134). Peripheral administration of neurotensin decreases food intake and grooming behaviour in rats only at large doses (135). Therefore at physiological levels, neurotensin is unlikely to play a major role in appetite regulation. Although neurotensin acutely reduces food intake when administered centrally in rats or peripherally in mice, chronic administration to mice has no significant effect on food intake or body weight (136). The lack of chronic effects on body weight suggests that NT is unlikely to be useful as a treatment for obesity.

Intracerebroventricular injection of glucagon-like peptide-2 (GLP-2) into rats inhibits food intake. In contrast, GLP-2 administered peripherally does not inhibit food intake in rodents or humans (137,138). GLP-2 appears to play a more important physiological role as an intestinal growth factor (138).

Amylin is a peptide co-secreted with insulin by pancreatic beta cells. Injection of amylin or amylin agonist has been shown to reduce food intake in a number of species, including humans (139-143). The amylin receptor agonist pramlintide has been shown to cause weight loss in diabetic humans (141,143).

Vasoactive intestinal polypeptide (VIP) has been shown to reduce appetite, in addition to its well-characterized effects on the cardiovascular system and gastrointestinal motility and secretion. Intracerebroventricular administration of VIP has been shown to cause a potent short-lived decrease in food intake and an increase in activity and energy expenditure in rats. Treatment of hypothalamic explants with VIP stimulated the release of the anorexigenic peptide α-MSH (144). These studies suggest a possible endogenous role for VIP in the hypothalamic control of energy homeostasis.

Gut hormones and the treatment of obesity

Lifestyle and dietary modification alone are inadequate for the successful treatment of the majority of obese individuals. However, despite an increasingly high demand for intervention, the field of obesity therapeutics has limited options to offer these patients. The history of obesity pharmacotherapy is littered with examples of drugs withdrawn from the market due to adverse effects outweighing the beneficial effects of weight loss. Recent examples include Sibutramine, a norepinephrine and serotonin reuptake inhibitor, and Rimonabant, which is a cannabinoid-1 receptor blocker. Sibutramine was withdrawn after it was found to increase heart rate and blood pressure in some subjects, and was associated with an increased risk of stroke and non-fatal myocardial infarction in patients with pre-existing cardiovascular conditions (145), whilst Rimonabant was withdrawn amidst concerns regarding adverse psychiatric events (146). The only currently licensed product in the UK is Orlistat, a pancreatic lipase inhibitor which prevents fat absorption and confers a modest weight loss of 2.9 kg more than placebo over the course of a year (147).

The only obesity treatment that has been shown to confer long-term, sustained weight loss and a decrease in overall mortality is bariatric surgical intervention (148,149). Several surgical procedures are available to achieve weight loss. Gastric banding restricts the amount of food that can be comfortably ingested and increases the satiating effect of food (150). A more efficient reduction in appetite and weight loss is seen with surgical procedures that involve gastrointestinal bypass, such as Roux-en-Y Gastric bypass (RYGB) (148,149,151). Weight loss is normally associated with reduced plasma levels of the adipocyte-derived anorectic hormone leptin, causing increased hunger (152). However, following RYGB, despite significant reductions in body weight and leptin levels, appetite is markedly reduced (151). It has now been demonstrated that RYGB is more effective than either standard or intensive medical therapy in achieving glycaemic control and remission in patients with

Type 2 diabetes (T2DM) (153,154). These seminal studies raise the question as to whether bariatric surgery could become a more important treatment for T2DM than medical therapy. Indeed, in a recent positional paper the International Diabetes Federation supported the selective use of various bariatric procedures for obese individuals with medically resistant T2DM (155). However, significant questions remain, not least is how long do these effects last? But also include when is the best time for surgical intervention? Does bariatric surgery work for everyone? What are the surgical/risk benefits in moderately obese patients? This list is by no means exhaustive. Given RYGB requires major surgery, which has inherent risk and is expensive, there is considerable effort aimed at determining how RYGB and other surgeries induce sustained weight loss and resolution of T2DM.

Of particular interest has been the suggestion that RYGB ameliorates coexistent type 2 diabetes mellitus before substantial weight loss has occurred and more rapidly than gastric banding. The differences between gastric banding and RYGB may be due to alterations in the anorectic and incretin gut hormone profile that is seen following RYGB, but not following gastric banding (156,157). Experimental evidence suggests that these anorectic gut hormones may mediate the effects of RYGB on appetite and body weight (157,158). Post-prandial PYY and GLP-1 levels begin to rise as early as 2 days following gastric bypass in humans (158), and secretory products of enteroendocrine L-cells, including PYY and GLP-1 remain elevated two years after bypass surgery (159). Inhibiting gut hormone release with somatostatin analogue octreotide increases the food intake after gastric bypass surgery but not following gastric banding (158), further suggesting that these hormones play a critical role. In rodent models of bariatric surgery increases in circulating GLP-1 (160) and PYY and a reduction in ghrelin (49), have been implicated in mediating the beneficial effects of these surgeries. Determining the mechanisms behind the sustained reduction in appetite may identify pathways that can be targeted by anti-obesity agents. To this end there has been recent concerted effort to mimic the rise in gut hormones following gut bypass by either the development of peptide based analogues or by the design of small molecule drugs which target nutrient sensing receptors on the enteroendocrine L-cell.

Long acting versions of PYY and OXM are being actively pursued by the pharmaceutical industry, such as Pfizer's OAP-189, we await the dissemination of data from ongoing trials. In addition Given that gut hormones are co-released one logical approach would be the development of combination therapies. Indeed data suggests that co-administration of gut hormones can have additive effects on food intake inhibition, for example PYY + GLP-1 (161) or PYY + OXM (162). Such combination approaches may prove more effective than individual administration. Very recently the development of chimeric agonists has emerged as a novel form of combination therapy (91,92). GLP-1/glucagon co-agonists combine the appetite suppressive effects of GLP-1 and glucagon with the energy expenditure promoting effects of glucagon. Whilst at the same time GLP-1's insulinotropic effects inhibit the detrimental hyperglycaemic effects of glucagon. Marcadia Ltd., now a subsidiary of Roche, first reported the beneficial effects of this approach and their compound is now undergoing clinical trials. In addition, Zealand Pharma is also developing a similar compound, ZP-2929, in partnership with Boehringer Mannheim. Time will tell if the promising pre-clinical data translates in to clinical benefit (163).

Considerable energy has also been directed toward the development of gut hormone secretagogues. The most well characterised class being agonists of GPR119. These compounds have been shown to release both GLP-1 and PYY (22). Their anti-diabetic effects are well defined; stimulation of GLP-1 and a direct insulinotropic action (22,164). It is less clear if these compounds will be effective as anti-obesity agents, but some agonists have been shown to significantly reduce food intake, for example PSN632408 (165). GPR119 is currently the only target for which synthetic modulators stimulate both incretin and insulin release. This highly beneficial profile has generated great industry interest with at least 9 companies actively working in this area. Initial clinical trials have been

successful with respect to the anti-diabetic indication (166,167).

Conclusion

Obesity has emerged as a major global healthcare challenge. The significant mortality and morbidity associated with obesity has inspired a vast amount of research directed towards developing safe and efficacious weight-loss agents. The beneficial effects of centrally acting weight-loss agents have been negated by their potentially hazardous effects on mood, reward, dependence and autonomic tone. Gut hormones, as outlined in this article, play an important role in the homeostatic control of food intake and offer an alternative to centrally acting drugs. We believe that in time these approaches will develop clinically useful compounds which will offer a real answer to the ever growing burden of obesity.

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Potential conflict of interest

None declared.

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10. PRENATAL AND NEWBORN SCREENING OF CYSTIC FIBROSIS

Dragica Radojković

10.1 INTRODUCTION

Cystic fibrosis (CF) is the most common life-threatening autosomal recessive condition affecting 1 in 2500 newborns among Caucasians (1). Anderson was the first who described the disease as «cystic fibrosis of pancreas» (2). Twenty-five years later Di Saint'Agnese et al demonstrated that excessive salt loss occurs in the sweat of CF patients, which led to the use of sweat electrolyte measurements as a diagnostic tool (3). Cystic fibrosis is a progressive multisystem disease that primarily affects pulmonary, pancreatic and gastrointestinal systems. The major clinical characteristics of CF include pancreatic insufficiency and progressive lung disease, caused by thick and dehydrated airway mucus frequently infected with *Pseudomonas* and *Staphylococcus*, leading to respiratory failure and CF mortality. In addition, most adult males with CF are infertile due to congenital absence of vas deferens (CBAVD). Patients with CF may also suffer from intestinal obstruction (meconium ileus, distal intestinal obstruction syndrome), diabetes, liver disease, growth retardation, dehydration due to excessive salt loss in sweat, nasal polyps and chronic sinusitis (4,5).

Our knowledge of underlying pathophysiology and molecular basis of the CF has grown exponentially over the years, driving the development of effective therapies. Consequently, improved treatment has increased the life expectancy of CF patients from what was a fatal childhood condition to a present median survival of 36.8 years. Predicted median survival for current newborns is at least 50 years and over 55% of patients in UK are adults (6).

10.2 GENETICS OF CYSTIC FIBROSIS

10.2.1 CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR (CFTR)

The cystic fibrosis gene was identified in 1989 by tremendous effort involving combination of positional cloning, gene walking and jumping and candidate gene analysis. The gene comprises of 27 coding exons, spanning over 250kb on chromosome 7q31.2 and encodes mRNA of 6.5kb. The protein encoded by the cystic fibrosis transmembrane conductance regulator (CFTR) gene is a chloride channel located in the apical membrane of exocrine epithelial cells and contains 1480 amino acids, with a calculated molecular weight of approximately 170kDa (7,8). The protein comprises of five domains: two membrane spanning domains (MSD1 and MSD2), each composed of six transmembrane segments (TM1 to TM12) that form the channel, two nucleotide binding domains (NBD1 and NBD2), capable of ATP hydrolysis and regulatory domain (R) which contains several phosphorylation sites. The CFTR belongs to ATP-binding cassette (ABC) transporter proteins and primarily finctions as ATP dependent chloride channel. In addition to this primary function, it also modifies the function and properties of other ion transporters including chloride, sodium and potassium channels and Cl^-/HCO_3 exchanger. Moreover, it has an effect on water permeability, ATP transport and mucus secretion (9,10).

10.2.2 MOLECULAR BASIS OF CYSTIC FIBROSIS

Initial molecular genetic characterization of the CFTR gene in affected patients revealed the major CF mutation, a 3-bp deletion causing a loss of phenylalanine at position 508 of the protein, designated as F508del (previously termed ΔF508) (11). The F508del is the most common mutation that accounts for approximately two thirds of all CFTR alleles in patients with CF, with a decreasing prevalence from Northwest to Southeast Europe (12). The remaining third of alleles are substantially heterogeneous, with fewer than 20 mutations occurring at worldwide frequency of more than 0.1%, but some can reach high prevalence in selected populations. The majority of CFTR mutations have been associated with European-derived populations. To date more than 1900 sequence alterations have been identified in the CFTR gene and their listing is continuously updated within Cystic Fibrosis Genetic Analysis Consortium (CFGAC) (13). The different CFTR mutations can be divided into 5 major classes according to their effect on CFTR function: defective protein (class I), defective processing and maturation (class II), defective regulation (class III), defective conductance (class IV) and reduced function/synthesis (class V). When patients who are homozygous or compound heterozygous for a class I-III CFTR mutations are compared to patients who carry at least one class IV-V CFTR mutation, the latter group tends to have less severe disease. The correlation between genotype and phenotype in CF is variable. Keeping in mind that CF phenotype is affected by CFTR genotype, as well as other genetic (modifier genes) and environmental factors (smoking, malnutrition), the functional classification of CFTR mutations in 5 classes should be considered as a research tool rather than a predictor of clinical outcome in individual patients (14).

10.3 CLASSICAL DIAGNOSIS OF CYSTIC FIBROSIS

Diagnosis of classical CF is based on a combination of consistent clinical features and evidence of CFTR channel dysfunction. Laboratory criteria include a positive sweat test (chloride value above 60mmol/l), and/or presence of two CF-causing mutations (in trans), and/or abnormal values of electrophysiological measurements of nasal potential difference and rectal chloride transport (15,16).

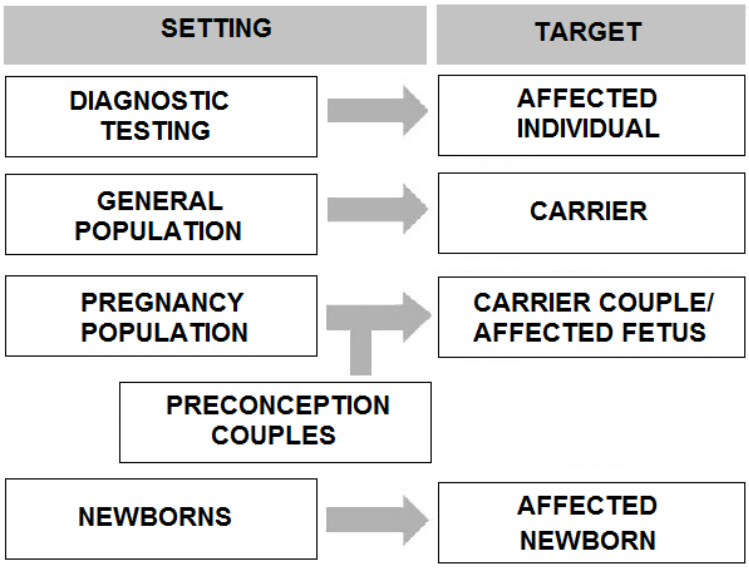

Figure 10.1 Clinical settings for cystic fibrosis genetic testing.

The sweat test with chloride determination is still the gold standard for the confirmation of CF diagnosis (sweet stimulation by pilocarpine iontophoresis and sweet chloride determination using coulometric titration method). In the majority of the cases the diagnosis of CF is straight forward, based on typical clinical features and abnormal sweat chloride test that supports the clinical diagnosis. In such situations, genetic analysis is not strictly necessary, although it is valuable for the confirmation of the diagnosis and also to enable carrier testing and prenatal diagnosis within the family (17). Genetic testing for CF could be performed in different clinical settings as illustrated by Figure 10.1.

