Jouko Laurila

SURGICALLY TREATED ACUTE ACALCULOUS CHOLECYSTITIS IN CRITICALLY ILL PATIENTS
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Abstract
Acute acalculous cholecystitis (AAC) is an insidious and increasingly recognized complication of critical illness, whose pathogenesis is poorly understood and clinical picture obscure. Diagnosis is difficult and there is no consensus on treatment.

The medical records of all ICU patients who had undergone open cholecystectomy due to AAC during the years 2000–2001 and 2003–2004 were examined for clinical and organ failure data. The indication for open cholecystectomy was a suspicion of AAC based on clinical signs and symptoms of sepsis or deteriorating multiple organ dysfunction without other obvious foci and/or radiological (computed tomography or ultrasound) findings indicative of cholecystitis.

A total of 73 patients had operatively treated AAC during the study periods, giving an incidence of 0.9% of all admissions (73/8184) and an incidence of 6.7% among the long-stayers (ICU stay >5 days). The hospital mortality of these patients was 43%. Infection was the most common admission diagnosis followed by cardiovascular surgery. The patients were severely ill, the mean (SD) APACHE II score being 25.5 (6.4) and the mean (SD) SOFA score 10.2 (3.5) on admission. In those patients who had AAC as the only intra-abdominal complication of multiple organ dysfunction, cholecystectomy was followed by a remarkable improvement of individual and total SOFA scores by the seventh postoperative day.

The AAC gallbladders were histologically and immunohistologically compared to normal gallbladders and to gallbladders of patients with acute calculous cholecystitis (ACC). The ACC patients were admitted into hospital because of primary acute gallbladder disease, were treated on a normal ward and did not have severe sepsis or multiple organ dysfunction. The typical histopathological features of AAC (34 cases) in the gallbladder wall were bile infiltration, lymphatic dilatation and leucocyte margination of blood vessels, while epithelial degeneration and defects, widespread occurrence of inflammatory cells and extensive and deep muscle layer necrosis were typical features of ACC (28 cases).

Tight junction proteins (claudin-1, -2, -3, -4, occludin, ZO-1 and E-cadherin) were uniformly expressed in normal gallbladder epithelium, with the exception of claudin-2, which was present in less than half of the cells. In AAC, the expression of cytoplasmic occludin and claudin-1 was decreased compared to control group. In ACC, the expression of claudin-2 was increased, but the expression of claudin-1, -3 and -4, occludin and ZO-1 was decreased compared to normal or AAC gallbladders.

In conclusion, AAC is associated with severe illness, infection, long intensive care unit stay and deteriorating multiple organ dysfunction. Open cholecystectomy is one important contributing factor to reverse the course of multiple organ dysfunction in these patients. Histological and immunohistological studies suggest that AAC is a manifestation of systemic inflammatory disease, while ACC is a local inflammatory and often infectious disease.

Keywords: acalculous cholecystitis, intensive care, multiple organ dysfunction, sequential organ failure assessment, surgery of gallbladder, tight junction
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Abbreviations

AAC   acute acalculous cholecystitis
ACC   acute calculous cholecystitis
APACHE acute physiology and chronic health evaluation score
CT    computed tomography
GI    gastrointestinal
IBD   inflammatory bowel disease
ICU   intensive care unit
JAM   junctional adhesion molecule
MODS  multiple organ dysfunction syndrome
MOF   multiple organ failure
SAPS  simplified acute physiology score
SIRS  systemic inflammatory response syndrome
SOFA  sequential organ failure assessment
TJ    tight junction
US    ultrasonography
WBC   white blood cell
List of original publications

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals. In addition, some unpublished data are presented.


