Laboratory evaluation of pancreatic diseases

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ABSTRACT

In this review an update of the contribution of Laboratory Medicine to the diagnosis and monitoring of pancreatic disease is given. Today, laboratory tests not only play a pivotal role in the diagnosis of acute pancreatitis, but also may help to assess the disease severity and its etiology. In the diagnosis of acute pancreatitis lipase measurement in serum is superior to (P-type) amylase in terms of cost-effectiveness and diagnostic performance. Laboratory is also important in detection and monitoring of pancreatic insufficiency (by elastase-1 and chymotrypsin) and in monitoring of pancreatic cancer (by carbohydrate antigen 19.9).

INTRODUCTION

The pancreas lies on the posterior wall of the abdomen. The pancreatic duct, in the head of the pancreas, enters the duodenal loop through the ampulla of Vater and the sphincter of Oddi, releasing its extensive exocrine secretions. The tail of the pancreas extends away from the duodenum toward the spleen. The pancreas is composed of the acini and islets of Langerhans. The islets are responsible for producing endocrine hormones (insulin, glucagon, somatostatin, pancreatic polypeptide). The acini secrete digestive enzymes (amylase, lipase, proteases) and other secretory products (bicarbonate, electrolytes) needed for breakdown of proteins, carbohydrates, and fats.

TESTS AND METHODOLOGIES USED FOR INVESTIGATION OF PANCREATIC DISEASE

Amylase

α-Amylase (1,4-α-D glucan glucanohydrolase, AMY) catalyzes the hydrolysis of complex carbohydrates at the 1,4-α-glucosidic linkages of adjacent glucose residues. The amylases occurring in human plasma have MW varying from 54,000 to 62,000 Da and are small enough to pass through the glomeruli of the kidneys. AMY is the only plasma enzyme that is normally found in urine. AMY is present in a number of organs and tissues (1). The greatest concentration is present in the salivary glands, which secrete a potent amylase (S-type) to initiate hydrolysis of starches while the food is still in the mouth and esophagus. In the pancreas, the enzyme (P-type) is synthesized by the acinar cells and then secreted into the intestinal tract by way of the pancreatic duct system. AMY activity is also found in extracts from semen, testes, ovaries, fallopian tubes, striated muscle, lungs, and adipose tissue. The enzyme present in normal serum and urine is predominantly of pancreatic and salivary gland origin. These two isoenzymes are products of two closely linked loci on chromosome 1. AMY isoenzymes also undergo post-translational modification of deamidation, glycosylation, and deglycosylation to form a number of isoforms.[1]

Macroamylase is a complex between immunoglobulin, usually IgG or IgA, and AMY, usually the salivary isoenzyme. Because of the high MW of this complex (>200,000 Da), macroamylase is not cleared by the kidneys and thus high enzymatic activity persists in the plasma. As for most macromolecular complexes of enzymes with immunoglobulins (e.g., macro-CK and LD), there is no clinical significance to macroamylasemia.

Methods for the Determination of Total AMY Activity

The use of defined substrates and auxiliary and indicator enzymes in the AMY assay has improved the reaction stoichiometry and has led to more controlled and consistent hydrolysis conditions. Substrates used include small oligosaccharides and 4-nitrophenyl (4-NP)-glycoside substrates. 4-NP-glycoside substrates are prepared by bonding 4-NP to the reducing end of a defined oligosaccharide. If the oligosaccharide is maltoheptaose (G7), the substrate is then 4-NP-G7. At the beginning, problems arose with the use of the 4-NP-glycoside assay with regard to the poor stability of the reconstituted assay mixture because of the slow hydrolysis of the 4-NP-glycoside by α-glucosidase, used as auxiliary enzyme in the reaction. This effect has been reduced by covalently linking a "blocking" group, i.e., a