10.3.1 PRENATAL TESTING IN CYSTIC FIBROSIS

Prenatal diagnostics is very important in detection and prevention of genetic diseases. In the past 40 years, there has been a marked improvement in this field primarily trough introduction of chorionic villi sampling (CVS) in the first trimester of gestation, development of preimplantation diagnostics, diagnostics based on fetal DNA in the peripheral blood of the pregnant women and molecular diagnostics of hereditary diseases (18).

Regarding the severity of the disease as well as its frequency, CF is a genetic disorder for which prenatal testing is an ultimate demand. Prenatal diagnosis for pregnancy at risk 1:4 is currently performed routinely by genetic testing. When CF was recognized as a clinical entity in the 40s of the last century, the only way to control the risk of not having affected child was to restrain from reproduction. The turning point in the field of prenatal diagnostics of cystic fibrosis was the measurement of the microvillar intestinal enzyme level in amniotic fluid (19). Since 1985, when CF locus was assigned to chromosome 7, indirect molecular genetic testing for CF was introduced, and in 1986 Farral performed the first prenatal diagnosis of CF, in the first trimester of gestation, using linked DNA polymorphic markers (20). The knowledge of the strong association between the presence of mutated gene and certain haplotype further spread the use of prenatal testing in CF, which was additionally speeded up by the implementation of PCR methodology (21,22). The important improvement in prenatal diagnostics of CF was the cloning of CFTR gene and the detection of most common F508del mutation. That was particularly important in genetic counseling of high-risk families lacking the index case.

Presently, prenatal diagnostics mainly relies on DNA diagnostics and high-risk couples have several reproductive options: termination of affected pregnancy, in vitro fertilization and preimplantation diagnostics of cystic fibrosis. With the development of a wide range of novel technologies available nowadays, the laboratory can choose the method that best suits the diagnostic requirements: Allele-Specific Nucleotide (ASO), Oligonucleotide Ligation Assay (OLA), Amplification Refractory Mutation System (ARMS), Invader assay, Nanochip microarray, Denaturing Gradient Gel Electrophoresis (DGGE), Denaturing High-Performance Liquid Chromatography (DHPLC), Single Strand Conformation Polymorphism (SSCP) and DNA sequencing. Methods used for CFTR testing can be divided in two groups: those targeted at known mutations (testing DNA samples for the presence or absence of specific mutations) and scanning methods (screening for any deviation from the standard sequence). These also include searching for large unknown CFTR rearrangements by multiplex ligation dependent probe amplification (MLPA) or quantitative fluorescent multiplex PCR. Such rearrangements, which can escape detection using conventional PCR assays, are present in 2% of alleles in CF patients (23). The majority of the laboratories use the commercial assays that cover the panel of 24-32 mutations (16). The combined use of all currently available techniques cannot guarantee detection of two disease-causing mutations in *trans* in all patients; 1-5% of alleles remain undetermined in CF patients with classical form of the disease. Best practice guidelines for molecular genetic diagnosis of CF recommend the

organization of testing laboratories in two levels of expertise: “level 1” (screening) and “level 2” (reference) laboratories. Laboratories should be aware of the heterogeneity in the distribution of CFTR mutations in different populations that consequently results in lower sensitivity of the test in regions of Central, Southern and Eastern Europe (14,16).

Prenatal testing for CF should be performed on request, following genetic counseling. It can be offered to (1) parents of a patient with the established diagnosis of CF where both parental mutations have been identified, preferably before ongoing pregnancy in order to minimize the anxiety; (2) carrier couples identified through carrier screening and (3) carrier couples identified through investigations for fetal bowel anomalies. Prenatal testing could be performed in the first (CVS) or the second trimester (amniocentesis) of gestation. Search for maternal cell contamination of the fetal samples should be carried out by a panel of microsatellite markers.

Fetal bowel anomalies are most often observed during the ultrasound examinations in the second trimester of pregnancy. They can be due to CF or other disease conditions or may not be associated with disease. Diagnostic investigations should therefore include search for frequent CFTR mutations, fetal karyotyping and screening for viral infections (24). Cystic fibrosis is confirmed in 2.5-10% of hyperechogenic bowel cases (25-27). Although the overall risk of CF varies between studies, a large French collaborative study performed on 641 pregnancies presenting fetal bowel anomalies determined the risk of CF as 3% (28).

Some laboratories offer preimplantation genetic diagnosis (PGD) to at-risk couples as an alternative to prenatal diagnosis. This procedure should be performed following the highest quality standards (29).

10.3.2 GENETIC SCREENING FOR CYSTIC FIBROSIS

Cystic fibrosis has been an obvious target for population screening ever since discovery of the CFTR gene. The reason for that is relatively high carrier frequency, especially in Caucasians (1 in 25-30). CF has recessive mode of inheritance, and most of the carriers have a negative family history and do not know that they are at risk of having an affected child.

Several pilot programs of various forms of population screening have been performed. Antenatal population based CF carrier screening has been carried out in Scotland, UK and USA. After 5 years of population screening in Scotland with 76% uptake, a decrease in the number of CF cases in pediatric population from 4.6 cases per year to 1.6 cases was observed. The report concluded that antenatal screening avoids the anxiety of newborn screening (NBS) and false-positive results. It also gives the opportunity to terminate any affected pregnancy and should be offered in the absence of pressure to proceed to prenatal diagnosis and pregnancy termination (30).

In USA, the National Institutes of Health (NIH) issued a recommendation to expand carrier testing to include couples seeking prenatal care or planning a pregnancy in which at least one person is Caucasian, and to make couples of other ethnicities aware of the existence of population screening for CF carrier status (31). In the following years, American College of Obstetricians and American Collage of Medical Genetics (ACMG) issued guidelines for implementation of CF carrier screening and laboratory standards for testing facilities (32). The ACMG also proposed a CFTR mutation panel for population-based carrier screening in USA (33).

As already mentioned, different models of CF carrier screening are available and they all have advantages and disadvantages. Preconception carrier screening is preferential to prenatal screening because it offers couples time to process their risk and the option to avoid

pregnancy if desired. However, it is recognized that the majority of the couples will be screened when the pregnancy is already under way (34). These couples are also more available to screening programs since they become part of the system of prenatal care. A high rate of unplanned pregnancies makes preconception screening unavailable to a significant proportion of target population.

The screening programs could be couple based or sequential. Couple based programs will only test if both members of the couple are available, and report the results and the risks in terms of the couple as a screening unit. Sequential screening usually begins with the female member of the couple, and test the male only if the female is a carrier. Generally speaking, sequential screening is preferred as it allows full disclosure of individual results, and it is possible to notify the extended family for additional carrier screening. On the other hand, full disclosure will identify couples with a minimally elevated risk (one individual tests positive and one tests negative), but provides no additional options for risk assessment or diagnosis (35). Couple screening will be performed only if both samples are available, which is not an option for pregnancies in which the male is unavailable and re-screening is required with each new partner. Brock evaluated both models and concluded that couple screening proved to be better theoretical model, as both members of the couple were needed to obtain information regarding the risk of CF in any pregnancy (36).

10.3.3 NEWBORN SCREENING FOR CYSTIC FIBROSIS

Newborn screening (NBS) has traditionally been performed to detect severe conditions that are, relatively prevalent, treatable, and for which a test exists and is measurable on a large scale and has been as an important and cost-effective health care provision since late 1960s (37).

The first NBS programs for CF were explored almost 40 years ago, and since than gradually established across USA, Oceania and Europe. In 2010, the number of European countries that perform NBS for CF as a national or regional service has increased to 14, while all states in US have started NBS for CF (38).

The first NBS test for CF was performed by measuring albumin content of meconium placed on filter paper, but it did not develop into widespread program and was replaced by the measurement of immunoreactive trypsin (IRT) introduced in 1979 in New Zealand (39). The test is performed on dried blood spots (Guthrie cards) already collected for other NBS tests such phenylketonuria and congenital hypothyroidism, and still represents the starting point for most CF NBS protocols. Later developments included incorporation of DNA testing from the bloodspots of newborns with elevated IRT, and recently the possibility of using pancreatitis-associated protein (PAP) (40).

The basis of IRT test is measurement of trypsinogen, a precursor for pancreatic enzyme. IRT level is usually persistently high in the blood of newborns with CF, even in cases with pancreatic sufficiency (41). This increase is the result of the pancreatic fibrosis in the majority of fetuses with CF that leads to a reflux of pancreatic enzymes into the circulation. The original method employed a radioimmunoassay with polyclonal antibodies, and was later replaced with enzymatic immunoassay with monoclonal antibodies, performed on ELISA plates, leading to increased sensitivity and shorter processing time (42).

All current CF NBS protocols relay on IRT as a primary test and on sweat test for confirming or excluding the diagnosis of CF. Intermediate tests are required to achieve an acceptable combination of sensitivity and specificity. The simplified algorithm for standard CF NBS procedure is shown in Figure 10.2. The choice of CF NBS protocol depends on various

factors, such as cost, collection time, ethnic mix of populations and variations in healthcare provision and resources.

10.3.4 SHORT DESCRIPTION OF PROTOCOL

Immunoreactive trypsinogen→immunioreactive trypsinogen protocol (IRT/IRT) was initially adopted as two-stage IRT. It measures the IRT level on a a second blood spot collected at 10-28 days of life from newborns with high IRT at birth. If the second IRT is also high, the sweat test is performed for the definitive diagnosis of cystic fibrosis. The main limitation of this protocol is a need for additional blood spot. With the IRT/IRT protocol, approximately one in 200 children requires a second IRT which causes great anxiety in the parents (42). Approximately 95% of children with cystic fibrosis are detected with IRT/IRT protocol, but with a high percentage of false-positive results.

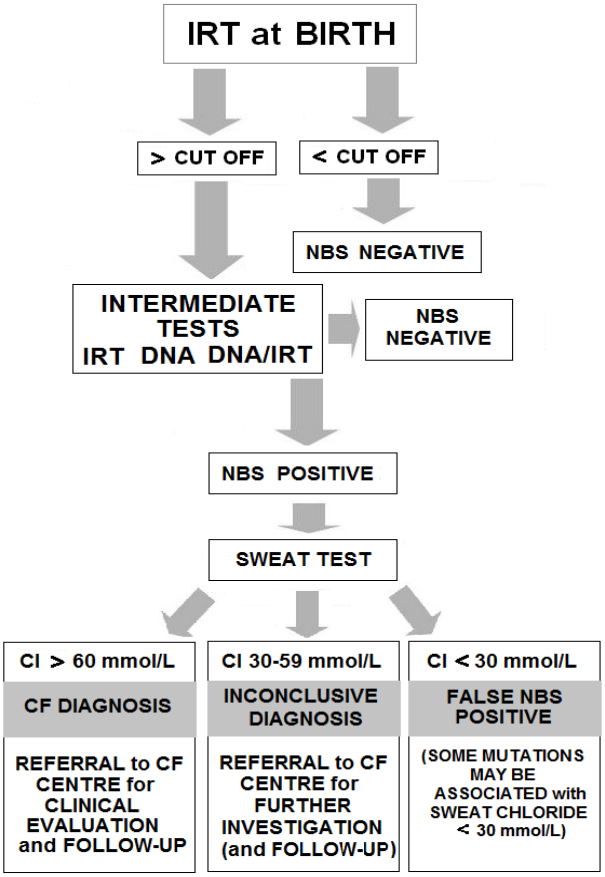

Figure 10.2 General CF NBS algorithm. The sweat test with chloride determination is still the gold standard for the confirmation of CF diagnosis (sweet stimulation by pilocarpine iontophoresis and sweet chloride determination using coulometric titration method).

Immunoreactive trypsinogen→CFTR mutation analysis protocol (IRT/DNA) utilizes CFTR mutation analysis as a second test. Identification of the CFTR gene and the most frequent mutation, F508del, allowed performing efficient NBS with a single blood sample. IRT is measured in routine newborn screening samples. In the case of elevated IRT, testing for a panel of selected "CF causing" mutation is performed using DNA isolated from blood eluted from the same Guthrie card. Newborns carrying one or two mutations are referred for a sweat test. The main limitation of this approach is identification of healthy carriers The DNA testing should always be linked to genetic counseling in order to provide sufficient advice to

the family and eventually offer testing to index' case relatives if they are within reproductive age. Australian group reported improved specificity of this combined protocol, fewer false-positives, and a 96% detection rate (43). The Wisconsin group reported an increase in diagnostic sensitivity up to 99% with the inclusion of the panel of 25 CFTR mutations (44). The program's costs were similar to those of other disorders included in NBS, such as phenylketonuria and congenital hypothyroidism (approximately 4US$).

Immunoreactive trypsinogen→CFTR mutation analysis→Immunoreactive trypsinogen (IRT/DNA/IRT) protocol combines the first IRT test with DNA testing and a second blood spot for IRT when the child has a mutant allele. Infants with persistently elevated IRT are referred for a sweat test, while those with second IRT bellow the cut-off are considers as healthy carriers. For this program, a reduction of 92% in the number of recalls for the second IRT test, as compared to the IRT/IRT protocol, and a reduction of 80% in recalls for the sweat test were reported (45). There are also variations of this protocol, such as: IRT/IRT/CFTR mutation analysis or IRT/CFTR mutation analysis/CFTR scanning or IRT/CFTR sequencing.

Pancreatitis-associated protein (PAP) is a secretory protein produced by the pancreas under stress conditions and can be measured in dried blood spots, which makes it a good candidate for the use in CF NBS protocols (46). Its use has been suggested as a second test, without recollecting the blood spot, as an alternative to DNA test, which eliminates outsourcing genetic analysis and may decrease the costs. The sensitivity of IRT/PAP protocol appears to be similar to that of IRT/CFTR mutation analysis protocol, but specificity is lower (47). A combined IRT/PAP assay kit is being developed and pilot studies are in progress in several European countries (48-50).

