CARBOXYLIC ESTER HYDROLASE IN ACUTE PANCREATITIS

A CLINICAL AND EXPERIMENTAL STUDY

P Jonas Blind
ABSTRACT

Diagnosis of acute pancreatitis (AP) is erroneous in up to one third of patients when based on clinical criteria and elevated serum amylase values. Furthermore, according to autopsy reports fatal pancreatitis remains clinically undiagnosed in 22 to 86 % of hospitalised patients. Consequently, search for better methods for the diagnosis of AP seems not only justified but urgent. The pancreas secretes a nonspecific lipase, the carboxylic ester hydrolase (CEH) with molecular properties different from other pancreatic secretory enzymes. These differences may imply that sites and rates of clearances from blood of pancreatic enzymes differ. Except for the pancreas this enzyme is secreted from the lactating mammary gland with milk.

A sensitive and reproducible sandwich-ELISA for quantitative determination of CEH was developed. When establishing referent values it was noted that in individuals aged 20 to 65 years serum concentrations of CEH did not depend on age, gender, the time of the day or duration from food intake to blood sampling, or use of nicotine. The mammary gland did not contribute significantly to basal serum levels of CEH; enzyme levels in lactating women or women with mammary tumours were identical to those of the reference population.

Seventy percent of patients with the diagnosis AP, based on elevated serum amylase levels and abdominal pain, had elevated CEH values. Among the patients with elevated amylase alone a probable cause of pancreatitis was lacking in the majority of patients. Contrastingly, a likely cause of AP could be identified in all patients presenting with abdominal pain and elevated CEH levels alone. These findings suggested that an elevated CEH level indicated AP more reliably than an elevated amylase level.

In patients with AP diagnosed by contrast enhanced computed tomography (CECT) alone, or combined with histopathological diagnosis, serum CEH levels were elevated on admission in all but one patient, and in all within the next 24 h. Furthermore, in patients with severe pancreatitis CEH levels remained at a raised level from the second to at least the 10th day following admission, whereas a significant decrease was noted in patients with mild pancreatitis. In contrast, serum amylase values were higher in patients with mild pancreatitis during the observation period than in those with severe pancreatitis. CEH levels were higher in patients with three or more Ranson signs than in those with less than three signs from the first day after admission. CEH levels were within referent range in 164 patients without known pancreatic disease admitted due to abdominal emergency conditions, or due to planned surgery for chronic extrapancreatic gastrointestinal diseases, and 16 patients having CECT without pathological findings in the pancreas. This suggests that AP can be excluded with very high degree of probability in presence of non-elevated CEH levels.

A sandwich ELISA for determination of Guinea pig CEH and a model for graded pancreatitis in the same species were developed. CEH levels showed proportional to severity of inflammation, thus confirming previous clinical observations. CEH levels in bile were proportional to inflammation, while it was absent in urine. Amylase levels in urine were identical regardless of severity of inflammation, but low in bile. These results suggested differences in sites and rates of clearance between the two enzymes.

Seemingly elevated CEH levels allowed identification of clinically significant pancreatitis following ERCP, which amylase levels did not.

The presented studies have shown that quantitative determination in serum of CEH by the described method is a more reliable test for the diagnosis of AP than determination of amylase activity. The differences between CEH and amylase are, at least partly, due to differences in molecular properties determining rates and routes of clearances of the two enzymes from serum.

Key words: Pancreatitis, carboxylic ester hydrolase, bile salt-stimulated lipase, lipase, amylase, endoscopic retrograde choledochopancreatography, Guinea pig
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P Jonas Blind
Either you create or you are reduced to a tool
P Jonas Blind, 1983
To Viktoria, Anna, Ina and Diana
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## ABBREVIATIONS

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AP</td>
<td>acute pancreatitis</td>
</tr>
<tr>
<td>AIP</td>
<td>acute interstitial pancreatitis</td>
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<tr>
<td>BSSL</td>
<td>bile salt-stimulated lipase</td>
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<tr>
<td>CEH</td>
<td>carboxylic ester hydrolase</td>
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<tr>
<td>CECT</td>
<td>contrast-enhanced computed tomography</td>
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<tr>
<td>CDL</td>
<td>colipase-dependent lipase</td>
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<tr>
<td>CRP</td>
<td>C - reactive protein</td>
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<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<tr>
<td>ERCP</td>
<td>endoscopic retrograde choledochopancreatography</td>
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<tr>
<td>GP</td>
<td>Guinea pig</td>
</tr>
<tr>
<td>NP</td>
<td>narcotising pancreatitis</td>
</tr>
<tr>
<td>Ranson signs</td>
<td>Ranson’s early objective prognostic signs for acute pancreatitis</td>
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<tr>
<td>WBC</td>
<td>white blood cell count</td>
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Abstract
Diagnosis of acute pancreatitis (AP) is erroneous in up to one third of patients when based on clinical criteria and elevated serum amylase values. Furthermore, according to autopsy reports fatal pancreatitis remains clinically undiagnosed in 22 to 86 % of hospitalised patients. Consequently, search for better methods for the diagnosis of AP seems not only justified but urgent. The pancreas secretes an non-specific lipase, the carboxylic ester hydrolase (CEH) with molecular properties different from other pancreatic secretory enzymes. These differences may imply that sites and rates of clearances from blood of pancreatic enzymes differ. Except for the pancreas this enzyme is secreted from the lactating mammary gland with milk.

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Papers
This thesis is based on the following papers which will be referred to by their Roman numerals.