## Contents

Abstract
Acknowledgements
Abbreviations
List of original publications
Contents

1 Introduction ................................................................................................................... 13
2 Review of the literature ................................................................................................. 15
   2.1 Anatomy, physiology and histology of the gallbladder...........................................15
   2.2 Acute acalculous cholecystitis (AAC)....................................................................16
      2.2.1 Historical aspects and incidence ....................................................................16
      2.2.2 Etiology and pathophysiology .......................................................................16
         2.2.2.1 Visceral hypoperfusion and ischemia .......................................................17
         2.2.2.2 Systemic inflammatory response .............................................................18
         2.2.2.3 Bile stasis ..................................................................................................19
      2.2.3 Diagnosis of AAC............................................................................................20
         2.2.3.1 Clinical signs, symptoms and laboratory investigations...........................20
         2.2.3.2 Ultrasonography .......................................................................................20
         2.2.3.3 Computed tomography .............................................................................21
         2.2.3.4 Hepatobiliary scintigraphy........................................................................21
         2.2.3.5 Percutaneous aspiration of gallbladder .....................................................22
         2.2.3.6 Histological findings in cholecystitis........................................................22
      2.2.4 Treatment.........................................................................................................22
         2.2.4.1 Cholecystectomy ......................................................................................22
         2.2.4.2 Percutaneous cholecystostomy .................................................................23
         2.2.4.3 Transpapillary endoscopic cholecystostomy .............................................23
         2.2.4.4 Antimicrobial therapy ...............................................................................23
   2.3 Tight junctions.......................................................................................................24
      2.3.1 General structure of the tight junctions............................................................24
      2.3.2 TJ proteins .......................................................................................................26
         2.3.2.1 Occludin ....................................................................................................27
         2.3.2.2 Claudins ....................................................................................................27
2.3.2.3 JAMs  ........................................................................................................28
2.3.2.4 ZO family .................................................................................................28
2.3.2.5 E-cadherin ..............................................................................................28
2.3.3 Tight junctions and diseases ............................................................................29

3 Aims of the study........................................................................................................... 30

4 Patients and methods ..................................................................................................... 31
4.1 Patients ................................................................................................................... 31
4.2 Clinical data (I, II) ...............................................................................................32
4.3 SOFA score (I, II) ..................................................................................................33
4.4 ICU treatment ...........................................................................................................34
4.5 Blinding (III, IV) ....................................................................................................36
4.6 Histological examination (III) ...............................................................................36
4.7 Immunohistochemical examination (IV) ..............................................................37
4.8 Statistical methods .................................................................................................37

5 Results ........................................................................................................................... 38
5.1 Patients ................................................................................................................... 38
5.2 Incidence of AAC (I, II) .......................................................................................39
5.3 Admission diagnoses (I, II) ..................................................................................39
5.4 Diagnostic findings (I, II) .....................................................................................40
5.5 Organ system dysfunction (I, II) ...........................................................................40
  5.5.1 Total SOFA scores .........................................................................................41
  5.5.2 Organ-specific SOFA scores ..........................................................................42
5.6 Mortality (I, II) .......................................................................................................44

6 Discussion ...................................................................................................................53
6.1 General discussion .................................................................................................53
6.2 Incidence and concomitant factors of AAC ..........................................................53
6.3 Diagnostic and treatment challenges .....................................................................54
6.4 Multiple organ failure and outcome ......................................................................55
6.5 Histological findings ..............................................................................................56
6.6 Tight junction proteins in AAC and ACC ...............................................................56

7 Conclusions ..................................................................................................................58

References

Original publications
1 Introduction

Acute acalculous cholecystitis (AAC) refers to acute inflammation of the gallbladder in the absence of gallstones (Glenn & Becker 1982, Barie & Eachempati 2003). Acute calculous cholecystitis (ACC) is caused by gallstones that obstruct the cystic duct and lead to distension, edema, bile stasis, inflammation and, often, bacterial infection of the gallbladder (Crawford 1999).

To date, the literature concerning AAC in critically ill patients is quite limited. There are some retrospective reports on patient series focusing mainly on predicting factors and diagnostic findings (Flancbaum et al. 1985, Shapiro et al. 1994, Molenat et al. 1996, Mariat et al. 2000, Puc et al. 2002, Ryu et al. 2003, Wang et al. 2003) but there are no studies comparing the different treatment modalities. In most cases, AAC has been reported as a complication of trauma or in postoperative patients. During the last few decades, AAC has been diagnosed with increasing frequency in ICU patients, probably because more and more seriously ill patients are treated for longer times in ICU (Glenn & Becker 1982, Barie & Eachempati 2003). The growing awareness of AAC and the improved imaging technologies have also led to an increasing number of AAC diagnoses.

There are no generally accepted diagnostic criteria for AAC, and the treatment of choice of AAC is also controversial (Melin et al. 1995, Boggi et al. 1999, Shapiro 1999, McChesney et al. 2003). There are, for example, no prospective controlled studies available to compare the conservative, percutaneous and surgical treatments of AAC. Furthermore, there are only a few studies concerning the histological changes in AAC, and the histopathology described so far has mostly been considered similar in acalculous and calculous cholecystitis (Glenn 1979, Jessurun & Albores-Saavedra 2001).