[1]*Isoenzyme is the term used to refer to genetically distinct forms of an enzyme: here, the pancreatic (P) and salivary (S) types of AMY. Isoform refers to variants of the isoenzymes that arise from variability in post-translational processing of the two isoenzymes P and S.*

4,6-ethylidene group (ethylidene-protected substrate), to the nonreducing end of the molecule. The blocked substrate also shows a different and more advantageous hydrolysis pattern. As a result, the cleavage of one α-glucosidic linkage by AMY results in the release of one molecule of 4-NP (Figure 1). The IFCC has optimized this method at 37 °C, recommending it as a reference measurement procedure for AMY (2). Using assays traceable to this IFCC recommended method, the serum reference interval is 31 to 107 U/L.

Methods for the Determination of Pancreatic AMY Isoenzyme

The lack of clinical specificity of total AMY measurement has led to the interest in the direct measurement of pancreatic amylase (P-AMY) instead of total enzyme activity for the diagnosis of patients with suspected acute pancreatitis. A double monoclonal antibody assay is commercially available that uses the synergistic action of two immunoinhibitory monoclonal antibodies to salivary amylase. After the salivary isoenzyme activity is inhibited by the addition of the antibodies, the uninhibited P-AMY activity is measured using 4,6-ethylidene-4-NP-G7 as a substrate. This assay is today available in full automation on clinical chemistry platforms with reagent costs similar to total AMY assays, which permits to abandon the latter (3). In healthy adults, P-AMY represents approximately 40% to 50% of the total AMY activity in serum. Using the immunoinhibition method, the reference interval for P-AMY activity in sera from adults is 13 to 53 U/L (4). False-positive P-AMY results have been reported in subjects with macroamylasemia, in whom the immunoglobulin complexed to AMY forms diminishes or voids the ability of monoclonal antibodies included in the test to efficiently inhibit salivary isoenzyme. Upon electrophoresis, macroamylase usually forms a broad migrating band, clearly different from the homogeneous bands that are produced by AMY isoenzymes present in serum (Figure 2). If electrophoretic separation is not available, precipitation of the macrocomplex by a polyethylene glycol (PEG) 6000 solution represents a good alternative. Residual AMY activity of less than 30% in the supernatant is indicative of macroamylasemia (Figure 3).

Pancreatic lipase

Lipases (triacylglycerol acylhydrolases) catalyze the hydrolysis of glycerol esters of long-chain fatty acids (triglycerides) sequentially into β-monoglyceride and two free fatty acids. With spontaneous isomerization to the α-form, the third fatty acid is also hydrolyzed, but at a slower rate. Human pancreatic lipase is a single-chain glycoprotein with a MW of 48,000 Da. Its concentration in the pancreas is ~9000-fold greater than in other tissues, and the concentration gradient between pancreas and serum is ~20,000-fold (5). Lipase activity depends on the substrate being present as an emulsion. The presence of bile salts (such as sodium deoxycholate) and a cofactor called colipase, a small

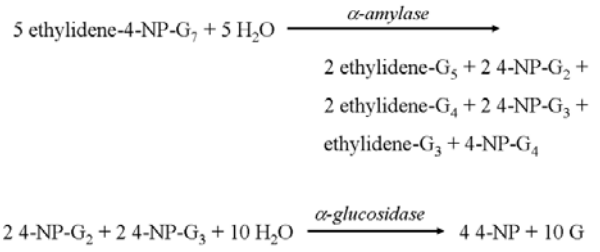

Figure 1
Principle of the assay for the determination of amylase activity using blocked 4-nitrophenyl-maltoheptaose (4-NP-G7) as substrate.