V. Blind PJ, Bläckberg L, Wirell S, Dahlgren ST, Hernell O. Serum carboxylic ester hydrolase is a more reliable indicator of pancreatitis following endoscopic retrograde choledochopancreatography than is serum amylase. Manuscript
Introduction

According to Robson (Robson 1904) anecdotal cases of inflammatory changes in the pancreas have been described by Tulpius in 1672, Portal in 1804, Percival in 1818 and by Haller in 1859. Balser in 1882 first described acute pancreatitis with fat necrosis (Balser 1882). The same year Senn pointed out that suppurative lesions of the pancreas were of special interest to the surgeon (Senn 1882). Reginald Fitz of Boston characterised hemorrhagic, suppurative and gangrenous pancreatitis by systematically reviewing 53 cases of histopathologically confirmed pancreatic inflammation (Fitz 1889).

Acute pancreatitis (AP) remains a common and potentially lethal condition without pathognomonic symptoms or signs. In clinical practice the rule is that a patient with abdominal pain and an elevated serum amylase level is considered to suffer from AP, whereas the diagnosis is excluded in a patient with similar pain but non-elevated amylase (Levitt & Eckfeldt 1986). However, relying on presence of abdominal pain and elevated total amylase in serum leads to a falsely positive diagnosis in up to one third of the patients as judged from isoamylase determinations (Weaver et al 1982, Koehler et al 1982). On the other hand, the severity of the disease is often underestimated as only one out of three patients suffering from severe AP is diagnosed correctly at an early stage of the disease (McMahon et al 1980).

Moreover, a significant rate of falsely negative diagnoses is likely because, according to autopsy reports, fatal pancreatitis remains clinically undiagnosed in 22 to 86% of hospitalised patients (McWhorter 1932, Corfield et al 1985, Choi et al 1987, Wilson & Imrie 1988).

Indicators in serum of AP

Amylase - 'the gold standard'

Diagnosis of AP by aid of biochemical tests is founded on the discovery by Payen and Persoz of a starch splitting substance from malt given the name diastase (Payen & Persoz 1803). Diastatic activity of the blood was first observed by Magendie (Magendie 1846). An assay admitting quantification of diastatic activity in animal tissues was designed by Foster (Foster 1867). Wohlgemuth and later Stocks suggested that determination of amylase activity in blood (Wohlgemuth 1908) and urine (Wohlgemuth 1909, Stocks 1916) is useful for diagnosis of various pancreatic disorders. Since a report in 1929 by Elman et al on hyperamylasemia in patients with AP (Elman et al 1929), elevated serum amylase has been regarded the cornerstone, or "gold standard", for the diagnosis of this disease.

Other proteins secreted from the pancreas

Measurements of other serum proteins, more or less specific for the pancreas, have been evaluated as indicators for the diagnosis of AP, e.g. elastase 1, immunoreactive trypsin (IRT) and phospholipase A2. Serum elastase 1 and IRT do not correlate to severity of AP (Clavien et al 1989, Büchler et al 1989). Furthermore, elevation in serum of elastase 1 or phospholipase A2 is not specific for AP (Büchler et al 1989). Hence, none of these proteins have proven superior to amylase for the diagnosis of AP. Consequently, search for better methods for the diagnosis of AP seems not only justified but urgent.

Besides amylase serum lipase activity is probably the most frequently used enzymatic parameter in the diagnosis of AP (Loevenhart 1907, Myrick 1976, Leybold & Junge 1986). Cumbersome assay techniques, and highly inconsistent results have however, impeded widespread use of lipase determination for this purpose. At time of initiation of the present study most lipase assays were based on enzymatic techniques. Such methods suffer from three main drawbacks. Firstly, the presence of, and hence interference by, lipases of extrapancreatic
origin, e.g. lipoprotein lipase from various tissues and hepatic lipase, cannot be ruled out. Secondly, the exocrine pancreas secretes more than one lipolytic enzyme (se below). It is thus difficult to estimate the relative contribution of each of them in different assays. Thirdly, serum may contain factors which inhibit or stimulate the various lipases to different degrees (Bläckberg et al 1985).

Pancreatic lipolytic enzymes
To date at least three different lipolytic enzymes have been characterised in human exocrine pancreatic tissue and/or pancreatic juice. One of them, phospholipase A$_2$, does not hydrolyse the typical long-chain triglyceride substrates of lipase assays. The remaining two are both triglyceride hydrolysing enzymes. The well characterised “classical” pancreatic lipase, which depends on a small protein cofactor named colipase, for its optimal activity will be referred to as colipase dependent lipase (CDL) (Verger 1984). In addition the pancreas secretes a much less specific lipase which has been given many different names, e.g. carboxylyl ester lipase, cholesterol esterase, sterol ester hydrolyase, monoglyceride lipase, nonspecific lipase, bile salt activated lipase, bile salt-stimulated lipase (BSSL), carboxylyc ester hydrolyase (CEH), micelle lipase, secondary ester hydrolyse and lipase A (Bläckberg 1981, Borgström 1991) reflecting its non-specific properties and the fact that different substrates have been used in different laboratories. There is now, however, consensus that all these different activities can be attributed to one and the same enzyme. In this thesis the name carboxylic ester hydrolyse (CEH) will be used to denote this enzyme (Lombardo 1978). CEH and CDL differ greatly with respect to functional properties and molecular structure (Verger 1984, Rudd & Brockman 1984). Whereas the main function of CDL is to digest dietary tri- and diglycerides, CEH plays important roles also for the use of other dietary lipid components, e.g. cholesteryl - and fat soluble vitamin esters. Regarding structure it suffices to say that CEH is about twice the molecular size of CDL, and that only minor amino acid homology can be found between the two enzymes (Winkler 1990, Reue et al 1991). Based on immunochemical and functional studies it was first suggested that CEH is very similar, if not identical, to the bile salt stimulated lipase (BSSL) present in human milk, (Bläckberg et al 1981) and milk from a limited number of other species (Hernell et al 1989). Later, studies elucidating the primary structure of the enzyme from both sources (Nilsson et al 1991, Reue et al 1991) as well as the characterisation of the gene (Lidberg et al 1992) have shown that their amino acid sequence indeed is identical, and thus that they are coded for by the same gene. Apart from the lactating mammary gland, there is today no evidence that CEH/BSSL is synthesised in extrapancreatic tissues. In the pancreas of Leopard shark, Triacus semifasciata (Patton et al 1977), and the cod (Gadus morhua) (Gjellesvik et al 1992) only one lipolytic enzyme has been found. This enzyme requires bile salts for activity. Therefore, phylogenetically, CEH has evolved earlier than the CDL. For the sake of simplicity, disregarding the source of the enzyme, below the name CEH will be used to denote both the pancreatic (CEH) and milk (BSSL) enzyme.