Although CF screening has been available for nearly four decades, there are few nationwide programs, with the majority of programs implemented at the regional or local level. In 2003, six European countries and five states in USA employed some type of newborn CF screening programs. Some countries, such as Denmark, Germany and the Netherlands, performed pilot studies, but decided not to implement the program (51). In England, six centers performed screening, with coverage of 22% of newborns, which was followed by implementation of nationwide program in 2004.The first detailed report on CF NBS screening in Europe, was published in 2007, and based on the results of 26 centers from seven countries (52). These centers have performed CF neonatal screening for an average of ten years (varying from nine months to 31 years), screening 1.600.000 newborns/year and detecting some 400 cases/year. Median age at diagnosis is reported at 37 days. In 2010, the number of European countries performing CF NBS doubled, and in the USA, all 50 states implemented screening program (http://genes-r-us.uthscsa.edu/).

The increasing introduction of CF NBS programs in recent years follows the growing evidence of the benefits of CF NBS regarding nutritional, gastrointestinal and cognitive aspects (48, 53). Furthermore, early diagnosis by NBS allows genetic counseling for carrier parents and testing of family members. Conversely, there are also risks associated with CF NBS that include anxiety for false positive results, identification of carriers and inconclusive diagnostic results (48). On balance, however, the evidence favors screening for CF at birth. In 2003, the U.S. Centers for Disease Control and Prevention (CDC) published guidelines for cystic fibrosis screening (54). The report concluded that CF screening is justified on grounds of evidence of moderate benefit and low risk of harm. The Consensus Conference organized in 2008 by European Cystic Fibrosis Society concluded that the available evidence supports CF screening with respect to clinical outcomes and health economics (48).

NBS programs have been associated with the reduced live birth prevalence of CF, attributable to the use of genetic counseling and prenatal diagnosis on subsequent pregnancies. Prenatal or

preconceptual population based carrier screening may also influence the prevalence in newborns. The New England NBS program reported a halving of homozygous F508del babies detected over a relatively short stretch of time (55), while a 40% drop in CF incidence in Brittany over a period of 35 years was observed (56).

10.4 CONCLUSION

Cystic fibrosis is diagnosed in a growing number of patients in the newborn period due to the increasing number of newborn screening programs. The benefit of early diagnosis in the newborn has been supported by randomized clinical trials, observational studies and the analyses of data from cystic fibrosis databases. CF NBS has beneficial effect on nutritional status, and coupled with early treatment limits lung damage in childhood, reduces the burden of care for families and may improve survival It The emphasis of health care system is on early diagnosis of CF and the provision of families with the opportunity to avoid the birth of additional affected children. Though CF alleles will always be present, current population based carrier screening and NBS may have a significant effect on the incidence of CF in the future.

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11. SEROLOGICAL DIAGNOSIS OF CELIAC DISEASE IN THE NEW DIAGNOSTIC CRITERIA

Zrinjka Mišak

11.1 INTRODUCTION

Celiac disease (CD) is an immune mediated systemic disorder elicited by gluten and related prolamines in genetically susceptible individuals (1,2). The term gluten indicates a broad group of prolamins (gliadins and glutenins) – water insoluble proteins found in wheat. Other prolamins showing similar immunogenic properties, also harmful to patients with CD, are found in rye (secalins) and barley (hordeins) (3).

In recent decades, our understanding of CD has improved considerably. It is nowadays widely accepted that CD is a common autoimmune-based disorder characterized by the presence of a variable combination of gluten dependent clinical manifestations, CD-specific antibodies, human leukocyte antigen (HLA) DQ2 and DQ8 haplotypes and enteropathy (1,2). Patients may present with a wide spectrum of symptoms, that may appear in infancy, childhood, adolescence or adulthood, or may be asymptomatic (1,2,4). Intestinal symptoms are common particularly in children diagnosed within the first two years of life (failure to thrive, chronic diarrhea, vomiting, abdominal distension, muscle wasting, anorexia and irritability). However, symptoms may also be mild and heterogeneous (e.g., short stature, infertility, hepatitis and liver failure, depression, and behavioral and neurological problems) or even absent (2,4-6). Also, some diseases, many with an autoimmune pathogenesis, are found with a higher than normal frequency in patients with CD, such as: type 1 diabetes, autoimmune thyroid diseases, Addison's disease, Sjögren's syndrome, autoimmune cholangitis, autoimmune hepatitis and primary biliary cirrhosis. Other associated conditions include selective IgA deficiency, Down's syndrome, Turner syndrome and Williams syndrome (2,5,7).

Traditionally, CD has been considered a fairly uncommon gastrointestinal disorder affecting mainly children, but, after the development of highly specific serological tests, recent screening studies have revealed the prevalence to be as high as 1–2% in the population, placing CD among the most common lifelong food-related disorders (4,5,8).

The disease etiology is multifactorial with very strong genetic influence (1). It is associated with major histocompatibility complex class II genes and the alleles encoding HLA-DQ2 and HLA-DQ8 that are major risk factors carried by almost all patients with CD. Individuals having neither DQ2 nor DQ8 are very unlikely to have CD and, therefore, the main role of HLA-DQ typing in the diagnosis of CD is to exclude the disease (1,2,5). However, 30–40% of healthy individuals also carry the DQ2 and DQ8 alleles, but the majority of these individuals never develop the disorder (2,5).

CD-specific antibodies comprise autoantibodies against transglutaminase type-2 (TG2, 'tissue' transglutaminase) including endomysial antibodies (EMA), and antibodies against deamidated forms of gliadin peptides (DGP) (1). A gluten free diet (GFD), currently the only lasting treatment for CD, improves or eliminates symptoms and normalizes the specific CD antibodies and histological findings (1,6). Early diagnosis and treatment with GFD can prevent severe, sometimes life-threatening complications (9,10).

This review gives an overview of the available tests for CD, including antibodies against gliadin, endomysium, tissue transglutaminase, and deamidated gliadin, and discusses their role in the diagnostic procedure.

11.2 ANTIGLIADIN ANTIBODIES

Among the first serum-based antibody tests applied in CD diagnostics are the antigliadin antibody (AGA) assays (5,8,11). The identification of AGA in patients with CD revolutionized the view of the disease, as it became evident that the measurement of these antibodies could allow an easier diagnosis of the disease, and a convenient follow-up by dietary change (7).

IgA-class antibodies have significantly better specificity compared with IgG-class antibodies, resulting in a higher accuracy. However, AGA levels can be elevated in some healthy individuals without celiac-type genetics or those suffering from gastrointestinal conditions other than CD. Indeed, the positive predictive value of AGA testing in most populations is less than 30% (5,7,8). On the other side, data suggest that AGA testing may still be useful in celiac children younger than 2 years of age (4). Lagerqvist et al. emphasised their high sensitivity found in Swedish children younger than 18 months and concluded that 17% of the children with CD in the youngest age group would have remained undiagnosed if IgA-antiTG2 had been used alone (12). However, this was not observed in a recent study performed by Foucher et al (13). According to the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) recommendations, these tests are no longer used as diagnostic aids because their sensitivities and specificities are fairly poor (1,5,11).

11.3 ENDOMYSIAL AUTOANTIBODY

The problems with the AGA tests were overcome by the advent of the gluten-dependent EMA tests (5). EMA binds to endomysium, the connective tissue around smooth muscle, producing a charateristic staining pattern that is visualised by indirect immunofluorescence. These tests use primate tissues as antigens and require microscopical evaluation that may be subject to interobserver variability (1,4,5,7,14). It is also labour intensive, and the availability of the substrates (monkey oesophagus, umbilicus) is limited (4,8,14). Despite of that, its high sensitivity and specificity in untreated CD have been repeatedly evidenced in a number of studies and it is currently considered as the reference standard of CD-specific antibody detection (1,4,14). However, even though a positive serum EMA has almost 100% specific association with CD, approximately 10–20% of patients with untreated CD remain EMA negative, making the test less sensitive, especially among children with CD under 2 years of age as well as in elderly patients suffering from more severe symptoms (14,15,16). Concerning children, studies showed that the specificity of EMA was nearly 100%, while the sensitivity was >90% (9). Burgin-Wolff et al. were the first to report that in children less than two years of age the sensitivity of EMA was significantly lower than in older children (80% vs 97%, $p<0.008$) while the specificity was 98% (17). More recently, similar results (sensitivity 89%) were reported by Wolters et al. (16). On the contrary, some authors did not observe any lack of antibody sensitivity in this age group (18, 19). Apart from the age of the patient, the other possible causes of antibody negative CD may be selective IgA deficiency, self-imposed GFD prior to testing or concomitant use of immunosuppressant drugs (14).

Nonetheless, EMA has the highest specificity and positive likelihood ratio for CD among currently available serology tools. It is more likely that CD is present if the EMA result is positive than if another CD antibody result is positive (1,9). Nevertheless, reports on EMA results should contain the specification of the investigated immunoglobulin class, the interpretation of the result (positive or negative), the cut-off dilution, the specification of the substrate tissue and, if possible, the information on the highest dilution still positive (1).

11.4 TISSUE TRANSGLUTAMINASE

In 1997, Dieterich et al. found that the endomysial antigen involved in the autoimmune response in CD was the enzyme tissue transglutaminase or Type 2 transglutaminase (tTG or TG2) (1, 4, 7, 20). TG2 occurs abundantly in the gut and functions as to deamidate proteins and peptides including gliadin or gliadin fragments leading to increased T cell reactivity in CD patients. Antibodies against TG2 bind *in vivo* to the patient's own TG2 expressed in the small bowel or in other tissues (e.g. liver, muscles, central nervous system) (1, 16, 21). Early assays were based on tTG derived from guinea pig liver, leading to false-positive results due to impurities in these preparations. Assays using human recombinant tTG showed improved accuracy, with higher sensitivity and similar specificity as compared with EMA, but being generally cheaper than EMA (8, 14). IgA and IgG class anti-TG2 antibodies can be detected in blood samples of patients by various immunoassays, but currently, TG2 Enzyme Linked ImmunoSorbent Assay (ELISA) tests are widely used. Weaknesses of the tTG test are that the accuracy of the assay varies between manufacturers and that different anti-TG2 test kits may have different measurement principles, calibrators and calculation modes meaning that the result and numerical values obtained cannot be directly compared. A large multicenter study demonstrated significant variability in sensitivity (69% to 93%) and specificity (96% to 100%) for the transglutaminase antibody among 20 participant laboratories. That supports the necessity of better standardization of the assays, so that all assays could be comparable from laboratory to laboratory (22).

Despite of these differences, many commercial anti-TG2 antibody tests have equally high sensitivity and specificity on the same blood samples (1,5,14).

According to two recent systematic reviews the pooled sensitivities of the human recombinant anti-TG2 tests have been 98% and 100% and specificities 98% and 97%, respectively. However, despite being a highly sensitive and specific markers of CD, both EMA and anti-TG2 alone are not good markers in younger children (<2 years) (4,23,24). The concordance rate of anti-TG2 and EMA tests is particularly high, although the specificity and positive predictive value of the EMA tests was higher in many studies (1,4,9,25). Therefore, IgA anti-TG2 could work better as an initial CD detection test, whereas the highly specific EMA test could be used as a confirmatory test to identify test positives as true patients with CD (4,8,9).

Several studies confirmed that high concentrations of anti-TG2 antibodies in serum predict villous atrophy better than low or borderline values (1,7,26-29). For those tests that use calibration curves to express antibody concentration, high specific CD antibody levels can be defined as those exceeding 10 times the upper limit of normal values (ULN) (1). A study in adults by Hill et al. reported that human-TG2 antigen–based serum anti-TG2 values always were associated with villous atrophy when they exceeded 10 times the ULN (27). Similar was found by both Dahlbom and Alessio (26,29). Recently published systematic review and meta-analysis of studies that enrolled mostly pediatric patients with suspected CD indicated that IgA-anti-TG2 and EMA were the best laboratory tests predicting CD (9).

As with EMA, TG2 antibodies may not be detected in the blood of all CD patients, although, TG2-specific antibodies may be present in small intestinal or other tissues of seronegative patients (1,16,21). Total IgA level should be determined with IgA anti-tTG to assess for selective IgA deficiency as this can result in false negative results (8). On the contrary, isolated positivity for anti-TG2, especially low levels, have been described in a number of conditions unrelated to CD, such as other autoimmune diseases, infections, tumours, myocardial damage, liver disorders and psoriasis (1,14). These antibodies are not associated with the EMA reaction which explains why EMA has higher reliability for the diagnosis of CD (1,5).

In addition to standard laboratory anti-TG2 assays, a tissue transglutaminase based point-of-care, i.e., fingerstick test, is now available. The major limitations, however, are a lower sensitivity and specificity than with most ELISA-based assays, and being the semiquantitative test, there is no "titer" that can be followed over time. Moreover, the evaluation of rapid tests is less reliable if done by untrained persons or lay people (1,8). Therefore, POC tests can not substitute laboratory-based tests, especially not in the hands of inadequately trained readers (9).

Anti-TG2 antibodies can also be detected in saliva, but accuracy of available diagnostic tests is lower compared to serological tests (1).

11.5 DEAMIDATED GLIADIN PEPTIDES

CD antibodies can also be detected by the use of synthetic peptides corresponding to deamidated gliadin sequences (30). The rationale behind the test is based on the conversion of certain gluten peptides to deamidated peptides by the action of intestinal tissue transglutaminase (5,8,11). These peptides bind with high affinity to HLA DQ2 or DQ8 on celiac patients' antigen-presenting cells to potently stimulate the inflammatory T-cell response observed in the intestinal mucosa of patients with CD (8). The result is an antibody response to these deamidated gliadin peptides that displays a higher specificity for CD than antibodies to native gluten (AGAs) (4, 8). Both IgG and IgA tests are available (11).

Although tests for anti-DGP antibodies performed favourably and much better than antibodies against native gliadin their performance was inferior compared to anti-TG2 or EMA assays unless special patient characteristics are present (IgA deficiency, age below 2 years) (1,9,31). According to a recent meta-analysis DGP-AGA assays had pooled sensitivity and specificity of 88 and 94%, respectively (32). It seems that IgG antibodies against DGP is a very good tool for identifying CD in children younger than two years and in patients with IgA deficiency (1,8,9,11,33,34).