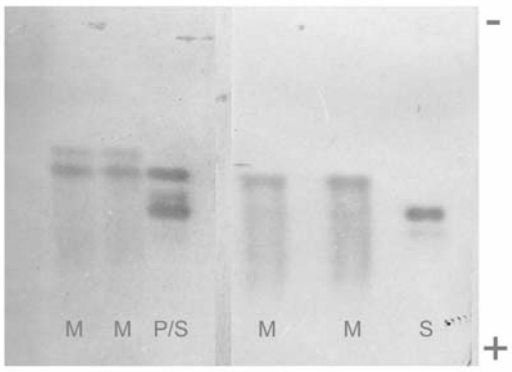

Figure 2
Cellulose acetate electrophoresis of amylase isoenzymes. M, macroamylasemia; P/S, mixture of two samples containing, respectively, pancreatic juice and saliva; S, saliva. The anodal direction is downward.

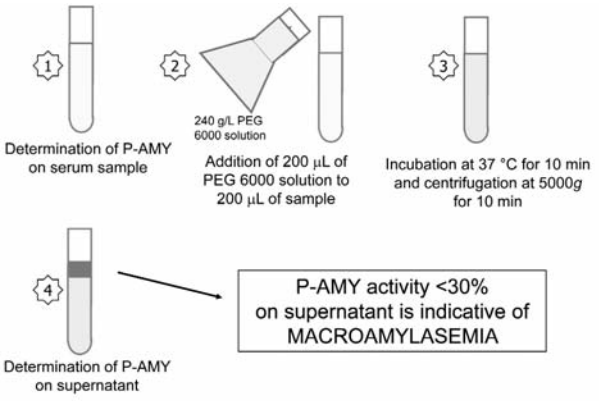

Figure 3
Demonstration of macroamylasemia by polyethylene glycol (PEG) 6000 solution.
P-AMY, pancreatic amylase.

protein secreted by the pancreas, increases the reaction rate and analytical specificity, eliminating lipoprotein lipase interference. Colipase, aided by the addition of bile salts to stabilize the triglyceride emulsion, binds to lipase to form a complex. This association produces a conformational change for lipase, such that the latter can now more efficiently bind to the substrate to produce lipolytic products (Figure 4). Other lipolytic enzymes

released into blood, including intestinal lipase and nonspecific hepatic triacylglyceride lipase, are more specific for short-chain fatty acid substrates.

Methods for the Determination of Pancreatic Lipase Activity

A number of substrates and complex auxiliary and indicator systems are currently used in lipase methods. In general, long-chain triglyceride (and some diglyceride) substrates have demonstrated a correlation of results with the clinical state that is superior to that with methods using other substrates (6). In the Vitros dry film technology, a mixed-chain fatty acid substrate is used, which reduces the specificity of the assay for pancreatic lipase. In the enzymatic reaction rate diglyceride assay, a sequence of indicator and auxiliary enzymes is used to form a quinonediimine dye, which is measured at 550 nm (7). More recently, the synthetic substrate 1,2-O-dilauryl-rac-glycero-3-glutaric acid-(4-methyl-resorufin)-ester, consisting of two glycerol ether and one ester bonds, has been proposed and assays based on its use are currently gaining widespread use (Figure 5). Using this assay, the upper reference limit is 38 UL at 37 °C, with no gender- or age-related differences (8).

Trypsin

Trypsin is a pancreatic proteolytic enzyme that specifically cleaves peptide bonds at the carboxyl groups of lysine and arginine. The human pancreas synthesizes two trypsins (1 and 2) in the form of inactive trypsinogens. Activation of enzymes occurs in the intestinal tract by enterokinase. Inhibitors, such as α_1-antitrypsin and α_2-macroglobulin, protect other plasma proteins against hydrolysis by trypsin if the enzyme enters the vascular system, so that free enzyme is not usually found in serum (9). Because of the existence of these antiproteases in the blood, trypsin is measured by immunoassays also detecting trypsinogen and complexed trypsin ("immunoreactive trypsin"). The utility of these assays has been examined for diagnosis of pancreatic injury. However, as there is no distinct role of trypsin estimation in patients with suspected acute pancreatitis, this test is considered of limited clinical value. Measurement of trypsin from dried blood spots can be used as a screening marker for cystic fibrosis.