For reasons of specificity, use of immunochemical methods allowing quantitative determination of pancreatic secretory proteins for the diagnosis of AP has been advocated (Dati & Grenner 1984). At the start of the present study such method had been described for determination of CDL but not yet been evaluated with respect to diagnosis of AP (Dati & Grenner 1982).

A prerequisite for diagnosis of AP by determination in serum of a substance synthesised in the pancreas is its release from the inflamed acinar cell and subsequent uptake into blood at a rate exceeding the corresponding phenomenon at basal conditions, and a clearance rate from the
blood that admits its detection. As both conditions are met for other pancreatic enzymes, one could assume that the same is true for CEH. Decreased and evenly dispersed immunostaining of CEH has been documented in necrotic acinar cells and interstitial tissue around necrotic pancreatic lobules in areas of fat necrosis in human AP (Aho et al 1989). Finally, staining pattern with antiserum against pancreatic secretory trypsin inhibitor differed distinctly from that with antiserum against CEH in pancreatitis specimens, although the staining pattern in normal pancreas was identical to that of CEH (Aho et al 1989). Evaluation of CEH in serum determined by an immunochemical technique, as indicator of AP seemed to be of decisive interest.
Aims of the study
The principal aim of this study was to evaluate if CEH, as determined quantitatively, could be used as a reliable indicator of AP.

The steps taken to achieve this goal were:

- to develop an assay based on an immunochemical method for quantitative determination of CEH
- to determine whether the milk enzyme (BSSL) is synthesised within the mammary gland or transported to this gland from the pancreas
- to establish referent values for CEH in serum
- to determine the tissue origin of CEH in serum
- to compare sensitivity and specificity of serum CEH and amylase as markers of AP
- to develop an assay allowing quantification of CEH in the Guinea pig (GP)
- to develop an experimental model allowing grading of pancreatitis in the GP
- to investigate any relationship between severity of AP and serum levels of CEH in this model
- to find an explanatory model for observations on CEH in clinical studies
- to study mild AP in a semiexperimental human model (ERCP, endoscopic retrograde cholangiopancreatography)

1 The term referent value is used rather than the term reference value, because it emphasizes the process of refering to a bench mark rather than placing emphasis on the benchmark itself (Galen & Gambino 1975). The concept normal value was not used because it defies definition and has many meanings in common use in science and colloquial language (Holland & Whitehead 1974).
Establishment of basal serum CEH levels

Sandwich-ELISA for determination of serum CEH

As a first step it was essential to determine basal levels and origin of CEH in serum. Except for the pancreas, a possible source of CEH in serum is the lactating mammary gland. For the determination of BSSL in serum an assay based on enzyme concentration, rather than on enzyme activity had to be designed; the reason being that normal serum inhibits the enzyme activity in the routine assay using triglyceride as substrate (I). The chosen method was based on the sandwich-ELISA principle (Engvall 1980). This principle admits detection of an enzyme as long its immunochemical properties are retained. Hence, inactive forms, or enzyme in absence of its coenzyme, is determined equally well as the native enzyme. Antiserum to purified BSSL was raised in rabbits (I). The assay proved sensitive (detection limit = 0.5 μg/L serum) and reproducible (intra- and inter-assay variations were less than 6 % and 17 %, respectively). Thus, the precision of the assay was in parity with others based on an immunochemical techniques (Engwall 1980, Dati et al 1984, Rizotti et al 1985, Uhl et al 1992). The applicability of the BSSL-ELISA for determination of the pancreatic counterpart CEH was proven by the fact that expected results were obtained when analysing samples with known concentrations of purified CEH. Consequently, the ELISA measures the sum of BSSL and CEH (I). Hence, an assay allowing quantitative determination of a pancreatic enzyme not formerly evaluated as means of diagnosis of AP was available. Adherence to the principle of quantitative determination of pancreatic enzymes for the diagnosis of AP, as postulated already in 1934, was possible (McCaughan 1934).

Referent values

As basis for interpretation of further studies baseline data concerning CEH had to be established. The finding of significant circadian changes in acinar cell volume, volumes and surface areas of secretory granules and several organelles involved in enzyme secretion in the rat (Müller et al 1985) urged for establishment of diurnal profile of CEH which was done in ostensibly healthy medical students (II). Our finding of median CEH levels being independent of time of the day and duration of fasting prior to blood sampling is in concordance with the finding of only minor diurnal variations of both salivary type and pancreatic type of isoamylase, none of which correlated to food intake (Skude 1975). Consequently, the time of day and duration from food intake to blood sampling could be disregarded in the further evaluation of CEH and amylase levels.