11.6 THE ROLE OF ANTIBODIES IN DIAGNOSTIC CRITERIA

The first diagnostic criteria for CD in children were proposed by ESPGHAN in 1969. According to these original criteria the diagnosis of CD was made upon the sequence of three biopsies if there was: 1. structurally abnormal jejunal mucosa when taking a diet containing gluten; 2. clear improvement of villous structure when taking a gluten free diet; 3. deterioration of the mucosa during the gluten challenge (35). The development of simple and reliable serological tests prepared the ground in 1980s for the revision of the diagnostic criteria. According to these, the diagnosis of CD is based on: 1. the typical histological finding of hyperplastic villous atrophy while the patient is eating adequate amounts of gluten; and 2. unequivocal and full clinical remission after withdrawal of gluten from the diet (36). In

2001, the first diagnostic criteria for CD in adults were published (37). According to all these, until recently, conventional diagnosis of CD required documentation of villous atrophy on intestinal biopsy (8). However, during the last decades evidence has accumulated on the diagnostic value of specific CD antibodies and HLA typing diagnostic purposes. As a result, in 2012, a working group within ESPGHAN published new evidence-based guidelines for the diagnosis of CD in children and adolescents. Two algorithms have been proposed. The first algorithm can be applied to children and adolescents with signs and symptoms suggestive of CD. The first step is to test for anti-TG2 IgA antibodies and for total IgA to exclude IgA deficiency. If anti-TG2 antibody testing is positive the patients should be referred to a paediatric gastroenterologist for further diagnostic work up. However, it is important to keep in mind that seronegativity in patients consuming gluten does not rule out the possibility of CD (5). Furthermore, autoantibody assays may vary greatly qualitatively from laboratory to laboratory (22, 38). However, in patients with positive anti-TG2 antibody levels at or higher than 10 times the ULN, there is an option to omit the biopsy. The patient is tested for EMA and HLA DQ2/DQ8 and if positive for both, the diagnosis of CD is confirmed. A gluten free diet is started and the patient is followed for improvement of symptoms and decline of antibodies (1). Omitting biopsies would reduce the burden of endoscopy and of general anaesthesia for the affected children, save costs, and avoid potential adverse effects of these procedures (9). Patients with positive anti-TG2 antibody levels lower than 10 times the ULN and asymptomatic children should undergo upper endoscopy with multiple biopsies (1).

If IgA class CD antibodies are negative in an IgA-competent patients, it is unlikely that CD is present. Further testing for CD is not recommended unless special medical circumstances (age below 2 years, restricted gluten consumption, severe symptoms, family predisposition or other predisposing disease, immunosuppressive medication) are present. However, in seronegative cases for anti-TG2, EMA and anti-DGP, but with severe symptoms and a strong clinical suspicion of CD small intestinal biopsies and HLA-DQ testing are recommended. If histology shows lesions compatible with CD and DQ2/DQ8 heterodimers are negative, CD is not likely and an enteropathy caused by a diagnosis other than CD should be considered. In these cases the diagnosis of CD can only be made after a positive challenge procedure with repeated biopsies.

On the contrary, when duodenal biopsies, taken during routine diagnostic work-up for gastrointestinal symptoms, disclose a histological pattern indicative of CD, antibody determinations and HLA-typing should be performed. In the absence of CD specific antibodies and/or HLA-DQ2/DQ8 heterodimers CD is unlikely and other causes of enteropathy should be considered.

The second algorithm in ESPGHAN diagnostic criteria should be applied to children and adolescents with no signs or symptoms suggestive of CD, who are investigated due to their increased risk for the disease (first degree relatives of CD patients or other immune-mediated or chromosomal diseases associated with CD). In this group of patients CD should always be diagnosed using duodenal biopsies (1).

11.7 CONCLUSION

In conclusion, serological tests have become invaluable in the identification and diagnosis of patients with CD. Yet, the diagnosis of CD depends on gluten-dependent symptoms, CD specific antibody levels, the presence of HLA DQ2 and/or DQ8 and characteristic histological changes (villous atrophy and crypt hyperplasia) in the duodenal biopsy. According to the recently published ESPGHAN guidelines, the duodenal biopsy can be omitted in the presence

of high antibody levels, symptoms and genetic predisposition. However, large, prospective studies are needed to further evaluate these guidelines and the role of serology.

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12. CHRONIC PANCREATIC INSUFFICIENCY IN CHILDREN: ETIOLOGY, CLINICAL PRESENTATION AND DIAGNOSIS

Oleg Jadrešin

12.1 INTRODUCTION

Exocrine pancreatic insufficiency (PI) should be considered in all children presenting with malabsorption: failure to thrive, chronic diarrhea, hypoalbuminemia, or symptoms of trace vitamin and mineral deficiencies. Since pancreas has a large reserve capacity, more than 95% of the function of the pancreas must be lost before maldigestion develops. Therefore children can have a severe pancreatic problem without experiencing any problems with digestion. The exocrine pancreas is not fully developed at birth and all healthy infants show some degree of maldigestion of starch and fat. By two years of age pancreas matures and functions as an adult organ (1,2).

Although the PI may be caused by a variety of clinical conditions, majority of children have one of two inherited pancreatic disorders: cystic fibrosis (CF) or Shwachman-Diamond syndrome (SDS). CF is the most common inherited pancreatic disease of childhood and SDS is much less common (1). Chronic pancreatitis can cause exocrine insufficiency due to gradual loss of pancreatic function, but this happens more frequently in adults (3). Also, there are some very rare syndromes that should be considered when other clinical features are present. Johanson-Blizzard syndrome is an autosomal recessive disorder presenting with pancreatic exocrine dysfunction, deafness, hypothyroidism, microcephaly, abnormal hair pattern, nasal cartilage hypoplasia, and small or absent permanent teeth. Pearson's bone marrow syndrome is a mitochondrial disorder and presents with pancreatic insufficiency, sideroblastic anaemia, and hematopoietic precursor vacuolization. Selected enzyme deficiencies (lipase, colipase, trypsin, amylase, enterokinase) are extremely rare (1).

Malabsorption in PI results from inadequate secretion of approximately 25 enzymes and cofactors from the pancreatic acini and from insufficient bicarbonate secretion from the ductal epithelium. Without adequate bicarbonate secretion pancreatic lipase is also inactivated by gastric acid and bile salt micellization of dietary fat droplets is impaired. Therefore fat malabsorption is the most prominent clinical feature of PI. Protein maldigestion and malabsorption are also important but they are more commonly subclinical. Significant fat malabsorption does not occur until lipase or colipase secretion is below 1% of normal values (1,2).

This review will mostly deal with clinical features of the most frequent inherited disorders of pancreas: cystic fibrosis and Shwachman-Diamond syndrome.

12.2 CYSTIC FIBROSIS

Cistic fibrosis is the most common lethal genetic disease in the white population. The disease is caused by a mutation in a gene that encodes cystic fibrosis transmembrane conductance regulator (CFTR) protein, which is expressed in many epithelial cells and blood cells. CFTR functions mainly as a chloride channel but has also other functions, particularly in regulation of sodium and bicarbonate transport through membranes, regulation of ATP channels and

regulation of intracellular vesicle transport. Life expectancy for patients with CF has increased from 31 years to 37 years over the past decade in US and even better prognosis for children being diagnosed today seem to be realistic (4,5).

The incidence of CF is most common in populations of northern European descent where the disease occurs in approximately 1 in 3000 births. The incidence varies from country to country and with ethnic background, from 1 in 2500 to 1 in 20000 (African Americans). The disease is uncommon in Africa and Asia (4,6).

12.2.1 GENETICS

CF is inherited in an autosomal recessive trait. More than 1600 CFTR mutations have been described so far, but the role of only part of them has been understood. Less than 10 mutations occur with frequency of more than 1% and the absence of phenyl alanine at position 508 (F508del, Phe508del, ΔF508) accounts for about two-thirds of mutated alleles in northern European and North American populations. In Europe the frequency of F508del mutation decreases towards the southeast. Testing for 40 most frequent mutation detects about 90% of affected persons in most populations. Frequency of carriers of the mutated gene is around 1 in 25-30 persons (4,6). Possible consequences of CTFR gene mutations are lack of protein production (class I mutations), protein trafficking defect (class II), defective regulation without CFTR activation (class III), reduced transport of chloride (class IV), reduced production of normal CFTR (class V) and accelerated degradation of CFTR (class VI). Pancreatic insufficiency is closely associated with class I–III mutations. As a result of F508del mutation CFTR is misfolded and proteolytically degraded in the proteasome. Only small amounts of F508del CFTR reach the plasma membrane and have a reduced half life (4,5).

12.2.2 PATHOGENESIS

CFTR belongs to a family of transmembrane adenosine triphosphate (ATP) binding proteins. It functions primarily as a chloride channel in apical membranes but also regulates transmembrane transport of sodium and bicarbonates.

In a most widely accepted hypothesis, lack of CFTR activity will cause less chloride secretion in the extracellular space and less water transport into the epithelial surface layer. As CFTR also inhibits sodium absorption, loss of its activity causes excessive sodium (and water) absorption through the epithelial sodium channel (5,7,8).Consequent airway-surface-liquid depletion leads to ciliary collapse, loss of mucociliary clearance and bacterial lung infections (Pseudomonas aeruginosa, Staphylococcus aureus, Burkholderia cepacia). Deficiency in bicarbonate secretion may lead to poor solubility and aggregation of luminal antibacterial mucins and low oxygen concentration may trigger P. aeruginosa to switch to mucoid cell types, resistant to host defences. However, colonisation of CF airways with these particular pathogens is not readily explained (5,9,10).

12.2.3 DIAGNOSIS

The diagnosis of cystic fibrosis should be considered in a child or an adult presenting with typical signs or symptoms such as:

a) chronic respiratory disease: bacterial infections typical for CF (S. aureus, P. aeruginosa, Burkholderia cepacia), chronic cough with sputum production, persistent infiltrates on chest radiographs, bronchiectasis, chronic sinuitis or nasal polyposis, clubbing of fingers and toe;
b) gastrointestinal symptoms: meconium ileus, distal ileal obstruction syndrome, rectal

prolapse, malnutrition, chronic diarrhea and steatorrhea, hypoproteinaemia, vitamin deficiencies (exocrine pancreatic insufficiency), recurrent or chronic pancreatitis, chronic liver/biliary disease; c) salt loss syndrome and d) obstructive azoospermia (4).

According to the European and US diagnostic guidelines cystic fibrosis consists of specific clinical (phenotypic) characteristics in combination with biochemical or genetic markers of CFTR dysfunction (11,12). The sweat test is the most clinically useful way of diagnosing cystic fibrosis. The test should be done with pilocarpine iontophoresis and a quantitative determination of chloride concentration (13). A diagnosis of cystic fibrosis can be made in a presence of clinical features if the concentration of chloride in sweat is greater than 60 mmol/L or if it is in the intermediate range (30–59 mmol/L for infants less than 6 months of age, 40–59 mmol/L for older individuals), and two disease-causing CFTR mutations are identified (12,14). There are certain conditions that may cause false-positive result of the sweat test, such as atopic eczema, malnutrition, congenital adrenal hyperplasia, ectodermal dysplasia, Klinefelter's syndrome, nephrogenic diabetes insipidus, adrenal insufficiency, hypothyroidism and autonomic dysfunction. A clinician should also be aware of the possible false-negative result in case of a sample dilution, malnutrition, peripheral oedema, low sweatrate, hypoproteinaemia, dehydration or in presence of CFTR mutations with preserved sweat duct function. The test is not accurate in newborn children, or children with a body mass below 3 kg (4,15).

Measurement of nasal transepithelial potential difference may be helpful method in persons who do not meet classic diagnostic criteria but it is difficult to perform and is not available in all centres. Also, full sequence analysis will detect most CFTR mutations but it may also reveal polymorphisms and new mutations of unknown importance (4,11,12).

Persons with milder problems associated with CFTR dysfunction may have male infertility, recurrent pancreatitis, chronic sinuitis and biliary disease. They usually have borderline chloride concentrations and the term "CFTR-related diseases" should be used for this group of disorders (4,16).

Almost 40% of children with CF have sufficient pancreatic function at birth in contrast to 10-15% at the age of five. Presence of F508del mutation practically determinates pancreatic disease: 99% homozygotes and 70% double heterozygotes have pancreatic insufficiency (PI). Most of class I-III mutations have also PI as a consequence, and most of mild mutations pancreatic sufficiency (PS), even in a presence of one severe mutation (4,15,17). Symptoms of malabsorption (chronic diarrhea, steatorrhea, failure to thrive), malnutrition and fat-soluble vitamin deficiency (bleeding disorder, night blindness, anemia, neuropathy, rickets, osteopenia) characterize PI (4,15). PS patients mostly have a milder course of the disease, better pulmonary function and lower sweat chloride levels. Recurrent pancreatitis is a manifestation of CF only in PS patients (17).

Low serum albumin, prolonged prothrombin time and/or low serum levels of fat soluble vitamins are features of PI. All patients with CF should be tested for presence of PI. Determination of the coefficient of fat malabsorption (CFA) during 3 or 5 days (fat stool content in relation to a dietary fat recorded) is the most accurate indirect test of pancreatic function. CFA above 7% in children older than 6 months, and above 10-15% in children below 6 months are pathologic (4,15). Difficulties in performance of this test lead to development of other diagnostic methods. Repeated analysis of acid steatocrit (percentage of fat in a stool sample) is less sensitive but a more simple method. NMR method is a relatively new method for the estimation of fat content of the stool (18). Determination of fecal elastase -1 in a stool sample is a relatively simple immunoassay that has a high sensitivity and specificity in diagnosing severe PI. The test is not so accurate in mild or moderate PI.

Pancreatic elastase is resistant to proteolysis in the bowel and therapy with pancreatic enzymes does not affect the test results (19).