Elastase-1

Human pancreatic elastase-1 is a carboxyendopeptidase that catalyzes hydrolysis of native elastin, the major structural fibrous protein in connective tissue, with a special affinity for the carboxyl group of alanine, valine, and leucine (10). The enzyme is synthesized by the acinar cells of the pancreas as a preproelastase. After processing to proelastase, it is stored in the zymogen granules and later it is activated to elastase by trypsin in the duodenum, undergoing minimal degradation during intestinal transit. An ELISA method in a microplate sandwich format is commercially

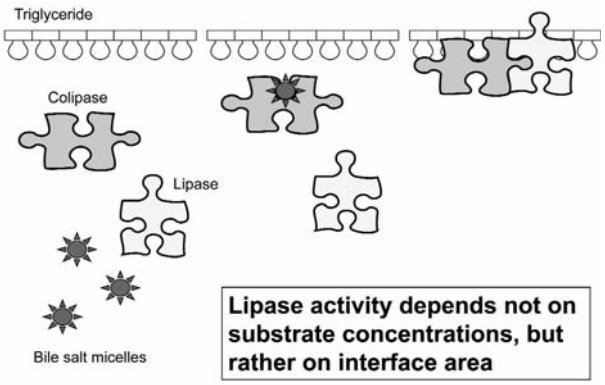

Figure 4
Mechanism of action for pancreatic lipase with colipase and bile salts. The pancreas secretes both lipase and colipase as separate proteins. In the intestine, the two proteins are bound in a complex, the binding being favored by the presence of bile salt micelles. Lipase is conformationally altered by colipase to favor binding to the substrate interface, where triglycerides can be hydrolyzed.

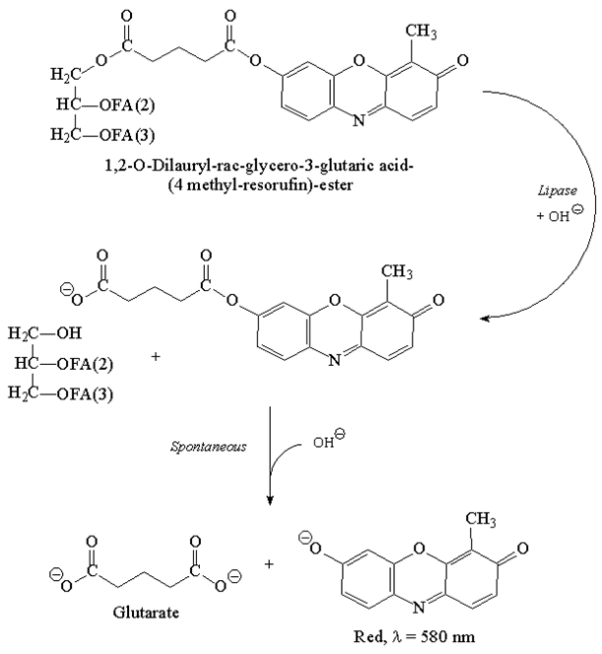

Figure 5
Reaction sequence of the chromogenic method using 1,2-O-dilauryl-rac-glycero-3-glutaric acid-(4-methyl-resorufin)-ester as substrate for measurement of pancreatic lipase activity.

available to measure elastase-1 mass concentrations in stool samples. The enzyme has been found to be stable in stool samples for up to 1 week at room temperature. The lower reference limit of fecal elastase-1 concentration was found to be 200 μg/g stool.