Furthermore, referent values for CEH had to be established. No strict rules for choosing reference population can be laid down, but the population and the way in which it is chosen must be clearly described (Holland & Whitehead 1974). Blood donors seemed suitable as referent material. An advantage is that they are uniformly managed in connection with collection of blood, which may be of importance for the outcome of subsequent analyses (Tryding 1979). The percentile method for determining referent values was used because it requires no assumption about distribution shape of the population from which it is drawn (Herrera 1958) (II and V). We adhered to the conventions using the 99:th (II) or 97.5:th (V) percentiles to determine cut-off levels.

The differences in recorded CEH concentrations between different sets of referent values could, at least partly, be explained by use of different polyclonal antibodies (I versus II, III and V). The CEH concentration corresponding to 97.5 percentile according to II is well within the 90 % confidence interval for the same percentile in III. The slight variation of the outcome of the assay was overcome by establishment of separate sets of referent values for
each study. It is likely that part of the variation will be controlled by use of an assay based on use of monoclonal antibodies. Development of such an assay is in progress. Unexpectedly, high concentrations of CEH were found in about 1% of blood donors; these extreme values fulfilled conventional criteria for outliers (Herrera 1958) in relation to the frequency distribution of CEH of the remaining individuals. The used protocol impeded follow up of these individuals. Hence, exclusion of presence of pancreatic pathology in them was impossible.

Higher basal levels of CEH than recorded in the present studies have been reported (Aho et al 1989). This may be explained by the fact that different antisera were used, but not by the fact that our ELISA was based on antibodies raised against BSSL (the milk enzyme), rather than CEH (the pancreatic enzyme), since purified proteins from both tissues were assayed with identical precision (see above).

The age span of blood donors is no different from that of the majority of patients undergoing investigative procedures due to suspicion of pancreatic disease. No differences in CEH concentrations between males and females were observed when stratified according to gender (II). This is in concordance with CDL as determined immunochemically (Dati & Grenner 1984), but contrasting with the finding of significantly higher serum lipolytic activity in males than females (Goldstein et al 1948), or significantly lower serum elastase levels in females than males (Hall 1966). Contrary to the finding of age related increase of some pancreatic secretory proteins in serum, e.g. pancreatic polypeptide (Floyd et al 1977), trypsin (Koehn 1981, Ammann 1982), immunoreactive trypsin and CDL (Mohiuddin 1984, Floyd et al 1977, Dati & Grenner 1984), CEH levels remained constant when stratifying individuals in decades (third to seventh). The reason for the observed discrepancy is unknown but might be due to a considerable proportion of individuals older than 70 years in the referred studies. Finally, CEH concentrations in blood donors were also compared after stratification in users and non-users of nicotine, as use of nicotine is debated as one etiologic factor of pancreatic disease (Andrén-Sandberg 1983, Cavallini et al 1994). Use of nicotine did not influence CEH levels (II)

Thus, within the range of 20 to 65 years serum concentrations of CEH did not depend on age, gender or use of nicotine habits. Also these parameters could therefore be disconsidered in the further evaluation of CEH levels.

**Tissue origine of the enzyme in serum**

Pancreatic origin of CEH is conceivable, as in basal conditions, leakage into blood of enzymes secreted in the pancreas occurs at a ratio of about 1 to 1000 of that secreted into pancreatic juice (Rohr 1986). Preliminary studies were carried out revealing that CEH indeed could be detected in serum and that elevated levels were found in patients presenting as abdominal emergency cases clinically diagnosed as suffering from AP (Blind et al 1983), and in patients with presumed AP after endoscopic retrograde choledochopancreatography (ERCP) (Blind et al 1984). Furthermore, postoperative raise of serum CEH was indeed related to surgical handling of the pancreas as patients having pancreatic surgical procedures showed elevated levels contrasting to non-elevated levels in patients undergoing at most pancreatic palpation via an opened omental bursa (II).

A second possible source of the enzyme in serum is the mammary gland, provided that the enzyme in milk is synthesised within the gland. Another possibility was that the enzyme is synthetised in the pancreas, transported to the breast before secretion with milk. One hypothetical route would be uptake of the enzyme from the gut to blood and subsequently
secretion of the intact enzyme with milk, in some analogy to the notion of entero-mammary
circulation of IgE (Hanson et al 1980). If so, then one would expect high enzyme levels in
breast-fed newborn infants as macromolecular absorption is far more pronounced in the
neonatal period than later in life (Udall & Walker 1982, Axelsson et al 1989). This hypothesis
was strongly contradicted by the fact that CEH levels were below detection limit in all
investigated breastfed infants (I). Another possible route would be uptake into blood of the
enzyme from the pancreas before secretion into the duodenum. This would imply higher
serum CEH levels in lactating than in non-lactating women. Such explanation is contradicted
by the finding of equal levels of serum CEH in lactating women and non-lactating women.
Considering the recorded low concentrations in serum of CEH in lactating women relative to
that found in milk, the prerequisite for BSSL being of pancreatic origin would be passage of
the entire serum volume through the mammary gland at a rate exceeding renal blood flow 10 -
20 times (Guyton 71), and a complete clearance of the enzyme during first passage. Such span
of change from basal to lactating state should be beyond adaptive possibility of human
physiology. Thus, data at hand unambiguously suggested that the enzyme secreted with milk
is in fact synthesised within the mammary gland. Later Bläckberg et al have given direct
evidence for this (Bläckberg et al 1987). As pathological conditions in an exocrine gland may
be associated with elevated serum levels of its secretory proteins, e.g. amylase in pancreatic
cancer (Nelson et al 1963, Gambill 1971, Allan et al 1973) and α-lactalbumin in mammary
tumours (Schultz & Ebner 1977) the possibility that pathological conditions in the mammary
gland would also be associated with elevated BSSL levels in serum had to be considered. We
therefore assayed CEH in patients with malignant or benign mammary tumours (I). Presence
of a mammary tumour is an unlikely cause of elevated CEH as all patients had
nonpathological levels.