Gut hypoperfusion has a pivotal role in the development of multiple organ dysfunction syndrome (MODS) in septic patients (Doig et al. 1998, Hassoun et al. 2001). It is also proposed to be an important factor in the pathogenesis of AAC (McChesney et al. 2003). Recently, there has been growing interest in the study of the epithelial barrier function and its role in the pathophysiology of MODS in critically ill patients (Fink & Delude 2005). In this respect, tight junctions (TJ), i.e. the apical cell-cell contacts in epithelial and endothelial cell sheets, may be important. So far, however, tight junctions have been investigated only in experimental sepsis models, but not in septic or critically ill patients or even in normal or inflamed gallbladders.
This study was carried out to evaluate the incidence, predisposing factors, clinical picture and diagnostic and histological features of surgically treated AAC and to find out the effect of surgical treatment on organ function in critically ill patients having AAC.
2 Review of the literature

2.1 Anatomy, physiology and histology of the gallbladder

Bile is formed in hepatic cells, and it is composed of bile salts, cholesterol, lecithin, bilirubin and electrolytes. The main function of bile is to facilitate the absorption of fatty acids and other lipids from the intestinal tract.

The gallbladder of a normal adult is a pear-shaped sac, 10 cm long and 3-4 cm wide, with a volume of 40 – 70 ml, lying in the depression of the posterior aspect of the right lobe of the liver. Its wall is 1-2 mm thick, depending on whether the organ is in a relaxed or contracted state. The primary functions of the gallbladder are to concentrate, store and release bile. It also liberates mucosubstances from its surface epithelial cells and the mucous glands. Approximately 800 – 1000 ml of bile flows daily into the gallbladder from the liver. The contraction and evacuation of the gallbladder are promoted mainly by cholecystokinin liberated from the proximal small intestine. (Frierson 1992).

The gallbladder absorbs water from bile. If too much water or bile acids are absorbed, or too much cholesterol is secreted in bile, or if the evacuation of the gallbladder is impaired, gallstones may develop. About 90 percent of gallstones are composed of cholesterol, while the remainder are made of pigments, such as calcium salts and bilirubin. Biliary sludge is a mixture of cholesterol crystals and calcium salts present in bile and may progress to gallstones or completely disappear. Biliary sludge may also lead to complications, including acute cholecystitis and acute pancreatitis (Lee et al.1992, Ko et al. 1999).

The blood supply to the gallbladder comes from the celiac trunk, through the common hepatic and right hepatic arteries into the cystic artery, which again divides into two arteries, one in the free surface of the gallbladder and the other between the gallbladder and the liver. However, significant individual variation exists. The superior mesenteric artery supplies the whole small bowel from the duodenum to the caecum, the ascending colon and a great part of the transverse colon. The inferior mesenteric artery supplies the remainder of the colon and the rectum. The splanchnic venous circulation passes through the portal venous system, and the venous drainage of the gallbladder leads partly directly to the liver and partly to the portal venous system. (Frierson 1992).
Lymphatic channels from the gallbladder lead to lymph nodes on the gallbladder neck or the cystic duct, and these channels then lead superiorly to lymph nodes near the hepatic hilum or inferiorly to lymph nodes of the hepatoduodenal ligament and then to lymph nodes on the celiac axis (Frierson 1992).

The gallbladder is innervated from the hepatic branch of the celiac nerve, which consists primarily of sympathetic fibres, and from the hepatic branch of the vagus nerve, which consists primarily of parasympathetic fibres (Tabata 1994). The gallbladder wall consists of surface epithelium, lamina propria, smooth muscle, subserosal fat and peritoneal surface. The folds surrounding the lumen of the gallbladder are lined by a single row of tall columnar cells with basally located nuclei. These cells are supported by fine lamina and lamina propria with numerous small blood vessels. The lamina propria contains, in addition to small blood vessels, loose connective tissue, elastic fibres, nerve fibres and lymphatic channels. The smooth muscle consists of loosely arranged bundles of circular, longitudinal and oblique fibres that do not form well-developed layers. The subserosal layer contains adipocytes, collagen fibres, fibroblasts, blood vessels, nerves and lymphatics. Rokitansky-Aschoff sinuses represent herniations of epithelium into lamina propria, smooth muscle or subserosal connective tissue. (Frierson 1992)

2.2 Acute acalculous cholecystitis (AAC)

2.2.1 Historical aspects and incidence


AAC rarely occurs as an isolated event, but normally accompanies MODS and most often develops on the second week of severe illness or later (Rady et al. 1998, McClesney et al. 2003). Most AAC patients are male (Thompson et al. 1962, Shapiro et al. 1994). In children, acute acalculous cholecystitis represents 30 - 70% of all cases of acute cholecystitis, and infection and trauma are common preceding factors (Tsakayannis et al. 1996, Imamoglu et al. 2002). In this work, we concentrate on AAC as a complication of severe illness in adult patients.