Chymotrypsin

Chymotrypsin hydrolyzes peptide bonds involving carboxyl groups of tryptophan, leucine, tyrosine, or

phenylalanine, with preference for the aromatic residues. The acinar cells of the human pancreas synthesize two different chymotrypsins (1 and 2, the latter being the major species) in the form of the inactive proenzymes, chymotrypsinogens-1 and -2. These zymogens are stored in granules and are secreted like trypsinogen into the pancreatic duct. In the intestinal tract, the chymotrypsinogens are converted to chymotrypsins by the action of trypsin. Chymotrypsin is more resistant than trypsin to degradation in the intestine; it is therefore the enzyme of choice for assay in feces. Synthetic substrates are commonly used for this application. A sensitive kinetic assay has been developed, that uses succ-ala-ala-pro-phe-4-nitroanilide as a substrate, and made commercially available. Prior treatment of the stool specimen with detergent to release particle-bound chymotrypsin is necessary. A cheap device enables a small stool sample to be homogenized, extracted, and centrifuged with minimal inconvenience. The extract is mixed with the substrate, which is hydrolyzed by chymotrypsin to produce free 4-nitroanilide, measured photometrically at 405 nm. The lower reference limit of fecal chymotrypsin activity, measured at 37 °C, was found to be 12 U/g stool (11).

Carbohydrate antigen 19-9

The carbohydrate antigen (CA) 19-9 is a tumor-associated antigen, first described in 1979, defined by a monoclonal antibody (1116 NS 19-9) produced against a human colorectal carcinoma cell line. It has been biochemically characterized as a sialylated lacto-N-fucopentaose II oligosaccharide related to the Lewis A blood group substance. The CA 19-9 epitope is carried out by mucins representing physiological secretion products found in exocrine body fluids like pancreatic juice. Accordingly, CA 19-9 can be detected in epithelial cells of the pancreas, bile ducts, gallbladder, and stomach. Pathobiochemical alterations in pancreatic cancer lead to increased serum concentrations of CA 19-9 mucins that acquire tumor marker properties. Fully automated immunoassays using non-competitive sandwich techniques are currently available showing fairly good analytical performance. However, significant differences have been shown in CA 19-9 values measured with different platforms, so that the goal of between-assay interchangeable CA 19-9 results has not yet been achieved and a single assay must be used in the monitoring of individual patients (12).

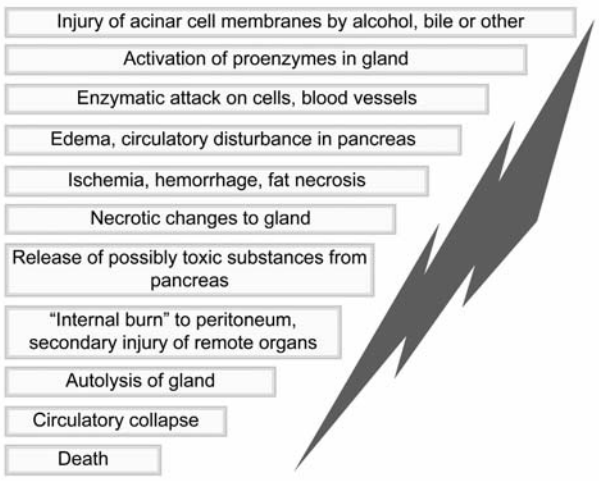

Figure 6
Pathogenic cascade of acute pancreatitis.

INTERPRETATIVE ASPECTS OF TESTS IN THE CONTEXT OF PANCREATIC DISEASE

Acute pancreatitis

Acute pancreatitis is an inflammatory disease of the pancreas. Acute abdominal pain is the most common symptom, and increased plasma concentrations of pancreatic enzymes confirm the diagnosis. Histopathologically, acute pancreatitis is further subclassified into interstitial pancreatitis (mild edema with signs of cellular inflammation) and necrotizing pancreatitis (microscopic and gross pancreatic necrosis) (approximately 85% vs. 15%, respectively). Pancreatitis results when digestive enzymes of the exocrine pancreas find their way into the parenchyma, and autodigestion of pancreatic tissue ensues (Figure 6). Key among these digestive enzymes is trypsin, which (together with other bile components) activates other proteolytic enzymes. Risk factors include excessive alcohol use, the presence of gallstones, and hypertriglyceridemia.