A view that most, if not all, of recorded basal serum level originates in the pancreas was
supported by nondetectable levels in of two pancreatectomized patients and one with cystic
fibrosis (I). Furthermore, the observation that lactating women had no higher levels of serum
CEH than non-lactating women supports the view that the mammary gland does not
contribute substantially to the level recorded in serum.

Clinical studies

Comparison of sensitivity and specificity of serum CEH and amylase for AP.
Because preliminary observations had revealed that pathology of or surgery on the pancreas
results in raised serum CEH levels the logical step was to investigate the consequence of using
CEH rather than amylase to corroborate the diagnosis of AP in a clinical context. For this
purpose blood samples were collected from patients admitted due to acute abdominal
conditions (II). The outcome of the CEH assay was related to diagnosis as stated in the
patients’ official records at discharge from the hospital and to serum amylase.

The advantage of this approach was that the contribution of CEH determinations for the
diagnosis of AP could be evaluated in patients with possibly false positive and false negative
diagnosis of AP. In the 27 patients given the diagnosis AP both enzymes were elevated in 20,
and amylase alone in seven patients (25 %). The latter figure corresponds surprisingly well to
the proportion of patients erroneously given the diagnosis of AP on basis of presence of
abdominal pain and elevated total amylase, as judged by isoamylase determinations (Koehler
et al 1982). Interestingly, five of these seven patients lacked an identifiable cause of AP.
Conversely, 11 patients with elevation of CEH only had clinical findings compatible with AP
and presence of a generally accepted etiologic factor for AP. One could speculate that
transient amylase elevations, having returned to non-pathological levels by the time of hospitalisation (Spechler & Schimmel 1985), explains this observation. A better compliance of CEH than of amylase to course of disease was suggested by persistence of elevated CEH levels in a patient with necrotizing pancreatitis (NP) at time of surgical intervention 12 days after admission, by which time amylase had returned to a non-pathological level (II).

It may also be speculated that elevation of CEH could have been an unspecific reaction to various diseases. Such assumption was strongly contradicted by finding of non-pathological levels of CEH in patients, without ostensible pancreatic disease, admitted due to acute abdominal conditions (n = 91), of which 27 had emergency surgery, or to elective surgery for chronic abdominal disease (n = 73) (II). However, laparotomy for extrapancreatic disease does not unambiguously rule out the possibility of AP, although it is unlikely that severe pancreatitis would have escaped detection. We therefore considered diagnostic means allowing exclusion of pathological changes in the pancreas at a higher level of probability than laparotomy indicated by non-pancreatic disease. CECT (contrast-enhanced computed tomography) probably is the non-invasive investigation technique with the highest specificity for pathological alterations in the pancreas presently available. We therefore determined CEH in 10 females and 6 male patients (median age 59 years, ranging from 36 to 84 years) undergoing investigation of the abdomen by CECT (5 mm slices). None of them had detectable pathological changes in the pancreas. Their median CEH was below detection limit of the assay; the highest value was 2.3 μg/L serum (Blind unpublished). Taken together, these observations suggested that serum CEH had both higher sensitivity and specificity for AP than amylase.

To reach definite conclusions on the relationship between serum level of any pancreatic secretory protein and the inflammatory state of the pancreas, objective proof of the severity and extent of inflammation is imperative. This principle was pointed out by Elman et al. already in 1929 (Elman et al. 1929), brought up again by Thistlethwaite & Hill in 1952 (Thistlethwaite & Hill 1952) and strictly adhered to by some more recent authors (Büchler et al. 1987), but often neglected. CECT and histological examination of pancreatic specimens obtained during laparotomy are presently regarded the most objective means for verification of AP. We therefore investigated daily CEH levels in patients with diagnosis based on CECT, alone or combined with histopathological confirmation of the diagnosis (III). All but one had elevated CEH on admission, and all within the next 24 h. Amylase was elevated in all patients on admission. It is arguable that the high sensitivity of CEH for pancreatitis only applies to patients with severe inflammation because all underwent CECT, which is an investigation usually only carried out on patients with suspicion of severe disease. Such bias was avoided by study design; all consecutive patients with suspicion of AP underwent CECT. According to the adopted criteria two thirds of the patients had acute interstitial pancreatitis (AIP) and the remaining ones necrotizing pancreatitis (NP).

Time course of serum CEH and severity of AP

A crucial criterium of any pancreatic enzyme suitable for the diagnosis and monitoring of patients with AP is that enzyme levels should be indicative not only of presence of, but also severity of inflammation. If CEH fulfils this criterium was investigated in the same patients with diagnosis of AP verified by CECT and histopathology (III). We recorded significantly higher daily CEH values from the second day after admission in patients with NP than in those with AIP. Therefore, persisting high CEH levels seem to indicate presence of NP, whereas diminishing levels suggest presence of AIP. This was not due to surgical intervention
in patients with NP as the difference remained after exclusion of all postoperative recordings of CEH. Missclassification of patients was possible by CECT as relatively good perfusion does not rule out pancreatic abscess formation (Balthazar 1989, Bradley et al 1989, Steinberg 1990), i.e. some patients with NP may have been referred to the AIP group. If so, that should, however, have lessened the difference in daily CEH values between the two groups.