AAC was first described 160 years ago by Duncan, who reported a fatal case of perforated acalculous cholecystitis in a patient who had undergone surgery for a femoral hernia (Duncan 1844). By 1970, altogether 80 sporadic cases of AAC had been reported in the English-language literature (Lindberg et al. 1970). This includes one series of 12 Vietnam war casualties who developed AAC during their recovery 10 to 35 days after the primary injury and after 2-11 surgical procedures prior to cholecystectomy. The incidence
of AAC in severely wounded US soldiers in USAF Hospital during Vietnam war was 0.5 \% (12/2412). (Lindberg et al. 1970)

Our present knowledge about AAC in intensive care patients is still based mainly on case reports and retrospective studies, and there are only a few prospective studies with small patient series. In addition, the verification of AAC in these studies has been based variably on clinical, imaging, laboratory and operative findings.

In a retrospective review of over 10,000 surgical intensive care patients, the incidence of operatively confirmed AAC was 0.2 \% (Kalliafas et al. 1998). After cardiovascular surgery, the incidence of AAC has been shown to be 0.3-0.5 \% (Sessions et al. 1993, Rady et al. 1998, Barie & Eachempati 2003, McChesney et al. 2003) and after aortic surgery 0.7 -0.9 \% (Ouriel et al. 1984, Hagino et al. 1997). In ICU long-stayers (> 7 days) the incidence of AAC after cardiovascular surgery was 3 \% (30 / 876) (Rady et al. 1998).

In a prospective study of 92 ICU patients with acute renal failure, the incidence of AAC based on ultrasonography (US) and laboratory criteria was 17 \% (Stevens et al. 1988). AAC was also screened in two studies of multiple trauma patients (injury severity score >11), where 10.6 \% and 18 \% of the patients developed US criteria of AAC and, respectively, 4.4 \% and 8.2 \% underwent cholecystectomy confirming the AAC diagnosis (Raunest et al. 1992, Pelinka et al. 2003). The incidence of AAC was recently studied in a mixed medical-surgical ICU, and 4 \% (14/346) of the patients developed operatively confirmed AAC (Mariat et al. 2000).

The reported mortality of ICU patients with AAC has been high (22 - 71 \%) reflecting the severe underlying diseases (Stevens et al. 1988, Hagino et al. 1997, Kalliafas et al. 1998, McChesney et al. 2003, Pelinka et al. 2003).

### 2.2.2 Etiology and pathophysiology

The etiology of AAC is considered to be multifactorial, and the precise pathophysiology is still unclear. Ischemia and reperfusion injury, systemic inflammatory response and biliary stasis are the most often proposed mechanisms for the disease.

#### 2.2.2.1 Visceral hypoperfusion and ischemia

Normally, the hepato-splanchnic blood flow accounts for 25-30 \% of cardiac output and the regional oxygen extraction in the splanchnic region is slightly higher than whole-body extraction (Dahn et al. 1987). In acute cardiogenic or hypovolemic shock, the sympathetic nervous system and other vasoactive mediators cause strong vasoconstriction in the mesenteric resistance and capacitance vessels providing a large blood volume to the vital organs reducing the splanchnic flow (Toung et al. 2000). In an experimental fat embolism model, decreased systemic oxygen delivery was followed by a decrease of mesenteric oxygen delivery, but intestinal oxygenation remained unaffected, probably due to increased oxygen extraction (Rautanen et al. 1996).
In septic shock, the splanchnic circulation is regulated by numerous inflammatory mediators, and the splanchnic flow can be normal, increased or decreased. In addition to the changes in flow, tissue integrity can be compromised by increased metabolism, impaired oxygen extraction and possible redistribution of flow (Tenhunen et al. 2003).

The assumption that visceral hypoperfusion and hypotension are important mechanisms for AAC is supported by the fact that AAC has mostly been described in association with hypovolemic shock, major surgery, multiple trauma or septic shock (Lindberg et al. 1970, Kalliafas et al. 1998, Rady et al. 1998, McChesney et al. 2003). Decreased arterial pressure combined with possibly increased intraluminal pressure impairs gallbladder perfusion and easily results in ischemia. Ischemia, on the other hand, renders the gallbladder mucosa vulnerable to the toxic effects of bile acids. Furthermore, the gallbladder vessels have been shown to be very sensitive to various inflammatory mediators (Becker et al. 1980, Cullen et al. 2000). Vasoactive drugs, which are commonly used in these situations, can also impair splanchnic circulation (De Backer et al. 2003, Martikainen et al. 2003). In addition, the increased sympathetic tone often associated with these situations, may be important. In animal studies sympathectomy has prevented experimental AAC in shock (Howard et al. 1952).