Diagnosis of Acute Pancreatitis

The diagnosis of acute pancreatitis is made on the basis of clinical presentation (presence of acute and constant pain in epigastric area or the right upper quadrant, radiate to the back, associated with nausea), combined with biochemical and/or imaging findings. The diagnosis is complicated by several factors including the presentation of nonspecific symptoms, the contraindication of pancreas visualization by laparotomy, false-negative results by trans-abdominal ultrasonography and abdominal computed tomography (CT) in up to 30% of cases, and the release of pancreatic enzymes in diseases of surrounding tissues.

The laboratory tests most commonly used include serum (P-type) AMY and pancreatic lipase. After an attack of acute pancreatitis, serum enzyme concentrations rise within a few hours and peak at about 24-36 h, remaining increased for 72–96 h (AMY), but up to 7–14 days for lipase (Figure 7). The extent of enzyme elevations in blood is not related to the severity of the disease; however, the greater the rise, the greater the probability of acute pancreatitis. The clinical sensitivity of serum enzymes is ~90% when blood is sampled in the first 36 h. Elevation of serum lipase activity greater than 3 times the upper reference limit, in the absence of renal failure, is a more specific diagnostic finding than

increases in serum AMY activity and should thus be preferred in the emergency department (Table 1) (13).

Biliary tract diseases, such as acute cholecystitis, may cause up to fivefold elevations of the serum pancreatic enzyme activities as a result of either primary or secondary pancreatic involvement. Some intraabdominal events other than pancreatitis can also lead to a significant increase in serum lipase, even if lipase has far less tissue distribution than P-AMY and, thus, its elevation in serum is less frequently associated with nonpancreatic disease states (Table 2). Finally, in subjects with a reduced glomerular filtration rate the activities of serum enzymes are increased in proportion to the extent of renal impairment (usually, no more than five times the upper reference limit).

To summarize, the diagnostic performance of pancreatic biomarkers for acute pancreatitis is greatly improved by restricting their use to a population with suspected disease. The lipase measurement is superior to (P-type) AMY in terms of cost-effectiveness and diagnostic performance; therefore, it is recommended that lipase replace AMY as initial test for acute pancreatitis. The obtaining of both serum (P-type) AMY and lipase is not warranted.

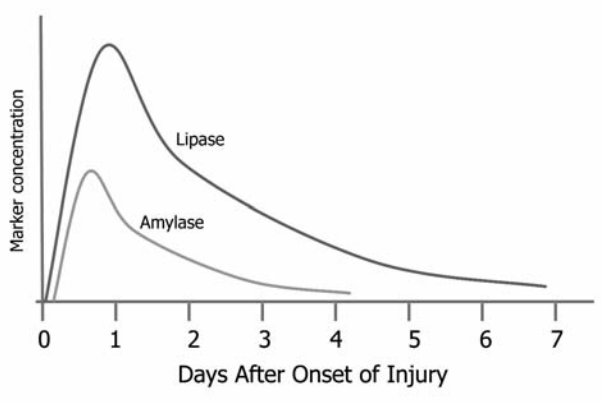

Figure 7
Time-dependent changes in serum amylase and lipase after acute pancreatitis.

Table 1
Diagnostic accuracy of serum pancreatic lipase for the diagnosis of acute pancreatitis in patients with acute abdominal pain (estimated disease prevalence in emergency department, 10%) (modified from ref. 6)

Cut-off	Sensitivity	Specificity	Negative predictive value	Positive predictive value
URL	90-100%	84-87%	99.5%	47.1%
3 x URL	79-82%	91-97%	97.7%	81.4%
5 x URL	69-74%	94-98%	96.6%	88.5%

URL, upper reference limit.