**Time course of serum CEH and Ranson’s early objective prognostic signs for AP**

Compound scoring systems for prediction of the course of AP have been developed. Their reliability may be questioned as they are partly based on data from patients without objectively verified diagnosis. Indeed, most scoring systems were developed before the era of CECT. Nevertheless, patients referred to above were stratified according to one such system (III), the Ranson’s early objective prognostic signs for acute pancreatitis (Ranson signs), into two groups; i.e. one with three signs or more and another with less than three signs. According to Ranson such stratification predicts a mortality of 62% and 3%, respectively (Ranson et al 1974). CEH levels seemed to reflect the general condition of the patient as average daily CEH concentrations were significantly higher in patients with three or more Ranson signs than in patients with less than three signs from one day after admission. These findings suggest that determination of CEH, i.e. a single laboratory test, would allow stratification of patients into a group running a high risk of septic complications and ultimately mortality, and another one implying low risk. Assessment of the prognostic value of a compound scoring system based on data from patients with objective confirmation of AP remains to be done. Such system should include assays admitting quantitative determination of pancreatic secretory enzymes, e.g. CEH, and laboratory parameters quantifying inflammatory reaction, e.g. serum CRP and white blood cell concentrations.

Our finding that amylase levels were inversely related to severity of disease is in agreement with previous findings (Adams et al 1968, Satiani & Stone 1979, Clavien et al 1989). The view that neither the maximal value nor the variability of serum amylase during hospitalisation have any prognostic value regarding the course of acute pancreatitis (Moosa 1984, Brooks 1972, Clavien et al 1989, Jacobs et al 1977) was confirmed in our study.

**Time course of serum CEH and etiology of AP**

A biochemical test admitting differentiation between pancreatitis of various etiology could guide patient treatment (Mimoz et al 1993). For example, patients with AP related to biliary lithiasis could benefit from intervention by endoscopic sphincterotomy and stone extraction. Some authors have reported that the relative rise of serum amylase and lipase depends on etiology of AP (Croton et al 1981, Ventrucci et al 1989, Gumaste et al 1991). We therefore related CEH concentrations to etiology in the patients having diagnosis confirmed by CECT and histopathology. CEH values did not discriminate between patients with pancreatitis of alcoholic, biliary or unknown etiologies which is in concordance with results from estimations of biochemical parameters in plasma and peritoneal fluid in patients with pancreatitis (Dubick et al 1987). Thus, possible differences in the pathophysiological mechanisms associated with respective etiology are not manifested as differences in serum levels of CEH.

**Experimental study in the Guinea pig**

In man the exact relationship between histopathological course of AP and time course of biochemical markers of the disease is difficult to assess, the reason being that the inflammation in the majority of patients subsides on non-operative treatment precluding access to tissue for histopathological examination at time of blood sampling. Furthermore,
exact duration of disease prior to hospitalisation is unknown. Therefore, further study on the relationship between severity of pancreatitis and serum levels of CEH obligated an experimental study.

**GP pancreatitis model**
Graded inflammation was achieved by varying duration of pancreatic ischemia followed by reperfusion. The body and tail (∼70% of pancreas) were made ischemic by appropriate placement of an arterial vascular clamp and ligation of collateral blood vessels. Choice of model was ruled by previous observations pertaining to the pathophysiology of AP. It is, for example, generally recognised that the pancreas is susceptible to ischemic injury (Panum 1862, Popper 1948, Rattner *et al* 1989), and in man development of pancreatitis due to interrupted blood supply to the pancreas is well documented (Feiner 1976). Distortion of the capillary circulation and thereby impaired pancreatic blood supply is considered triggering or an early event in the development of AP (McEntee *et al* 1989, Klar *et al* 1990). Experimentally induced pancreatic ischemia followed by reperfusion causes pancreatitis in the rat, with severity of inflammation being proportional to duration of ischemia (Slater *et al* 1975). The choice of model was further encouraged by observation of very high serum CEH concentrations in a patient with gangrene of the entire small bowel and the ascending colon due to acute superior mesenteric artery (SMA) embolism (Blind unpublished). It is most likely that the blood supply of the pancreatic head in this patient was, at least partly, impeded because its arterial supply is in part derived from the SMA (Falconer & Griffiths 1950). The present model was also attractive because of simplicity.

Duration of pancreatic ischemia and postischemic observation time was a compromise between time needed for development of unequivocal inflammatory changes *versus* survival (Slater *et al* 1975, Letko *et al* 1984, Brackett *et al* 1983). Absence of total necrosis distal to clamping site indicated that reperfusion was successful.

Enzyme levels were then related to extent of pancreatic inflammatory changes as determined histopathologically. There was a highly positive correlation between the extent of morphological changes and duration of pancreatic ischemia was ($r = 0.7, p < 10^{-6}$). Exudation of fluid containing pancreatic secretory proteins into the peritoneal cavity is a significant characteristic of AP (Geokas *et al* 1978), and the fact that the number of animals with inflammatory exudate in the peritoneal cavity was proportional to duration of ischemia further indicated that the model allowed graded inflammation. Indeed, a form showing mainly interstitial inflammation in animals exposed to ischemia for 20 and 30 min, and another showing acinar cell necrosis to various extent in those exposed to ischemia for 60 min could be discerned. Animals having no ischemia and those exposed to ischemia for 10 min had practically no inflammatory changes (referred to as controls).