The assumption of ischemia as an important pathogenetic factor in AAC is also supported by microangiographic findings. Severe microcirculatory disturbances and small vessel occlusion were shown in AAC in contrast to ACC, where strongly dilated arterioles and regular filling of the capillary network was found (Warren 1992, Hakala et al. 1997). In addition to ischemia, reperfusion injury can be a significant pathogenetic factor as well. Taoka produced macroscopic and histologic cholecystitis in dogs by 45 minutes of ischemia followed by 90 minutes of reperfusion, whereas ischemia alone did not induce cholecystitis (Taoka 1991). The autonomic nervous system may also have a role in the development of AAC, because an animal study showed that ischemia-reperfusion after autonomic denervation caused more severe cholecystitis than ischemia-reperfusion alone (Tabata 1994). This could explain the increased risk of AAC after gastrectomy and esophagectomy with vagotomy (Inoue & Mishima 1988, Paull 2001).

2.2.2.2 Systemic inflammatory response

The systemic response to injury, trauma or infection includes a huge number of different mediators, including pro- and anti-inflammatory cytokines, activated neutrophils, monocytes, microvascular endothelial cells, neuroendocrine reflex and complement, coagulation and fibrinolytic system (Bone et al. 1992, Levy et al. 2003, Hotchkiss & Karl 2003, Annane et al. 2005). This response functions to save the organism and results in recovery and rehabilitation. The response can also be overwhelming and break out of control, leading to multiple organ failure and death. AAC may represent one end-organ phenomenon in this response. In animal studies, endotoxin and inflammatory mediators, such as activated factor XII and platelet-activating factor, have been shown to induce acute inflammation in the gallbladder wall similar to that seen in clinical AAC (Becker et al. 1980, Kaminski et al. 1990, Kaminski et al. 1994, Cullen et al. 2000). Subsequently, endotoxin has been demonstrated to decrease bile acid synthesis and secretion, and this,
together with gallbladder hypomotility, has been suggested to predispose to bacterial proliferation, translocation and endotoxin absorption in the small intestine, resulting in a vicious circle. These studies have also suggested that the gallbladder wall may be more sensitive to the effects of inflammatory mediators than other areas of the alimentary tract or other organs (Becker et al. 1980, Cullen et al. 2000). The mechanism of how inflammatory mediators influence epithelial cells and the barrier function in critical illness is not completely clear. Alterations in the structure and function of epithelial and endothelial tight junctions may be important in the pathogenesis of end-organ failures in MODS (McConnell & Coopersmith 2005) (see chapter 2.3. Tight junctions).

2.2.2.3 Bile stasis

Bile stasis has been implicated in the pathogenesis of AAC for decades, and bile stasis may also alter the chemical composition of bile, which can promote local injury in gallbladder mucosa (Glenn & Becker 1982, Lee 1990, Barie & Eachempati 2003). Normally, a fatty meal causes vagal stimulation and cholecystokinin release from intestinal mucosa, which leads to contraction of the gallbladder and relaxation of the sphincter of Oddi, resulting in evacuation of the gallbladder. Dysfunction of gallbladder smooth muscle and dysfunction of the sphincter of Oddi have been shown to be associated with bile stasis and inflammation of the gallbladder wall similar to AAC (Parkman et al. 2000, Cullen et al. 2000, Barnes et al. 2004, Gomez-Pinilla et al. 2005). Endotoxin has also been shown to decrease the contractile responses of the gallbladder to hormonal and neural stimuli, leading to bile stasis (Cullen et al. 2000).

Fever, dehydration and fasting have been suggested to increase bile viscosity and to obstruct the cystic duct (Glenn 1947). Prolonged total parenteral nutrition has similarly been reported to result in AAC (Petersen & Sheldon 1979, Roslyn et al. 1983), but later studies have shown that AAC also developed in patients on enteral feeding and in patients not receiving parenteral nutrition (Savoca et al. 1990b, Shapiro et al. 1994, Rady et al. 1998).

Opioid analgesics may increase biliary pressure due to spasm of the sphincter of Oddi and the effect of morphine has been shown to be stronger than that of pethidine (Joehl et al. 1984, Wu et al. 2004). The role of opioids in the development of AAC has been emphasized in many reports, partly because most of the described patients have been trauma or postoperative patients in need of analgesics (Flanebaum et al. 1985, Pelinka et al. 2003). A notable proportion of them have also received multiple blood transfusions, and it has been suggested that transfusions lead to an increased bile pigment load and cause injury to the gallbladder wall (Lindberg et al. 1970). However, others have found no difference in the amount of blood transfused into AAC patients as compared with control patients (Long et al. 1978).