12.3 SHWACHMAN-DIAMOND SYNDROME

Shwachman-Diamond syndrome (SDS) is a multi-system disease with the affection of the bone marrow, pancreas, skeleton, and other organs. Bone marrow failure, pancreatic exocrine dysfunction and skeletal abnormalities are the major features but the liver, kidneys, teeth, brain, and immune system may also be affected (20-22). Patients are prone to development of myelodysplastic syndrome (MDS) and leukaemia. Estimated incidence is 1/76.000. Although SDS is an autosomal recessive disorder, the ratio of males to females is 1,7 to 1 (20). Approximately 90% of patients meeting clinical criteria for the diagnosis of SDS harbour mutations in the SBDS gene (Shwachman-Bodian-Diamond syndrome). SBDS maps to the 7q11 centromeric region of chromosome 7 (23,24). The SBDS gene is highly conserved throughout evolution and is widely expressed in human tissues (24). SBDS encodes a predicted protein of 28.8 kd, and functions in 60S large ribosomal subunit maturation and in mitotic spindle stabilization. Recent data suggest that it may also affect actin polymerization, vacuolar pH regulation, and DNA metabolism (25).

12.3.1 HEMATOLOGICAL MANIFESTATIONS

Neutropenia is the most common hematological abnormality, occurring in nearly all patients. It can be persistent or intermittent, and some patients may have defects in migration and chemotaxis of neutrophils. Up to 80% of the patients have also normochromic and normocytic (or macrocytic) anemia with low reticulocytes. Fetal hemoglobin may also be elevated. Thrombocytopenia or severe aplasia with pancytopenia may also occur. Bone marrow is usually hypoplastic with increased fat deposition but normal or increased cellularity have also been observed (20-22).

12.3.2 PANCREATIC DISEASE

SDS is characterized with variably severe exocrine pancreatic dysfunction. Pancreatic acini are replaced with fat and islets of Langerhans and ductal architecture are preserved. Pancreatic dysfunction is usually diagnosed within the first six months of life and in almost all other patients during the first year. While secretion of enzymes is severely decreased, ductular electrolyte and fluid secretion remains normal (20-22). Also, improvement in pancreatic function may be observed in later childhood and by 4 years of age almost 50% of patients may no longer require pancreatic enzyme supplements (26).

12.3.4 LIVER DISEASE

Hepatomegaly is common and elevated serum liver enzymes are seen in up to 75% of young children but tend to resolve with age (26,27).

12.3.5 GROWTH FAILURE

Growth failure with malnutrition is usual in the first year of life, due to inadequate nutrient intake, feeding difficulties, pancreatic insufficiency and recurrent infections (20). During the first year over half of patients have both height and weight below the 3rd percentile. After starting pancreatic enzyme supplementation most children show normal growth velocity, but

remain consistently below the 3rd percentile for height and weight in half of patients (21,28,29).

12.3.6 BONE DISEASE

SDS-associated bone disease includes skeletal dysplasia and low-turnover osteoporosis. Children may have metaphyseal changes in the long bones and costochondral junctions and ess frequently other bone anomalies.

Delayed dentition of permanent teeth, dental dysplasia, increased risk of dental caries, and periodontal disease may also occur (20-22).

12.3.7 DIAGNOSIS

The clinical diagnosis is established by documenting exocrine pancreatic dysfunction and hematological abnormalities and excluding other causes of PI and bone marrow failure (cystic fibrosis, Pearson syndrome, cartilage hair hypoplasia and other inherited bone marrow failure syndromes such as dyskeratosis congenita)(20-22). Since indirect tests of pancreatic function may be normal despite a significant defect in pancreatic acinar function, serum pancreatic enzyme concentrations may serve as useful markers of the pancreatic phenotype in patients with SDS (20,21). Serum immunoreactive trypsinogen concentrations are low (<6 g/L) in patients with SDS and PI, and are usually above 6 g/L or normal in patients with PS patients (20,21,30). However, normal serum trypsinogen does not exclude impaired exocrine pancreatic function (20). Concentrations of fecal elastase less than 200 μg/g are indicative of severe pancreatic dysfunction, while values below 100 μg/g are suggestive of exocrine pancreatic insufficiency (20).

Hematologic work-up reveals intermittent or persistent neutropenia, or cytopenias of other blood cell lineages. Bone marrow aspiration and biopsy should include assessment of cellularity, iron stain and cytogenetics. Bone marrow cytogenetic finding of I(7q) or del(20q) is highly associated with SDS (20).

As the clinical diagnosis of SDS is usually difficult, it is advisable to test most or all suspected cases for mutations in the SBDS gene. About 10% of the SDS patients may be negative for mutations, and de novo SBDS mutations have been identified in some families (20,21).

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Review

Serological markers of inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD) is a heterogeneous group of chronic inflammatory disorders of the gastrointestinal tract with two main distinguishable entities, Crohn's disease (CD) and ulcerative colitis (UC). IBD-unclassified (IBD-U) is a diagnosis that covers the "grey" zone of diagnostic uncertainty between UC and CD. Current diagnosis of IBD relies on the clinical, endoscopic, radiological, histological and biochemical features, but this approach has shortcomings especially in cases of overlapping symptoms of CD and UC. The need for a diagnostic tool that would improve the conventional methods in IBD diagnosis directed the search towards potential immunological markers, since an aberrant immune response against microbial or endogenous antigens in a genetically susceptible host seems to be implicated in IBD pathogenesis. The spectrum of antibodies to different microbial antigens and autoantibodies associated with IBD is rapidly expanding. Most of these antibodies are associated with CD like anti-glycan antibodies: anti-Saccharomices cerevisiae (ASCA) and the recently described anti-laminaribioside (ALCA), anti-chitobioside (ACCA), anti-mannobioside (AMCA), anti-laminarin (anti-L) and anti-chitin (anti-C) antibodies; in addition to other antibodies that target microbial antigens: anti-outer membrane porin C (anti-OmpC), anti-Cbir1 flagellin and anti-I2 antibody. Also, autoantibodies targeting the exocrine pancreas (PAB) were shown to be highly specific for CD. In contrast, UC has been associated with anti-neutrophil cytoplasmic autoantibodies (pANCA) and antibodies against goblet cells (GAB). Current evidence suggests that serologic panels of multiple antibodies are useful in differential diagnosis of CD versus UC and can be a valuable aid in stratifying patients according to disease phenotype and risk of complications.

Key words: inflammatory bowel disease; colitis, ulcerative; Crohn disease; serological tests

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Introduction

The term IBD refers to a chronic and relapsing inflammatory disorder of the gastrointestinal tract (GIT) accompanied by abdominal pain, rectal bleeding and malabsorption. It comprises two major entities, ulcerative colitis (UC) and Crohn's disease (CD). Despite sharing similar clinical features, these diseases have significant clinical, endoscopic and histopathological differences (Table 1) (1-4).

Typically, IBD manifests between adolescence and the third decade of life, with approximately 10% of cases in individuals younger than 18 years (5). IBD diagnosis is based upon the coevaluation of clinical findings, endoscopic, radiological, histological and laboratory investigations with the main goal of excluding other conditions with similar presentations and defining the extent and severity of inflammation. In the majority of cases, endoscopic findings and histological examination of tissue biopsies provides a specific diagnosis of UC or CD (3,6-8). However, despite all available diagnostic methods, approximately 5 to 15% of patients with IBD affecting the colon are unclassifiable, as they present features of both conditions. Such patients are diagnosed with indeterminate colitis (IC) or IBD unclassified (IBDU), which is considered to be a temporary diagnosis since about 80% of these pa-

Table 1. The key features of Crohn's disease and ulcerative colitis.

Feature	Crohn's disease	Ulcerative colitis
Clinical features		
Diarrhea	Fairly common	Very common
Rectal bleeding	Fairly common	Very common
Sites of involevment		
Ileum and colon (ileocolonic region)	50% of patients	Never
Ileum	30% of patients	Never
Colon	20% of patients	Exclusively
Upper parts of GIT	Infrequent	Never
Endoscopic findings	Discontinuous lesions, cobblestoning, aphthous and linear ulcerations, strictures	Continous lesions, pseudopolyps
Histologic findings	Transmural inflammation	Mucosal/submucosal inflammation

tients will eventually be diagnosed with either UC or CD (2,9,10).

The growing body of evidence suggests that IBD evolved as a result of inappropriate and ongoing activation of the mucosal immune system driven by the commensal luminal microflora in a genetically susceptible host (2,4,11-14). The triggering factor for disturbance of the tightly regulated balance between immune tolerance and defensive inflammatory response to intestinal microbiota is yet to be discovered. The serological immune response in IBD patients, which includes antibodies against the yeast *Saccharomyces cerevisiae* (ASCA), *Escherichia coli* outer membrane porin C (Omp-C), flagelin (cBir1) and *Pseudomonas fluorescens* – associated sequence I-2 (I2), suggests that commensal flora or a dietary antigen is the triggering factor. On the other hand, the autoimmune concept has its base in the autoimmune extraintestinal manifestations of IBD (inflammation of the skin, eyes and joints), successful immunosupressive therapy and a variety of autoantibodies including antineutrophil cytoplasmic antibodies (ANCA), antibodies against exocrine pancreas (PAB) or intestinal goblet cells (GAB) (15-17).

The objective of this review is to give an overview of the current knowledge of the serological markers in IBD with regard to their use in differentiating IBD from other conditions with similar presentation, in differentiating UC from CD, in disease stratification and prediction and, finally, their response to therapeutic interventions in IBD.

Serologic markers of IBD

Generally, antibodies related to IBD encompasses two main groups: antibodies targeting microbial antigens and autoantibodies (Table 2.).

Table 2. Serological markers of IBD.

Antibodies to microbial antigens	Autoantibodies
Anti-glycan antibodies (ASCA, ACCA, ALCA, AMCA, Anti-L, Anti-C)	pANCA
Anti-OmpC	PAB
Anti-I2	GAB
Anti-Cbir1	

ASCA - Anti-Saccharomyces cerevisiae antibodies; ACCA - antichitobioside carbohydrate antibodies; ALCA - antilaminaribioside carbohydrate antibodies; AMCA - anti-mannobioside carbohydrate antibodes; Anti-L - anti-laminarin antibodies; Anti-C - anti-chitin antibodies; Anti-OmpC - antibody to outer membrane porin C; Anti-I2 - antibody to *Pseudomonas fluorescens* - associated sequence I2; Anti-Cbir1 - antibody to bacterial flagellin; pANCA - anti-neutrophil cytoplasmic antibodies; PAB - antibodies against exocrine pancreas; GAB - antibodies to goblet cells.

Anti-glycan antibodies

These antibodies targets cell wall carbohydrate epitopes found in microbiota such as yeasts and bacteria (18,19). The most prominent member of this group of antibodies are anti-*Saccharomyces cerevisiae* antibodies (ASCA). The major antigen targeted with ASCA antibodies is the 200 kDa phosphopeptidomannan (PPM), a cell wall mannan of the common baker's or brewer's yeast *Saccharomyces cerevisiae*. The greatest discrimination among patients with Crohn's disease, ulcerative colitis, and controls was obtained with the Su1 strain of *S. cerevisiae* used in beer brewing and mannotetraose was identified as the most important polysaccharide epitope within PPM (16,20). Regarding the widespread distribution of oligomannosides, three theories have been presented in an attempt to explain the mannose-induced immunological response. The first theory assumed that ASCA antibodies originate from immunization by dietary yeasts or yeasts that colonize the digestive tract, as a consequence of increased exposure of yeast antigens to immune reactive cells due to increased intestinal permeability (20-23). The second theory considers the epitopes shared by other microorganisms (*Mycobacterium* species), and the third presupposes structural homologies between *S. cerevisiae* oligomannosides and oligomannosides expressed on human glycoconjugates as autoantigens or neoautoantigens (20,24). ASCA was shown to have a high specificity for CD, and both IgA and IgG antibodies are formed. Methods used for the detection of these antibodies are indirect immunofluorescence (IIF) using smears of *Saccharomyces cerevisiae* or standardized enzyme linked immunosorbent (ELISA) assays with an antigen derived from disrupted or boiled *S. cerevisiae* and phosphopeptidomannan purified from the cell wall (gASCA assay) coated on microtiter plates (25-30).

In an attempt to identify novel antibodies associated with inflammatory bowel disease, Dotan *et al.* (18) profiled sugar-binding antibodies from the serum of patients with diagnosed CD or UC using glycan array technology and ELISA. The newly identified antibodies were antilaminaribioside carbohydrate IgG antibodies (ALCA), antichitobioside carbohydrate IgA antibodies (ACCA) and anti-mannobioside carbohydrate IgG antibodes (AMCA) (18,31,32). Laminaribioside is a building block of the glucose-based glycan laminarin, while chitobioside is a building block of the N-acetyl-glucosamine-based glycan chitin. Both laminaribioside and chitobioside, as well as mannose and mannan, are components of the cell walls of microorganisms such as bacteria, fungi and yeast and are capable of stimulating the immune system, specifically innate immunity (33). The most recently discovered members of the anti-glycan family of antibodies in IBD were anti-laminarin (anti-L) IgA antibodies and anti-chitin (anti-C) IgA antibodies (34). Similarly to ASCA, these antibodies have proven to be specific for CD, though with significantly lower sensitivity.

Antibody to outer membrane porin (anti-OmpC)

OmpC is an outer membrane porin C isolated from *Escherichia coli.* Originally, this protein was identified as a pANCA cross-reactive antigen using the library of colonic bacteria (35). The ELISA assay demonstrated an excessive secretion of IgA anti-OmpC antibodies in CD patients (36,37).

Antibody to Pseudomonas fluorescens - associated sequence I2 (anti-I2)

In 2000, the novel DNA sequence (I2) with homology to the ptxR and tetR bacterial transcription factor family was isolated from CD colonic lesional mucosa, suggesting that the microorganism expressing the I2 gene product may be related to CD pathogenesis (38). This bacterial sequence has been shown to derive from *Pseudomonas fluorescens* (39). An ELISA assay showed frequent immunoglobulin A seroreactivity in CD as opposed to UC or other inflammatory enteric diseases and healthy individuals (37,38).