**Sandwich-ELISA for GP CEH.**
Previous purification and characterisation of the GP pancreatic counterpart of human CEH (Svensson & Bläckberg 1994) constituted the basis for the first study of the enzyme in an experimental model (IV). A sandwich-ELISA designed essentially as described for the human counterpart (I) using rabbit polyclonal antibodies to purified GP CEH. The sensitivity (detection limit = 0.5 μg/L) and precision of the assay proved satisfactory in ranges relevant for the study.

**CEH levels and severity of inflammation**
In contrast to serum amylase, CEH levels were elevated already in animals with mainly
interstitial inflammation as compared to controls. Significant elevation of serum amylase levels, as compared to all other groups, were observed only in animals showing acinar cells necroses. Thus elevation of serum CEH concentration in the GP occurred at an earlier disease stage than did elevation of amylase activity. This finding supports our previous results suggesting that patients with elevated serum CEH, in presence of etiologic factors associated with AP and clinical findings compatible with mild inflammation, could in fact have suffered from pancreatitis, albeit their amylase levels were normal (II).

Furthermore, elevation of CEH was proportional to severity of inflammation as animals with interstitial pancreatitis and those with presence of necroses had approximately ten- and 60-fold increase of serum CEH levels as compared to controls, respectively. This confirms our observation of higher CEH levels in patients with NP than those with AIP (III).

Interestingly, the level of amylase in animals with mainly acinar cell necroses was similar (1.5 times that of controls) to that recorded when AP of identical severity was induced by injection of sodium taurocholate and cephalothin into the pancreatic parenchyma in the same species (Orda 1976). This supports the view that CEH data obtained were not restricted to type of pancreatitis model but rather to severity of inflammation.

**Explanatory model for clinical observations**

When designing the experimental model we considered the possibility to explain previous clinical observations on CEH in pancreatitis (II and III). About 97% of radiolabeled GP pancreatic enzymes injected intravenously into non-pancreaticic rats were taken up by various tissues (Rohr & Scheele 1983). Both time-courses of uptake, and disappearance of the enzymes varied between tissues. The kidney and liver were documented as sites of clearance of intact pancreatic enzymes at a proportion of 1 - 2% and 0.3 - 0.5%, respectively (Rohr & Scheele 1983). For this reason, presence of CEH and amylase in urine and bile was investigated.

A CEH level in urine exceeding detection level of the assay was noted in only a single animal. In contrast, significantly higher level of urinary amylase as compared to controls was recorded in all groups of animals exposed to ischemia. No differences of amylase levels between ischemic groups were observed. The difference in serum levels between the two enzymes might be explained by the fact that the molecular weight of GP CEH (80 kD) (Svensson & Bläckberg 1994) exceeds the threshold size for permeability of the normal glomerular membrane (65 kD) (Stroeber & Waldman, 1974), whereas the size of amylase (55 kD) does not. It is however noteworthy that urine amylase levels were raised already when only interstitial inflammatory changes were present. Hence, elevated urinary amylase seems to be a more sensitive indicator of mild pancreatitis than elevated serum amylase. Absence of CEH in urine in all but one animal raises the interesting hypothesis whether occurrence of the enzyme in urine would be suggestive of pancreatitis associated with increased vascular and glomerular membrane permeability, *i.e.* an indicator of a potential extrapancreatic complication during the course of AP.

The median biliary CEH level was significantly higher in animals with acinar cell necroses than in those with mainly interstitial inflammation and controls, whereas the median amylase level was not. As differences in transport rate from blood to bile in the rat of intravenously injected GP pancreatic proteins have been reported (Rohr & Scheele 1983), the differences in hepatic clearances of the two enzymes could offer a further explanation of the dissimilarity in serum levels. The fact that CEH levels in bile were proportional to CEH levels in serum, in contrast to uniformly low levels of amylase in all animals, is compatible with this explanation.
The observation of contrastingly low levels of amylase activity in bile is in accord with previously suggested low leakage rate of amylase from the hepatic blood circulation to bile (Donaldson et al 1979).

**ERCP as model for mild AP in man**

Studies in the GP model showed that serum levels of CEH were proportional to severity of inflammation. This was in agreement with observations in previous clinical studies.

In spite of that, a relationship between mild pancreatitis and serum levels of CEH in man needed further elaboration. Therefore, a human model for mild AP was necessary. AP may be induced in a number of animal species by injection of harmful substances into the pancreatic duct (Bilchik et al 1990). Severity of the inflammatory reaction is dependent on type of substance, concentration and volume (Tulassay Z 1989, Bilchik et al 1990). A corresponding set up in man is endoscopic retrograde choledochopancreatography (ERCP). Indeed, incidence of AP after ERCP varies with type and volume of contrast medium used (Tulassy et al 1981, Sherman & Lehman 1991). The fact that the incidence of AP after ERCP requiring operative treatment is less than 1 % (Sherman & Lehman 1991) implies that the inflammation, if any, is mild in the majority of patients. Therefore, patients undergoing ERCP was assumed to provide a model for the studies of mild AP in man (V). On the other hand, figures on incidence of AP subsiding without operative treatment vary greatly (0 - 59 %) reflecting inconsistencies with respect to diagnostic criteria (Sherman & Lehman 1991, Sternlieb et al 1992). The latter figure appears unreasonably high considering the uneventful clinical course in most patients. Obviously, a method indicating presence and severity of AP following ERCP is required.