Bile stasis may also be induced by mechanical ventilation. In an animal study, ventilation with positive end-expiratory pressure of 7-10 cmH₂O increased hepatic venous pressure, decreased portal perfusion and increased resistance to flow through the choledochoduodenal junction (Johnson & Hedley-Whyte 1975). Different modes of mechanical ventilation may also have different effects on the gallbladder by decreasing or
increasing splanchnic perfusion and by inducing different hormonal and cytokine responses (Perttilä et al. 1989, Lee et al. 1998, Ranieri et al. 1999, Mutlu et al. 2001). On the other hand, many patients not on mechanical ventilation have developed AAC (Orlando et al. 1983, Flanbaum et al. 1985).

Most authors agree that the etiology of AAC can be multifactorial, and that the pathophysiology of the disease in any one patient can be a combination of the above mechanisms (Shapiro et al. 1994, Kalliafas et al. 1998, Rady et al. 1998). Bacterial invasion is generally regarded as a complication of an already diseased gallbladder (Boland et al. 1993).

### 2.2.3 Diagnosis of AAC

#### 2.2.3.1 Clinical signs, symptoms and laboratory investigations

The diagnosis of AAC is usually based on clinical, laboratory, radiologic and/or operative findings. The clinical diagnosis is difficult because the majority of patients are critically ill, sedated and unable to communicate about their symptoms. The frequently presented clinical signs and symptoms of AAC are pain, tenderness and mass in the right upper abdominal quadrant, fever, nausea, vomiting, jaundice, diarrhoea, abdominal discomfort, alteration of mental status and sudden deterioration of overall clinical condition. These symptoms have, however, been nonspecific in critically ill patients and may be present or absent in AAC patients. Leukocytosis, leukopenia and elevated values of liver function tests are widely used to prove or disprove a suspicion of AAC, but their diagnostic value is also low. (Howard 1981, Flanbaum et al. 1985, Shapiro et al. 1994, Kalliafas et al. 1998, Rady et al. 1998, Pelinka et al. 2003, Trowbridge et al. 2003).

#### 2.2.3.2 Ultrasonography

Ultrasonography is a widely recommended imaging modality of the gallbladder (Barie & Eachempati 2003, Wang et al. 2003). Sonographic findings in AAC include increased wall thickness (> 3.5 mm), increased gallbladder volume (hydrops), intraluminal sludge (increased echogenicity within the gallbladder lumen), pericholecystic fluid, sonographic Murphy’s sign (pain on inspiration while pressing on the gallbladder with the US probe) and presence of intramural gas. None of the above-mentioned findings alone are very sensitive, and a combination of three of them is generally needed for a diagnosis of AAC. (Raunest et al. 1992, Molenat et al. 1996, Mariat et al. 2000, Boland et al. 2000, Puc et al. 2002, Pelinka et al. 2003, Wang et al. 2003).

The sensitivity and specificity of US in AAC vary, depending on the patient population studied. In non-ICU patients the sensitivity and specificity of US in evaluating suspected acute cholecystitis is high, but in ICU patients diagnostic accuracy decreases remarkably (Boland et al. 1994, Shea et al. 1994, Puc et al. 2002, Pelinka et al. 2003). Imaging findings can be compromised by bowel gas, ascites and abdominal dressings and
drains. Several recent studies have documented the high incidence of abnormal gallbladder sonograms in ICU patients without evidence of the disease. In a study on 44 medical-surgical ICU patients, the gallbladder was systematically screened by ultrasound, and 57% of the patients had at least three sonographic abnormalities. Only one of these patients developed AAC during the hospital stay. No statistical correlation was found between sonographic findings suggestive of cholecystitis and clinical or laboratory parameters. (Boland et al. 2000) In another study of 62 critically ill trauma patients, US had a sensitivity of only 30% and specificity of 93% in the detection of AAC (Puc et al. 2002). In a recent prospective study of 255 severely traumatized patients requiring intensive care for more than four days, 27 patients developed highly pathologic ultrasound changes in the gallbladder. However, six of these patients (22%) were closely monitored without any interventions and their US findings normalized within 3 weeks. (Pelinka et al. 2003) Furthermore, US has not been able to identify the patients likely to develop complications, such as necrosis and perforation (Wang et al. 2003). Despite some promising results of color doppler ultrasound in the diagnosis of acute cholecystitis, the method has not proved very helpful in clinical practice (Jeffrey et al. 1995, Draghi et al. 2000, Boland et al. 2000).