Antibody to bacterial flagellin CBir1 (anti-CBir1)

Among bacterial antigens, flagellin is an interesting candidate to play a role in mucosal immune responses because it is a common bacterial antigen present on most motile bacteria in the gut and is highly antigenic. Multiple strains of colitic mice had elevated serum anti-flagellin IgG2a responses,

and flagellin CBir1 has been identified as an immunodominant colitogenic antigen. In line with this, it has high anti-CBir1 IgG reactivity in human CD patient sera, as detected with the ELISA assay, and only minor reactivity in the sera of patients with UC or other inflammatory GIT diseases. CBir1 flagellin is most closely related to the flagellins of bacteria in the genera *Butyrivibrio, Rosburia, Thermotoga*, and *Clostridium* and fall within the *Clostridium* subphylum XIVa cluster of Gram-positive bacteria (40).

Anti-neutrophil cytoplasmic antibodies (ANCA)

ANCAs are classically associated with small-vessel systemic vasculitis such as Wegener granulomatosis, Churg-Strauss syndrome, microscopic polyangiitis and its renal-limited variant (pauci-immune necrotizing and crescentic glomerulonephritis), where their measurement is used in the purposes of diagnosis, prognosis and in monitoring of inflammatory activity (41). In vasculitis, this heterogenous family of antibodies targets different proteins, mainly located in the azurophilic granules of neutrophils and in lysosomes of monocytes. According to the International Consensus Statement (41), ANCAs are screened by indirect immunofluorescence (IIF) on normal peripheral blood neutrophils where two main types of fluorescence pattern are obtained: cytoplasmic granular with accentuation between nuclear lobes (cANCA) and fine homogenous, diffuse rim-like staining of the perinuclear cytoplasm (or rim-accentuated fluorescence of the nuclei) designated as the pANCA pattern. The major ANCA antigen targets in inflammatory vasculitides are proteinase-3, mainly displaying cANCA pattern and myeloperoxidase associated with the pANCA pattern (41-43).

The distinct subset of ANCA associated with UC was first reported in 1990 (44,45). The pattern of staining on IIF exhibited broad inhomogeneous rim-like staining of the nuclear periphery, different from classical pANCA and was designated as atypical p-ANCA. Various target antigens of atypical pANCA have been intensively studied in IBD patients. These studies included proteins located in the granules of the neutrophils and monocytes such as: serine proteases cathepsin G and elastase, hydrolase β-glucuronidase, iron-binding protein lactoferrin and the natural antibiotic bactericidal permeability increasing protein (BPI); cytoplasmic proteins such as α-enolase and catalase; proteins distributed in the cytoplasm and nuclei of eukaryotic cells: high-mobility group of non-histone chromosomal proteins (HMG-1 and HMG-2), and finally proteins located in the nuclei, such as histone H1 (25,27). Overall, most studies supported the conclusion that IBD-associated ANCA specific antigens are not located within neutrophil granulas but rather within the nuclei. The immunoelectron microscopy finding of UC-associated pANCA reactivity localized over chromatin concentrated toward the periphery of the nuclei supports this thesis (46). However, UC sera did not react with double-stranded DNA. Vidrich *et al.* (47) demonstrated the loss of the UC-related pANCA staining pattern after digestion of substrate cells with DNAse, suggesting that the epitope recognized by this subset of antibodies is a protein-DNA complex or that the presence of intact DNA is necessary to maintain the integrity of the epitope. It is likely that the target antigen for UC-related atypical pANCA is a complex conformational epitope which comprises the previously reported nuclear proteins histone H1, HMG-1 and HMG-2. Since the target antigen for UC-associated pANCA is yet unrecognized, sensitive and specific solid-phase methods cannot be developed, and therefore IIF on normal peripheral blood neutrophils is still the most commonly employed method in use. Detection of DNase I - sensitive pANCA antibody is more specific for UC in differentiation from similar pANCA patterns in autoimmune liver diseases. Recently, lactoferrin was suggested as a major pANCA target in UC but it has to be bound to DNA to present epitope relevant for the reaction with the autoantibodies. Use of the lactoferrin reconstituted (LFR) granulocytes (granulocytes stripped of pANCA targets and then reconstituted with human lactoferrin) as a substrate in addition to standard ethanol-fixed granulocytes raised the sensitivity of the IIF assay from 71.8% to 87.2% (48).

Antibodies against exocrine pancreas (PAB)

These antibodies were first described in 1987 by Stöcker *et al.* (49) who tested sera of patients with

CD and UC for autoantibodies by the IIF method using 19 different human tissues as antigenic substrates. They demonstrated that autoantibodies against exocrine pancreas (PAB) were found almost exclusively in CD patients (although with rather low sensitivity) and suggested that this specific autoantigen is a component of normal pancreatic juice. In the IIF assay on sections of human or primate pancreatic tissue, PAB antibodies stain different structures of the exocrine pancreas and are divided into two subtypes accordingly: subtype I with a typical, extracellular, drop-like staining pattern in the acinar lumen of pancreatic tissue sections, and subtype II with speckled staining of the cytoplasm of pancreatic acinar cells (50). Recently, the major zymogen granule membrane glycoprotein (GP2), a glycosylphosphatidylinositol (GPI)-anchored protein of the pancreatic acinar cells was identified as the autoantigen of PAB in CD (51). Upon hormonal or neuronal stimulation of the pancreas, GP2 is transported to the apical compartment of acinar cells, from which it is released, together with zymogens, into the pancreatic duct. The two obtained fluorescence patterns of PAB on pancreatic tissue are consistent with the localization of GP2. In addition to pancreatic acinar cells, M cell-specific expression of GP2 in humans and mice was also seen among the intestinal epithelium (52,53) The characteristic of the M-cells as specialized epithelial cells of mucosa-associated lymphoid tissues (Peyer's patches) is their role in the transport of antigens from the lumen to the cells of the immune system (54). It was found that the GP2 expressed on M cells serves as an uptake receptor for a subset of commensal and pathogenic bacteria (53). Bearing in mind that Peyers patches are abundant in the distal part of the ileum, the predominant site of inflammatory onset in CD, Roggenbuck *et al.* (51) demonstrated GP2 expression at mRNA and protein levels in colon biopsies from patients with CD at a significantly higher level than in UC colon biopsies. This observation supports the hypothesis of a direct involvement of anti-GP2 in CD pathophysiology, rather than being merely an epiphenomenon as an antibody targeting non-intestinal antigen.

Antibodies to goblet cells (GAB)

Intestinal epithelial cells represent a physical barrier against the excessive entry of bacteria and other antigens from the intestinal lumen into the circulation. Goblet cells, as specialized intestinal epithelial cells, regulate the production of mucus and factors that contribute to epithelial repair and regulation of inflammation (4). GAB have been detected primarily in adult UC patients with prevalence varying from 15% to 46.6% while in CD patients observed prevalence of GAB showed even wider range from 1.4% to 33% (15,49,55,56). Obtained differences in GAB prevalence are likely attributed to methodological differences, such as the use of different origin of antigenic substrate for IIF: human or monkey intestinal tissue, rarely rat jejunum or human colonic cancer cell line HT29-18-N2, which differentiates into intestinal goblet cells. Tissue substrates are associated with problematic reproducibility due to the natural fluctuations of tissue quality. The next sources of variability in the obtained results lie in the evaluation of fluorescence patterns on IIF. Beside the IIF test, the ELISA assay using the HT29-18-N2 cell line assay is also in use for GAB detection (55).

Clinical usefulness of serological investigation of IBD

The main concerns regarding clinical usefulness of serological markers in IBD refers to: a) their efficiency in distinguishing IBD from other diseases with similar clinical presentation and in distinguishing subtypes of IBD (ulcerative colitis from Crohn's disease), b) their prognostic value in stratifying disease phenotypes, and c) monitoring disease activity and reflecting the response to therapeutic intervention.

Serological investigation for IBD diagnostic purposes

Use of serological markers in distinguishing IBD from other non-IBD gastrointestinal diseases

Currently, diagnosis of IBD is based on a combination of clinical, radiological, endoscopic and histological studies and, in most cases, the diagnosis

can be made with high certainty. Assessment of the currently known IBD-associated antibodies has not surpassed the diagnostic accuracy of the previously mentioned conventional methods, mainly due to their limited sensitivity (Table 3) (15,19,26,27, 29,33,55,56-59).

A positive test result for any individual antibody, even those with highest sensitivity like pANCA or ASCA, only modestly influences the pretest/post-test probability in distinguishing IBD from other GIT disorders with similar clinical presentation, while a negative test result has no clinical value. Although most studies have confirmed the high specificity of IBD-associated antibodies (75–99%), caution should be taken in considering the control groups of non-IBD GIT disorders that mainly included irritable bowel syndrome, infectious colitis or functional gut disorders (26). Namely, ASCA that was considered as a highly CD-specific antibody was observed in 30% to even 59% of patients with celiac disease prior introducing the gluten-free diet. ASCA antibodies were more often of the IgG class and were, unlike the ASCA-IgA, insensitive to gluten withdrawal (60,61). Another highly CD-specific antibody, PAB, showed a frequency of 22.3% in celiac patients at diagnosis (more often IgA class), and positivity demonstrated a tendency to be lower in patients on a strict gluten-free diet (56). This observation suggests that the presence of ASCA or PAB may be a marker for increased permeability of the small bowel and autoimmunity instead of a specific IBD (CD) marker.

Therefore, at the present, assessment of serological markers are not suitable for screening for IBD in patients with gastrointestinal symptoms, but rather as assistance in cases of a diagnostic dilemma.

The variation in the prevalence of individual serological markers across studies could be explained by differences in the methods used. For example, pANCA have been determined with standardized IIF on ethanol fixed neutrophils from healthy donors, or with fixed granulocytes ELISA, or using ELISA followed with IIF for ANCA-ELISA positive samples. Some studies were performed with IIF including the step with DNase I digestion of neutrophils. Variation in the sensitivity of pANCA as-

Table 3. Prevalence of individual serological markers in patients with IBD, non-IBD GIT disorders and healthy individuals (15,19,26, 27,29,33,55-59).

Antibody	Immunoglobulin class	Prevalence (%)			
		CD	UC	other GIT disorders	Healthy
ASCA	IgA and/or IgG	29–71	0–29	0–23 (37.9)*	0–16
ACCA	IgA	8–25	5–7	3–20	0.5–12
ALCA	IgG	17.7–27	3–8	9	2
AMCA	IgG	12–28	7	8	9
Anti-L	IgA	11–26	3–7	23	1–10
Anti-C	IgA	10–25	2–10	11	2–12
Anti-OmpC	IgA	24–55	2–24	5–11	5–20
Anti-I2	IgA	38–60	2–10	19	5–15
Anti-Cbir1	IgG	50–56	<6	14	8
PAB	IgA and IgG	26–39	0–22.7	0–11.5 (22.3)*	0–8
GAB	IgA and IgG	1.4–33	15.4–46.6	0–9.3	0
pANCA	IgG	2–38	24–85	8	0–8

* prevalence in active celiac disease. CD - Crohn's disease; UC - ulcerative colitis; ASCA - Anti-Saccharomyces cerevisiae antibodies; ACCA - antichitobioside carbohydrate antibodies; ALCA - antilaminaribioside carbohydrate antibodies; AMCA - anti-mannobioside carbohydrate antibodes; Anti-L - anti-laminarin antibodies; Anti-C - anti-chitin antibodies; Anti-OmpC - antibody to outer membrane porin C; Anti-I2 - antibody to *Pseudomonas fluorescens* - associated sequence I2; Anti-Cbir1 - antibody to bacterial flagellin; PAB - antibodies against exocrine pancreas; GAB - antibodies to goblet cells; pANCA - anti-neutrophil cytoplasmic antibodies.

says from 0–63% in UC samples across five different laboratories suggests that these assays obviously do not detect the same spectrum of antigens (28). Also, considerable lack of agreement exists within the same methodology due to differences in antigen preparation and quality, cut-off values based on the receiver operating curve, differences in evaluation of fluorescence patterns for IIF methods, cut-off titer or the origin of the substrate used for IIF.

Distinguishing CD from UC

The heterogeneity within the IBD group of disorders influences the clinical presentation with overlapping symptoms of CD and UC. In up to 15% of cases, no differentiation into a particular IBD subtype can be made, giving rise to a diagnosis of IBD-unclassified (IBD-U) or previously known as indetermined colitis (IC). Differentiation into either of the IBD subtypes in the early phase of the disease has an influence on the tailoring of drug therapy. According to retrospective database analysis of 250 children diagnosed with IBD, IBD-U appears to have a higher prevalence among paediatric patients (up to 29.6%) and is associated with early disease onset and rapidly progresses to pancolitis (62). In the same study, 66.2% patients maintained their diagnosis of IBD-U after a mean follow-up of 7 years, which favours the hypothesis of some investigators that IBD-U is a unique disease phenotype within the IBD group, with more a extensive disease, more severe clinical course and higher rate of complications. Paediatric IBD patients comprise a particular population in whom non-invasive testing is desirable and who would benefit the most from the early proper therapeutic approach. As the specificity of serological markers exceeds their sensitivity, serological profiles can be useful in the differentiation of IBD subtypes (62,63).

Most of the data pertaining to the usefulness of serological markers in distinguishing CD from UC refer to the ASCA and pANCA antibodies, while fewer data are available for other anti-glycan antibodies, anti-I2, anti-Omp-C, anti-Cbir1, PAB or GAB. Overall, ASCA has the best combined sensitivity and specificity for CD and pANCA for UC. Most other investigated serological markers are specific for CD, with the exception of GAB. Table 4. summarizes the data on diagnostic accuracy of individual and combined antibodies in the differential diagnosis of of CD and UC (10,15,26-30,56,64-66).

The general opinion is that combined testing instead of individual antibodies is more useful in obtaining a differential diagnosis of CD versus UC. Profile ASCA+/pANCA- increases specificity and positive predictive value (PPV) for diagnosis of CD comparing to ASCA+ as isolated result. In the same manner, the reverse profile pANCA+/ASCA- was shown to have a higher specificity and PPV for diagnosis of UC than pANCA+ alone (26-30). Profile pANCA-/ASCA- was found to have a strong positive correlation with IBD-U diagnosis. Prospective study of IBD-U patients revealed that half of the patients had pANCA-/ASCA- seroprofile and the vast majority of them did not change the initial diagnosis after 6 years. Therefore, pANCA-/ASCA- seems to be the serological marker closely associated with IBD-U as a separate disease entity (30,63).