Relying on presence of abdominal pain and elevation of amylase, the conventional criteria, or abdominal pain and elevated CEH would have implied an incidence of AP within 2 days after ERCP of 21 % and 3 % of the patients, respectively (V). It emerged that adherence to such conventional protocol precluded determination of the value of either enzyme for the diagnosis, the reason being that evidence, independent of enzyme levels, for the presence of an inflammatory reaction was missing. However, the difference in figures was a challenging observation, and prompted to the use of more objective criteria to assess presence of an inflammatory reaction following ERCP. Determination of WBC and serum CRP concentrations are conventionally used to confirm whether an inflammatory reaction present.

In 9 out of 41 consecutive patients presence of inflammatory reaction within two days after ERCP was confirmed by elevations of both WBC (Stanten & Frey 1990) and CRP (Viedma et al 1994) concentrations. Exclusion of cholangitis contributing to the inflammatory reaction was not possible; indeed three patients had elevated serum alanin - , aspartataminotransferases and total bilirubin levels. Elevations of WBC and CRP concentrations were modest, and the clinical course was indicative of mild inflammation in all except one patient who required prolonged hospitalisation. The proportion of patients with elevated CEH was significantly higher in the group of patients with presence of inflammatory reaction (5/9) than in those without (2/32). Daily amylase levels did not allow discrimination between the two groups. The proportion of patients with elevated amylase was significantly higher in the patients with presence of inflammatory reaction than in those without only on the first day after ERCP. Persistent elevation of CEH was seen only in the patient requiring prolonged hospitalisation. Four patients in the group with presence of inflammatory reaction had mild abdominal pain at clinical examination; and indeed all four had elevation of CEH.
Consequently, relying mainly on total serum amylase activity for the diagnosis of AP after ERCP would have implied considerable overdiagnosis as judged by elevated serum concentrations of WBC and CRP, and presence of abdominal pain at clinical examination, i.e. of clinically significant disease. Relying mainly on serum CEH concentrations would have diagnosed all patients with clinically significant disease and only slight overdiagnosis.

A partial explanation for the great difference in proportions of patients with elevated CEH and amylase could have been elevation of salivary isoamylase only, as rise of this isoenzyme is responsible to elevated total amylase in 7 to 10 % of patients undergoing ERCP (Skude et al 1976, Pelot et al 1978, Pelletier et al 1987). This was contradicted by the fact that none of the patients with elevated WBC and CRP had elevation of salivary amylase alone. Consequently, a genuine difference between serum levels of CEH and pancreatic isoamylase was present in our patients undergoing ERCP.
General conclusions

Acute pancreatitis (AP) remains a common and potentially lethal condition without pathognomonic symptoms or signs. Both falsely positive and falsely negative diagnosis is common. Consequently, search for better methods for the diagnosis seems not only justified but urgent. CEH, an nonspecific lipolytic enzyme is secreted from the human pancreas and the lactating mammary gland. A sandwich-ELISA admitting quantitative determination of CEH was designed. The pancreas was confirmed being responsible for recorded basal levels of CEH in ostensible healthy reference individuals. The mammary gland did not contribute significantly to serum levels of the enzyme. Clinical studies proved CEH to have high sensitivity and specificity for AP. The daily CEH levels were proportional to severity of inflammation when classified according to findings by CECT alone or combined with histopathological diagnosis. CEH was also proportional to severity of AP as stratified into a group predicting high risk of morbidity and mortality, and another predicting a low risk, according to Ranson signs.

An animal model for studies on AP was developed. Results in the clinical studies were confirmed experimentally. Moreover, an explanation model for observed differences between serum CEH and amylase levels was obtained, implying different routes and rates of clearances of the two enzymes. The model allowed grading of severity of AP.

Finally ERCP was used for the study of mild AP in man. Again CEH proved superior to amylase in identifying patients with clinically significant disease.

A significant proportion of patients admitted due abdominal emergency conditions suffer from AP. Means for immediate and correct identification of these patients would guide decisions on treatment and further investigations, i.e. optimise use of hospital resources. Determination of serum CEH seems to be an excellent tool for this purpose. This may be shown by the case of ERCP.

Half of patients undergoing ERCP have elevated amylase levels for at least three days, whereas at most 5% have elevated CEH levels persisting equally long. Confirming diagnosis of AP by elevated amylase levels would imply prolonged hospitalisation for about 150 days of 100 patients undergoing ERCP per year; the corresponding figure for elevated CEH would be 15 days. As our study has shown that elevated CEH concentrations are present predominantly in patients with clinically significant AP, then relying on determination of CEH to diagnose AP would imply a saving of about 135 days of hospitalisation in these patients. Assuming a cost of 3 000 SEK per day of hospitalisation this would imply reduction of expenses of about 400 000 SEK per year for hospitalisation only.

Future studies should include establishment of (i) time course of serum CEH in relationship to severity of disease in experimental studies, (ii) the relative contribution by CEH in thoracic duct lymph to serum levels of the enzyme, (iii) whether appearance of CEH in urine would be indicative renal complications, (iv) the relative contribution of CEH levels, as determined by multivariate analysis, in a scoring system for prediction of course AP, (v) whether CEH levels are increased in pancreatic cancer, (vi) whether CEH levels are decreased in chronic pancreatitis (vii) significance of CEH levels for the diagnosis of AP in patients undergoing surgery implying use of extracorporeal circulation.
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