### 2.2.3.3 Computed tomography

The most commonly described computed tomography (CT) findings in AAC are thickening of the gallbladder wall, mucosal irregularity, luminal distention, increased bile density (sludge), intramural or intraluminal gas, intraluminal hemorrhage, pericholecystic fluid collections and obscurity of the liver-gallbladder interface. The reported sensitivities and specificities of CT vary, but have generally been higher than for US. (Mirvis et al. 1986, Fidler et al. 1996, Harvey & Miller 1999, Bennett et al. 2002). In a recent study of CT findings in operatively confirmed acute cholecystitis (AAC and ACC), the sensitivity and specificity of preoperative CT were more than 90%, but the sensitivity to discover gangrenous cholecystitis was only 29.3% (Bennett et al. 2002). The disadvantages of CT are the need to transport the critically ill patient to the radiology department and the need for radiocontrast agent, which may be harmful for the renal function.

### 2.2.3.4 Hepatobiliary scintigraphy

Hepatobiliary scintigraphy is performed by administering intravenously iminodiacetic acid compounds labelled with radioactive technetium 99m, which are rapidly taken up and excreted by a normal liver. Isotope scanning is used to sequentially visualize the liver, extrahepatic bile ducts, gallbladder and duodenum. Nonvisualization of the gallbladder with normal visualization of the liver and bile ducts in 30 minutes – 4 hours supports the diagnosis of acute cholecystitis. (Prevot et al. 1999, Mariat et al. 2000, Barie & Eachempati 2003) In spite of some positive reports on the specificity and sensitivity of cholescintigraphy in critically ill patients (Shapiro et al. 1994, Flanebaum et al. 1995, Mariat et al. 2000), it has not gained wide popularity, partly because of the time-
consuming study design in a nuclear medicine laboratory, where the facilities are usually not intended for the treatment of severely ill patients.

### 2.2.3.5 Percutaneous aspiration of gallbladder

AAC is primarily a non-infectious disease, and bacterial invasion is regarded as a secondary event (Boland et al. 1993). In addition, many critically ill patients are on broad-spectrum antibiotics, which diminished the possibility of positive bacterial culture. That is why percutaneous aspiration of the gallbladder is of limited value and is not routinely recommended (Boland et al. 1993).

### 2.2.3.6 Histological findings in cholecystitis

The inflammatory reactions in the gallbladder wall in acalculous and calculous cholecystitis have been regarded histologically similar in both types of cholecystitis, including edema, mucosal ulceration, leukocytic infiltration, vascular congestion, abscess formation and gangrenous necrosis (Glenn 1979, Crawford 1999). In AAC, thrombosis and focal necrosis in blood vessels in the serosa and muscularis layers have also been shown to be characteristic features (Glenn & Becker 1982).

### 2.2.4 Treatment

The historical treatment of choice for AAC has been cholecystectomy, but during the last two decades percutaneous cholecystostomy has been suggested as an alternative to cholecystectomy (Melin et al. 1995, Davis et al. 1999, Spira et al. 2002). There are no randomized studies to compare percutaneous cholecystostomy and open or laparoscopic cholecystectomy in AAC patients. Medical treatment includes hemodynamic optimization with vasoactive drugs and antimicrobial treatment, in spite of the fact that only a small number of gallbladders in AAC have cultured positively (Barie & Eachempati 2003). The reported mortality rates of patients with AAC have been high (10-67 %) irrespective of the treatment, mostly due to the serious underlying diseases and the rapid progression to gangrene and perforation (Orlando et al. 1983, Johnson 1987, Shapiro et al. 1994, Rady et al. 1998).

#### 2.2.4.1 Cholecystectomy

The incidence of gangrene in AAC has been high (27 - 78 %) in many studies where the gallbladder has been removed (DuPriest et al. 1979, Orlando et al. 1983, Shapiro et al. 1992, Kalliafas et al. 1998, Rady et al. 1998, Wang et al. 2003). This makes direct inspection of the gallbladder and cholecystectomy very reasonable treatment. In the same procedure, other intra-abdominal problems mimicking AAC can also be identified and managed (Barie & Eachempati 2003). Furthermore, the lack of correlation between
symptomatology and US findings and the incidence of necrosis and gangrene of the
gallbladder has made many authors prefer cholecystectomy over cholecystostomy in the
cholecystectomy may cause less tissue damage than open cholecystectomy, but
ventilatory, cardiovascular or hormonal side-effects may be severe in critically ill
patients. There are, however, some reports on small patient series (2-10 patients) with
good results even in critically ill patients (Brandt et al. 1994, Almeida et al. 1995,
McClain et al. 1997).