Recent study assessed the reactivity of seven anti-glycan antibodies in a large cohort of 818 IBD patients, including gASCA IgA and IgG (gASCA is an improved ASCA assay based on immobilized purified mannan polysaccharide), ACCA, ALCA, AMCA, anti-L and anti-C (34). Within the CD patient population, 73% were positive for $\geq$ 1 anti-glycan antibody. All anti-glycan markers were specific for CD and were significantly more prevalent in CD than in UC. The most efficient discrimination between CD and UC was achieved by the addition of anti-L and anti-C to gASCA/pANCA panel while adding of anti-L to the same panel improved differentiation of colonic CD from UC.

Diagnostic accuracy for CD *versus* UC of the gASCA and ALCA antibodies was find out to be similar between adult and paediatric IBD cohorts, while discrepancies were found for AMCA and ACCA. In paediatric population, both serologic markers showed significantly higher specificity, while AMCA showed significantly lower sensitivity compared to adults (33).

The importance of combined testing was pointed out by the finding that about one third of ASCA negative CD patients may be positive for at least

Table 4. Diagnostic accuracy of individual serological markers and their combinations in differential diagnosis of CD and UC (10,15,26-30,56,64-66).

Diagnosis	Antibody	Sensitivity (%)	Specificity (%)	PPV	NPV
CD	ASCA +	37–72	82–100	87–95	36–68
	pANCA -	52	91	85	65
	ACCA	9–21	84–97	78–87	24–52
	ALCA	15–26	92–96	78–90	25–53
	AMCA	12–28	82–97	65–92	25–52
	Anti-C	10–25	90–98	87–88	29–39
	Anti-L	18–26	93–97	90–91	30–40
	Anti-OmpC	20–55	81–88	83	25
	Anti-I2	42	76	NR	NR
	PAB	22–46	77–100	69–100	48–75
	ASCA+/pANCA-	46–64	92–99	86–97	44–82
	PAB+/ANCA-	22–42	98–100	87–100	48–74
	PAB+/ASCA+/pANCA-	16–34	97–100	100	66–72
UC	pANCA	50–71	75–98	74–95	49–84
	pANCA+/ASCA-	42–58	81–100	93–100	43
	GAB	12*–46	98	75–93	70–74
	pANCA or GAB+/PAB-	82	98	96	89

* in pediatric population. PPV - positive predictive value; NPV - negative predictive value; ASCA - Anti-Saccharomyces cerevisiae antibodies; pANCA - anti-neutrophil cytoplasmic antibodies; ACCA - antichitobioside carbohydrate antibodies; ALCA - antilaminaribioside carbohydrate antibodies; AMCA - anti-mannobioside carbohydrate antibodes; Anti-C - anti-chitin antibodies; Anti-L - anti-laminarin antibodies, Anti-OmpC - antibody to outer membrane porin C; Anti-I2 - antibody to *Pseudomonas fluorescens* - associated sequence I2, PAB - antibodies against exocrine pancreas; GAB - antibodies to goblet cells; NR - not reported.

one of the previously mentioned anti-glycan antibodies (67).

Similarly, anti-CBir1 were positive in about half of ASCA-negative adult CD patients while the addition of the anti-CBir1 assay to the ASCA, pANCA and anti-OmpC panel halved the number of serologically negative paediatric CD patients (68,69).

PAB was confirmed as a highly specific marker of CD in several studies, but with low sensitivity (15,29,65). Therefore, the use of PAB in combination with pANCA and ASCA was suggested, particularly in the differentiation between isolated colonic CD and UC where the clinical difficulty lies in the differentiation between CD and UC. In another study, authors concluded that PAB detection could be useful only in clinically highly suspected patients without circulating ASCA, since they found PAB positivity in 14% of CD patients that were negative for ASCA (29). Several studies, however, showed lower specificity of PAB in distinguishing CD from UC both in adult and paediatric patients (56,63,66).

In majority of reports, GAB was confirmed as highly specific serological marker in distinguishing UC from CD, but due to the low sensitivity (especially in paediatric population, 12%) it is poorly usable in differential diagnosis of IBD subtypes, both in adults and children (15,56,65,66,70).

However, in a recent study by Homsak *et al.* (15), the combination of positive pANCA or GAB with negative PAB managed to detect the majority of UC patients.

In summary, testing for isolated antibody is of limited value in differential diagnosis of IBD subtypes, while the combined testing of several antibodies (serologic panels) significantly improves specificity

and PPV for certain IBD subtype. Furthermore, widening panel of antibodies can also improve the sensitivity. However, result of serological testing is not decisive but is an adjunctive tool in patients in whom all other clinical features does not allow a distinction between CD and UC.

Use of serological testing in disease phenotype stratification

The heterogenic nature of both CD and UC is reflected in the different phenotypes of the disease, according to location, clinical course and activity or behaviour patterns, and response to treatment within each IBD subtype. An ability to stratify IBD subtypes by the risk for disease progression and complications would most certainly improve overall disease outcomes through an early decision of the most appropriate treatment option available.

In addition to contributing to an improved IBD diagnosis, there is mounting evidence of a link between serum immune reactivity and specific clinical phenotypes in IBD.

In CD, disease extent can evolve with time from a non-stricturing, non-fistulizing, inflammatory phenotype to a more severe stricturing (fibrostenotic) or penetrating (with internal fistulae, fistulizing) phenotype (71). Numerous studies have examined the presence of different IBD-related antibodies and disease behaviour (9,19,26,64,68,69,72-80). Positive association of the most of the anti-microbial, especially anti-glycan antibodies with more complicated CD phenotype and a higher frequency of Crohn's disease-related abdominal surgery has been consistently demonstrated (Table 5). It is important to emphasize that this association becomes stronger with increasing diversity (multiple antibodies) and magnitude (higher titers) of the serologic response.

Table 5. Association of serological markers with CD phenotype.

Antibody	CD phenotype characteristics	Reference
ASCA (ASCA+/pANCA-)	disease located in small bowell (or ileocolonic) stricturing and/or penetrating higher risk for IBD-related surgical interventions early disease onset	9, 19, 26, 64, 72–76, 79, 80
pANCA (pANCA+/ASCA-)	benign (UC-like) with colonic involvment, non-stricturing, non-penetrating	9, 72, 77
anti-CBir1	disease located in small bowell, stricturing and/or penetrating early disease onset	68, 69, 81
anti-OmpC	disease located in small bowell, stricturing and/or penetrating higher risk for IBD-related surgical interventions	78 - 80
anti-I2	higher risk for stricturing phenotype higher risk for IBD-related surgical interventions	79
AMCA	stricturing and/or penetrating higher risk for IBD-related surgical interventions, early disease onset	19, 67, 78, 80
ACCA	stricturing and/or penetrating higher risk for IBD-related surgical interventions	19, 78, 80
ALCA	stricturing and/or penetrating higher risk for IBD-related surgical interventions	78, 80
anti-L	stricturing and/or penetrating strong association with IBD-related surgical interventions	19, 64
anti-C	strong association with IBD-related surgery	64

ASCA - Anti-Saccharomyces cerevisiae antibodies; pANCA - anti-neutrophil cytoplasmic antibodies; Anti-Cbir1 - antibody to bacterial flagellin; Anti-OmpC - antibody to outer membrane porin C; Anti-I2 - antibody to *Pseudomonas fluorescens* - associated sequence I2, AMCA - anti-mannobioside carbohydrate antibodes; ACCA - antichitobioside carbohydrate antibodies; ALCA - antilaminaribioside carbohydrate antibodies; Anti-L - anti-laminarin antibodies, Anti-C - anti-chitin antibodies.

Similar results were obtained in the paediatric population. Dubinsky *et al.* (81) evaluated associations between anti-I2, anti-OmpC, anti-CBir1 and ASCA immune response and clinical phenotype in 196 paediatric CD patients. Increased frequency of internal penetrating and/or stricturing disease with increasing diversity of immune response was demonstrated, with the highest odds in patients positive for all four antibodies. Also, patients positive for ≥1 antibody progressed to a complicated disease faster than those negative for all antibodies. These results were further confirmed for the same panel of antibodies in a later study performed on a 796 paediatric CD patients (82).

Data on the association of PAB with the CD phenotype are somewhat conflicting. In the Eastern European IBD cohort, antibody response to PAB was proven to be associated with complicated disease phenotype and extraintestinal manifestations. The presence of PAB was associated with perianal disease and extraintestinal manifestations such as arthritis, ocular or cutaneous manifestations. Also, the presence of PAB IgA antibodies was associated with penetrating disease behaviour (56). On the contrary, in the study of Joosens *et al.* (57) both PAB patterns were negatively associated with stricturing disease behaviour of CD. In the study conducted on Caucasian and Chinese IBD populations, PAB expression was not associated with stricturing or perforating CD, while in another study, the small differences in PAB prevalence in CD subtypes do not suggest that PAB detection is useful in the discrimination of CD phenotypes (65,83). In a study of PAB and GAB antibodies in paediatric IBD patients, there was a lack of correlation with the clinical phenotype (66).

In contrast to CD, UC has a less heterogeneous disease behaviour but can evolve into a more aggressive phenotype with regard to a higher number of surgical interventions, moderate to severe disease activity or larger disease extent. In the study performed on 366 IBD patients, no relation of pANCA in UC patients or in CD patients was found with disease activity, duration of illness, location, disease extent, previous bowel operations or medical treatment (84). In another study, a tendency of higher prevalence of pANCA+ or pANCA+/ASCA- reactivity in severe UC compared to remission cases according to the Montreal classification was demonstrated, though this difference was not statistically significant. The pANCA-/ASCA- pattern was observed less often in active UC when compared to remission phase although with borderline significance (9).

In other UC cohorts, pANCA expression was significantly associated with a higher relapse rate, more aggressive disease course requiring early colectomy or with the treatment-resistant left-sided disease (85-87). A possible association between pANCA and relative resistance to medical therapy in UC patients was also recently documented, with negative pANCA status as an independent positive predictor for response to treatment with infliximab (88).

Recent studies indicated that serological responses may identify patients with higher risk for postoperative complications in patients with UC or IBD-U. Patients who were pANCA-/ASCA+ were shown to have an increased risk for the development of fistulas after surgical intervention compared to patients who were pANCA+/ASCA-, and were also more likely to have their diagnosis changed postoperatively to CD (89). Another study confirmed preoperative ASCA-IgA seropositivity as a predictor of postoperative CD diagnosis in UC and IBD-U patients (90). There is an indication that high levels of pANCA prior to colectomy is significantly associated with the development of postoperative complications in UC patients (91).

In summary, an assessment of serological markers is useful as a predictor of complicated disease behaviour in CD or in predicting postoperative complications in UC or IBD-U patients. The presence of multiple antibodies and the magnitude of the immunological response appear to be the strongest predictors of disease progression.

Use of serologic markers in monitoring disease activity and response to drug therapy

According to the available data, there is no use of serial measurement of IBD serological markers, including ASCA, ALCA, ACCA, anti-OmpC or pANCA in monitoring disease activity (26,27,92).

Regarding the association with response to therapy, there was no relationship between ASCA or pANCA and response to therapy in a study conducted on 279 CD patients before starting anti-TNF therapy (infliximab). Although lower response rates were observed for patients with refractory intestinal disease carrying the pANCA+/ASCA- combination, this finding lacked significance (P = 0.067) (93). Possible association of pANCA with relative resistance to medical therapy was further documented in a study by Sandborn *et al*. (87) who found an increased frequency of pANCA in treatment-resistant left-sided ulcerative colitis. The report of a negative pANCA status as an independent positive predictor for response to therapy with infliximab in UC patients supports this (88). Other studies have not found an association between ALCA, ACCA, AMCA, anti-OmpC, anti-I2 or pANCA and treatment in CD (26).

Other aspects of IBD serological markers

Prevalence and diagnostic value of IBD serological markers have shown significant variation among different ethnic or geographic population. For example, in Chinese, Japanese and Iranian CD patients ASCA was shown to be less sensitive comparing to Caucasians. On the other hand, studies conducted on Tunisian, Korean and Brasilian patients yielded prevalences comparable to Caucasian CD patients The prevalence of pANCA was found to be lower in Chinese, Japanese, Korean, Thai and Romanian patients with UC but higher in Mexican-American compared to Caucasian UC patients (26,32). Therefore, these data suggest that ethnic background should be considered when applying IBD serological markers in clinical practice.

Family studies indicated ASCA as potential subclinical biomarker for population in risk for CD, as it has been reported that this antibody is present with significantly higher frequency (20-25%) in unaffected first-degree relatives of CD patients, as compared to general healthy population (0-10%) (94,95). Furthermore, in the retrospective study, ASCA reactivity was found at a median of 38 months before clinical diagnosis in 32% of CD patients (96). In contrast to ASCA, PAB seems to rarely occur in family members of patients with Crohn's disease (50).

pANCA was not proven as a marker of increased susceptibility for disease in first-degree relatives of patients with UC (26). Recent study assessed risk factors for CD in multicase families and a cumulative effect of number of first-degree affected relatives, and number of positive antimicrobial antibodies (ASCA, AMCA, ALCA, ACCA, anti-OmpC, anti-CBir1, Anti-I2) was found (97).

An interesting aspect of IBD-related serologic response is their possible role in bridging the genetic susceptibility and clinical disease. Several studies investigated the association of serological markers with IBD-susceptible gene mutations (26,98). In spite of some inconsistency, more studies found ASCA frequency significantly associated with greater frequency of mutant NOD2/CARD15 alleles and also the genotype-seroreactivity synergism in predicting complicated CD phenotypes (33,59, 72,99). In accordance with this, it is reported that association of other anti-glycan antibodies (ALCA, AMCA, ACCA and ASCA) with NOD/CARD15 mutations in a dose-effect manner is found where more mutations were associated with higher seroreactivity (78,80).