Assessment of Disease Severity

The assessment of severity is one of the most important issues in the management of acute pancreatitis. However, no test exists that can accurately estimate prognosis at admission. A refined prediction of severity can be achieved at 48 h after disease onset by use of the APACHE II scoring system (based on values of 12 routine physiological measurements, age, and previous health status), utilizing a cutoff of ≥8, and C-reactive protein concentrations (>150 mg/L) (13). CT scan can selectively provide additional prognostic information at 72 h in patients with predicted severe disease.

Determination of Etiology

Although there are many causes of acute pancreatitis in adult, the two most frequent are gallstone migration into the common bile duct and alcohol abuse (Table 3). Determination of the cause is important for guiding immediate management and preventing recurrence. If an attack of pancreatitis is associated with elevation of the serum alanine aminotrasferase (ALT) and alkaline phosphatase (ALP) activities, there is 100% likelihood that the source of the pancreatitis is biliary (Table 4). Laboratory testing may also reveal hypertriglyceridemia as possible causes of pancreatitis (typically with serum triglyceride concentrations >1000 mg/dL).

Chronic pancreatitis and pancreatic insufficiency

Chronic pancreatitis, defined as a continuing inflammatory disease of the pancreas, can be caused by either recurrent episodes of acute pancreatitis (the most common cause in adults) or continual subclinical

Table 2
Conditions associated with an elevation of pancreatic lipase in serum

Pancreatic disease:	– Acute pancreatitis, any cause
	– Pancreatic trauma
Intraabdominal diseases other than pancreatitis:	– Acute cholecystitis
	– Perforated peptic ulcer
	– Intestinal obstruction
	– Peritonitis
Renal insufficiency	

pancreatic damage occurring over a period of several years (as seen in cystic fibrosis, the most common cause in children), which typically cause permanent loss of exocrine function evolving into end-stage disease leading to steatorrhea. Major predisposing factors to chronic pancreatitis may be categorized as either a) toxic-metabolic (e.g., alcohol), b) idiopathic, c) genetic, d) autoimmune, e) recurrent and severe acute pancreatitis, or f) obstructive (e.g., duct obstruction by tumor) (TIGAR-O system) (14).

The abdominal CT scan (revealing pancreatic calcifications, dilated pancreatic duct, and parenchymal atrophy) is the initial test for the diagnosis of chronic pancreatitis, even if the pancreatic biopsy represents the gold standard. Functional abnormalities alone (e.g., abnormally low concentrations of exocrine pancreatic products in feces) cannot be diagnostic because, in case, they detect the presence of a pathophysiological sign (pancreatic insufficiency) without any differentiation of the causing mechanism. Invasive tests of pancreatic function (e.g., the secretin test) are considered the gold standard for determining exocrine pancreatic function. However, very few centers perform direct testing. Elastase-1 measurement in stool is currently the most reliable noninvasive procedure for the diagnosis of chronic pancreatic insufficiency because possible treatment with pancreatic enzyme supplements does not interfere with the test results. However, such a test does not consistently detect mild to moderate insufficiency cases (sensitivity from 22% to 77%). In chronic pancreatic insufficiency treated with oral pancreatic enzyme supplements, monitoring of elastase-1 provides no information helpful to the therapeutic management of these patients. On the contrary, the measurement of fecal chymotrypsin, which is included in oral supplements, may provide valuable information, revealing whether the therapy is adequate or whether increased supplementation is necessary (15). Measurements of serum enzyme concentrations are less useful. A decreased P-AMY activity in serum (less than lower reference limit) identifies with high specificity (~95%) patients with exocrine pancreatic insufficiency and steatorrhea. If, however, the enzyme activity is normal, a reduced pancreatic function cannot be excluded (sensitivity <60%) (16). In general, as the pancreas has significant functional reserve, subjects with low-grade functional disease will not be detectable by enzyme studies.

Cystic fibrosis is a genetic disorder that primarily affects the lungs and digestive system resulting in thick mucus production that blocks ducts in the pancreas preventing normal transport of trypsinogen. In this condition, plasma trypsin concentrations have been reported to be high in neonates; as the disease progresses, the concentration falls. Newborn screening is done by the measurement of immunoreactive trypsin in dried blood specimens. Infants who have high concentrations on initial testing undergo further assessment via a repeat test 1-3 weeks later or by analysis of the initial blood spot for specific DNA mutations.

Table 3
Causes of acute pancreatitis

Common causes (~75%)
- Acute or chronic alcohol abuse
- Biliary tract diseases (gallstones)

Uncommon causes (~25%)
- Surgery to pancreas or nearby organs
- Pancreatic tumors – Pancreatic calcification or fibrosis
- Trauma to abdomen
- Blockage of pancreatic duct (post-endoscopic retrograde cholangiopancreatography)
- Duodenal obstruction – Intra-abdominal inflammation
- Metabolic disorders (hypertriglyceridemia)
- Drugs or idiosyncratic reaction
- Hepatitis of any cause
- Renal failure of all types

Table 4
Diagnostic accuracy of liver enzymes for biliary etiology of acute pancreatitis

	ALT	ALP	ALT + ALP
Cut-off	URL	URL	URL
Sensitivity	95.8%	87.5%	85.4%
Specificity	81.3%	81.3%	100.0%
Positive predictive value	93.9%	93.3%	100.0%
Negative predictive value	86.7%	68.4%	69.6%

ALT, alanine aminotransferase; ALP, alkaline phosphatase; URL, upper reference limit.

Pancreatic cancer

Pancreatic cancer has a mortality rate that exceeds 95% after 5 years. Although surgery to remove the tumor is effective in curing pancreatic cancer, it requires early diagnosis. Unfortunately, there are no readily available combinations of symptoms, signs, and laboratory tests to identify pancreatic cancer at an early stage. Current pre-operative diagnosis of pancreatic cancer mainly relies on contrast-enhanced CT.

The only tumor biomarker currently accepted for clinical use in pancreatic adenocarcinoma is CA 19-9. Table 5 summarizes recommendations for use. The utility of serum CA 19-9 in the diagnosis of pancreatic cancer has been extensively investigated in numerous studies. A systematic review with pooled data from 2283 patients (prevalence of malignancy, 41%) from 22 reports, performed in 2006, reported serum CA 19-9 had a median sensitivity of 79% (range 70-90%) and a specificity of 82% (range 68-91%) (18). However, for small tumors (<3 cm) the sensitivity of CA 19-9 decreases significantly (~55%). Other limitations of the diagnostic usefulness of CA 19-9 include its elevation in a variety of benign conditions often resulting in obstructive jaundice: up to 50% of hepatobiliary disorders, 75% of acute pancreatitis, and 20% of chronic

Table 5
Recommendations for use of CA 19-9 in pancreatic cancer (modified from ref. 17)

Clinical goal	Timing of sampling	Interpretative criteria
Differentiation of benign disease from pancreatic cancer	Not applicable	Not applicable
Staging – Prognosis	Preoperative	>95% of tumors that result in CA 19-9 >1000 kU/L had unresectable cancers
Monitoring therapy	After 2 months therapy	A >20% reduction of CA 19-9 baseline concentrations indicates a good response
Detecting tumor recurrence	Not defined	Reference change value (~45%)

pancreatitis can increase CA 19-9 values (19). Pretreatment serum CA 19-9 concentrations carry independent predictive value for the determination of resectability of pancreatic cancer and of overall patient survival (20). Substantial evidence is also available regarding the role of serial CA 19-9 measurements in the monitoring of palliative chemotherapy for pancreatic cancer. Finally, serial CA 19-9 monitoring is recommended in the follow-up of patients after potentially curative surgery, although the utility of detecting early rises in CA 19-9 and instituting therapy prior to other evidence of relapse has yet to be demonstrated.

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