Conclusions

The current diagnostic approach based upon clinical, endoscopic, histological, radiological and biochemical criteria provides a reliable diagnosis in the majority of cases of IBD over other GIT disorders that share similar clinical presentation, as well as differentiation into IBD-subtypes, CD or UC. However, there are certain cases where a significant overlap is present in the results of conventional diagnostic tests, thereby makes differentiation of these two subtypes difficult. It is this particular point in the diagnostic algorithm of IBD where serological testing has the greatest benefit. Due to their lack of sensitivity, serological markers are not advisable for use in the diagnosis of IBD but rather in differentiating CD from UC, particularly with the use of a wide panel of antibodies. According to the growing evidence of an association between the magnitude of serological immune reactivity and specific clinical phenotypes, the most

important clinical utility of serological markers could be in stratifying patients according to risk for aggressive disease phenotype or postoperative complications. Such a "risk score" that would integrate markers of immune response, genetic markers and clinical characteristics might enable the application of personally-tailored therapeutic strategies and better surveillance of patients at risk. At the current time, there is insufficient evidence of usefulness of serological markers in monitoring the treatment of IBD patients.

Potential conflict of interest

None declared.

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14. LABORATORY FINDINGS AND CLINICAL INDICES AS INDICATORS FOR ENDOSCOPIC REMISSION IN LUMINAL CROHN'S DISEASE

Clas-Göran af Björkesten

14.1 INTRODUCTION

Crohn's disease (CD) is a disabling chronic inflammatory bowel disease with a relapsing and remitting course (1,2). Exacerbations are characterised by diarrhoea, abdominal pain and rectal bleeding. The assessment of disease activity has traditionally been based on symptoms and clinical signs. During the era of therapy with anti-tumour necrosis factor alpha antibodies (anti-TNF), a complete disappearance of mucosal ulcerations has been associated with significantly fewer hospitalizations and surgeries in patients receiving scheduled anti-TNF therapy, and a complete mucosal healing has been shown to be the only factor predicting long-term steroid-free remission after initiation with anti-TNF (3-5). Accordingly, determination of inflammatory activity should be essential for the assessment of disease activity, for tailoring therapy and measuring treatment response. Ideally, a disease activity marker should be disease specific, reflect disease activity, be usable in every-day clinical practice and identify patients at risk for relapse. No such disease activity markers have been identified so far.

Since endoscopic monitoring of the treatment response in CD is time-consuming, difficult to perform, invasive, expensive and unpleasant for the patient, surrogate biochemical markers are frequently used in assessing disease activity. There have been only a few attempts to develop combined clinical scores consisting of both clinical findings and symptoms and laboratory markers to improve the diagnostic accuracy. This review aims to summarise the literature on the power of indirect disease activity assessment methods detecting endoscopic remission, with a focus on combined scores comprising combinations of both clinical indices and laboratory findings.

14.2 CLINICAL ASSESSMENT OF DISEASE ACTIVITY

The Crohn's Disease Activity Index (CDAI) is the most common index used for assessment of disease activity (6,7). The index consists of eight components, each summed after adjustment with a weighing factor. The components are the following: number of liquid or soft stools each day for seven days (weighing factor 2), abdominal pain, graded 0 (no pain) to 3 (severe pain), each day for seven days (weighing factor 5), general well-being, subjectively assessed from 0 (well) to 4 (terrible) each day for seven days (weighing factor 7), presence of complications, with one point each added for each set of complications: arthralgia/arthritis, iritis/uveitis, erythema nodosum/pyoderma gangrenosum/oral aphtous ulcers, anal complications, other fistulae, fever (weighing factor 20), use of anti-diarrhoea medication (weighing factor 30), presence of palpable abdominal mass, graded 0 (none), 2 (questionable) and 5 (definite) (weighing factor 10), haematocrit of <0.47 in males and <0.42 in females (weighing factor 6), and percentage of deviation from standard weight (weighing factor 1). However, the correlation of the CDAI with ileocolonoscopy findings is weak and it seems to underestimate endoscopically determined inflammatory activity (8-10). The clinical remission

with the CDAI is usually set at scores <150 (7). When using this cut-off, the sensitivity of the CDAI to detect endoscopic remission is only 24-36% (11-13). With optimized CDAI cut-offs far below 150, the diagnostic accuracy has been slightly improved, with sensitivities 67-71% and specificities 64-70% (11,13). Even if the CDAI is mainly based on clinical signs and symptoms and therefore in general is regarded a clinical index, it is noteworthy that it also notices the haematocrit. However, compared to the other items in the CDAI, the weighing factor of haematocrit is small.

Due to the complexity of calculating the CDAI it has been suggested that the simpler Harvey-Bradshaw index (HBI) might offer an easier assessment for CD activity (14, 15). The HBI consists only of clinical parameters: general well being (0-4 points), abdominal pain (0-3 points), number of liquid stools per day, palpable abdominal mass (0-3 points), and complications/extraintestinal features (one point for each). For the HBI it has been suggested, that clinical remission would be set at scores ≤4 (15).

14.3 ENDOSCOPIC ASSESSMENT OF DISEASE ACTIVITY

Findings in ileocolonoscopy can be scored either by the Crohn's disease endoscopic index of severity (CDEIS), or more easily in clinical practice, by using the simple endoscopic score for Crohn's disease (SES-CD) (16-18). Nevertheless, measuring endoscopic response can be challenging, as there is no consensus on either the SES-CD or CDEIS definition for endoscopic remission or significant response. The most suggested definitions on endoscopic remission for the SES-CD have been a score of 0-2 and 0-3 (9,12,19).

14.4 BIOCHEMICAL MARKERS OF DISEASE ACTIVITY

The most used surrogate markers used to monitor disease activity in CD are C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), haemoglobin, haematocrit, white blood cells and albumin. CRP is an acute phase protein produced by the liver in response to tissue damage due to inflammation, infection or injury. Regrettably, its correlation with CD activity has been inconsistent (20-23). A proportion of patients with mildly active disease seem to systemically present low CRP values, which to some extent has been suggested to be a consequence of genetic polymorphism (24). The use of high sensitivity CRP may be a more sensitive method of detecting low-grade inflammatory luminal disease than standard CRP, but it is still in limited use in every-day clinical practice (25). Despite their changes during inflammation, ESR, haemoglobin, haematocrit, white blood cells and albumin are all in one way or another too unspecific and insensitive to alone function as reliable markers of endoscopic remission.

Faecal calprotectin, a neutrophil derived calcium and zinc binding protein, has repeatedly been shown to correlate with both SES-CD scored endoscopy and histological findings in luminal CD. A normal calprotectin concentration has also been found to be a reliable surrogate marker for endoscopically and histologically inactive disease (9,18,25,26). However, there are still open questions regarding the role of calprotectin as a marker of inflammatory activity. Despite the frequently used cut-off values <50 μg/g or <100 μg/g for calprotectin to discriminate between active and inactive CD, the optimal cut-off value for endoscopic remission in CD is still somewhat unclear, with suggested values of up to <200 μg/g for endoscopically inactive disesase (10,27). Additionally, a considerable intraindividual variance of calprotectin concentrations in stool samples has been reported in a population of patients with mild to moderate clinical activity (28). There are also study results suggesting that calprotectin concentrations are dependent on the inflamed bowel segment (12).

Lactoferrin is an iron-binding glycoprotein secreted by most mucosal membranes. It is a major component of the secondary granules of polymorphonuclear neutrophils, which are a primary component of the acute inflammatory response (29). Polymorphonuclear neutrophil elastase (PMN-e) is a neutral proteinase normally stored in the azurophil granules of polymorphonuclear neutrophils but released by activation of these cells as a mediator of inflammation (30).

14.5 COMBINING CLINICAL INDICES AND BIOCHEMICAL MARKERS TO DETECT ENDOSCOPIC REMISSION

It is indisputable, that the assessment of CD activity requires a combination of clinical observations, laboratory and endoscopy findings and in certain situations radiological imaging. Studies on the attempts to successfully combine systematically different noninvasive tests and clinical findings and symptoms to assess the presence or absence of endoscopically determined inflammation are rare (11,13). All four combined scores comprising different combinations of clinical indices and laboratory findings found in a literature search are presented below and compared in Table 14.1.

Table 14.1 Diagnostic accuracy of combined scores to discriminate endoscopically active Crohn's disease from inactive disease.

	Cut-off	Sensitivity %	Specificity %	Patients *n*	Measurements *n*	Author
Comprehensive index[a]	Categorical	79	70	43	43	Langhorst et al. 2008
HBI + 2 × ln[Calprotectin (µg/g)][b]	10	85	82	64	106[e]	Björkesten et al. 2012
Calprotectin (µg/g) + 60 × HBI[c]	155	86	82	64	106[e]	Björkesten et al. 2012
CRP (mg/l) + 3 × HBI[d]	4	80	59	64	150[e]	Björkesten et al. 2012

Legend**: a** - A categorical index consisting of the Crohn's disease activity index (CDAI) C-reactive protein (mg/l), calprotectin (µg/ml), lactoferrin (µg/ml) and polymorphonuclear neutrophil elastase (µg/ml). Endoscopically inactive disease was defined as a complete absence of inflammatory lesions in all observed segments of the colon and the terminal ileum; **b** - The Harvey-Bradshaw index combined with 2 × the natural logarithm of faecal calprotectin. Endoscopically inactive disease was defined as SES-CD ≤2; **c** - Faecal calprotecin combined with 60 × the Harvey-Bradshaw index. Endoscopically inactive disease was defined as SES-CD ≤2; **d** - C-reactive protein combined with 3 × the Harvey-Bradshaw index. Endoscopically inactive disease was defined as SES-CD ≤2; **e** - Inflammatory activity was assessed 1–3 times in each patient during follow-up.

14.5.1 COMBINING THE CDAI, CRP, AND THREE STOOL PARAMETERS

Langhorst et al. studied the performance of the **CDAI**, **CRP**, and **three stool parameters** (calprotectin, lactoferrin and polymorphonuclear neutrophil elastase [PMN-e]) at discriminating active inflammation from inactive inflammation in 43 patients with CD (11). Inactive inflammation was defined as a complete absence of inflammatory lesions in all observed segments of the colon and the terminal ileum. Extraintestinal and small bowel involvement was excluded by clinical examination and contrast-enhanced magnetic resonance imaging of the small bowel. Their comprehensive activity index was rated positive when at least two out of the following three conditions were fulfilled: (a) at least two stool parameters were elevated, (b) elevated CRP, and (c) elevated CDAI. CRP >7 mg/l and CDAI >80 were considered elevated whereas stool samples were classified as elevated based on following

optimized cut-off values: Calprotectin >48 μg/ml, lactoferrin >7.05 μg/ml, PMN-e ≥0.062 μg/ml. The sensitivity for this categorical index was 79% and the specificity 70%, which was superior to either CRP or the CDAI, but still inferior to calprotectin (sensitivity 82%, specificity 80%).

14.5.2 COMBINING THE HBI AND CALPROTECTIN

In an observational follow-up study on 64 anti-TNF treated patients with active luminal CD, with inflammatory activity assessed 1–3 times in each patient during follow-up, the sensitivity for calprotectin was 84% and specificity 74% to discriminate between endoscopically determined remission (SES-CD 0-2) and active disease (SES-CD ≥3) (13). For the HBI the corresponding sensitivity was 80% and specificity 56%. Further calculations were made to analyze whether the accuracy to identify endoscopically determined inflammation could be improved by combining noninvasive tests and clinical indices. Receiver-operating characteristic (ROC) curves based on the sums of differently emphasized HBI, CDAI, calprotectin, and CRP, assessed at the follow-up control, were constructed. The scores based on the HBI and calprotectin proved consequently to be superior to both other combinations of parameters and calprotectin alone. The highest area under the ROC curve (AUC), 0.900, was obtained when using the score: **HBI + 2 × ln[Calprotectin(μg/g)]**. The sensitivity for the score was 85% and the specificity was 82%, when using its resulting sum 10 as a cut-off value for endoscopic remission. The sensitivity and specificity of a simplified, non-logarithmic score in the form of **Calprotectin (μg/g) + [60 × HBI]** were almost identical, although it had a somewhat lower AUC at 0.880. The differences in diagnostic accuracy between these both scores and calprotectin alone were not statistically significant, though.

14.5.3 COMBINING THE HBI AND CRP

In the follow-up study on 64 anti-TNF treated patients with active luminal CD, the clinical indices and CRP, used alone or in combination, consistently proved to be clearly inferior to calprotectin alone (13). However, the score **CRP + [3 × HBI]** slightly improved the identification of endoscopic remission compared with either test used separately, with a sensitivity of 80% and specificity of 59%. The differences between this score and either test separately were not statistically significant either.

14.6 FUTURE PERSPECTIVES

The increasing need for reliable surrogate methods for discriminating active mucosal inflammation from mucosal healing will guarantee that new combinations, new surrogate markers and new techniques are further studied in the future. On basis of available studies on surrogate markers for mucosal inflammation, focusing on the power of the combination of high sensitivity CRP and faecal calprotectin and/or a clinical index could be a logical consequence. Additionally, radiological imaging, including high-resolution and contrast-enhanced ultrasound and magnetic resonance imaging, will probably have more important roles in the future non-invasive assessment of mucosal inflammation (31,32). Another attractive field of upcoming non-conventional, although invasive investigation of colonic inflammatory activity is capsule endoscopy developed for the assessment of the colon (33).

14.7 CONCLUSION

As endoscopy displays direct evidence of mucosal injury, but is time-consuming, invasive and expensive, there is an increasing need for reliable surrogate methods for discriminating active

mucosal inflammation from mucosal healing. Due to the superiority of faecal calprotectin to CRP and clinical indices commonly used in the assessment of disease activity in luminal CD, the use of calprotectin is consecutively increasing. However, as the sensitivity and specificity for calprotectin to detect endoscopic remission at its highest has been around 80%, there have been attempts to develop combined clinical scores consisting of both clinical findings and symptoms and surrogate markers to improve the diagnostic accuracy. These combined scores would be simple and cost-effective screening tools for detecting endoscopic remission in the treatment and surveillance of CD in the future and could partly replace the need for endoscopic monitoring in active luminal CD. However, the studies performed in the subject are very limited and so far no statistical superiority of the combined scores over calprotectin alone has been demonstrated.

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