2.2.4.2 Percutaneous cholecystostomy

Percutaneous cholecystostomy has gained popularity during the last decades. It is
minimally invasive and can be accomplished under local anesthesia even on severely ill
patients. The gallbladder is punctured under sonographic control via an anterior or
anterolateral transhepatic or transperitoneal approach through the bed of the gallbladder,
and a pigtail catheter is passed into the gallbladder over a guidewire. (Akhan et al. 2001).
Rapid improvement should take place if the procedure is performed properly. Reported
complications are catheter dislodgement, bile leakage causing peritonitis, hemorrhage,
cardiac arrhythmia and hypotension from procedure-related bacteremia (van Sonnenberg

2.2.4.3 Transpapillary endoscopic cholecystostomy

In this technique, the cystic duct is located during endoscopic retrograde
cholangiopancreatography (ERCP), and either a double pigtail biliary stent or a
nasobiliary catheter is placed into the gallbladder. Technical success has been achieved in
up to 90% of patients, but the technique has not been evaluated in ICU patients.
Advantages include the decreased risk of bleeding, bile peritonitis and injury to adjacent
organs. Disadvantages include the risk of pancreatitis and associated risks related to
ERCP (Johlin & Neil 1994).

2.2.4.4 Antimicrobial therapy

Bile is normally sterile. In AAC, bacterial invasion is not regarded as the triggering
etiologic factor, but rather a complication of an already diseased gallbladder (Raunest et
al. 1992, Boland et al. 1993). Bacteria can enter the gallbladder hematogenerously or
retrogradely from the duodenum. Retrograde bacterial colonization is more important
(Subhani et al. 1999). In AAC, bile culture has been positive in 16 - 66 % of patients, and
the commonly cultured bacteria have been E.coli, Klebsiella, Enterococcus, Enterobacter
species, Staphylococcus, Pseudomonas, Clostridium and Bacteroides fragilis. Fungi and
anaerobes are particularly likely in patients with diabetes and advanced age. (Howard
AAC has also been reported to develop during systemic infection of uncommon pathogens, such as salmonella, cholera, leptospirosis, dengue fever, brucellosis and malaria (McCarron & Love 1997, West et al. 1998, Vilaichone et al. 1999, Andriopoulos et al. 2003, Wu et al. 2003, Saha et al. 2005). Antibiotic treatment does not substitute for purification of the necrotic focus, but remains an important adjunct and should be targeted against the likely organisms covering at least gram-negative rods and enterococci (Barie & Eachempati 2003).

2.3 Tight junctions

The major physiological manifestations in multi-organ dysfunction and severe sepsis are increased vascular and intestinal permeability leading to a loss of cell barrier function and significant oedema formation. Tight junctions (TJ) are important apical intercellular contacts in epithelial and endothelial cell sheets, and they were first described in electron microscopy in 1963 (Farquhar & Palade 1963). Although increased vascular and epithelial permeability is a fundamental feature in the pathophysiology of severe multi-organ failure in critically ill patients, TJ proteins have, however, not yet been studied in critically ill patients. Detailed information of TJ molecular components and structure has only begun to accumulate during the past two decades. The normal function of many organs, such as the lungs, intestines, liver and kidneys depends on the barrier function of epithelial and endothelial cells.

2.3.1 General structure of the tight junctions

In columnar epithelial cells (e.g. those present in the gallbladder wall), TJs clearly subdivide the plasma membrane into apical and basolateral domains, which face the lumen and connective tissue. (Fig.1)
In endothelial cell sheets, TJs separate the membrane into apical and basolateral regions facing blood and the perivascular space, respectively. Tight junctions have two functions; the barrier function and the fence function. The barrier function means that they selectively open and close in response to various signals both inside and outside cells. This allows selective passage of ions, water and various macromolecules (inflammatory cells) and even cancer cells through paracellular spaces and is thus relevant to permeability disorders, such as edema formation and diarrhea (Madara 1998, Schneeberger & Lynch 2004). It also prevents both bacteria and toxins in the intestinal lumen from penetrating deeper into tissues through the paracellular pathway. The fence function maintains cell polarity by restricting the diffusion of lipids and proteins between the apical and basolateral plasma membranes (van Meer & Simons 1986). The adherens junction and desmosome are more basal components of the junctional complex than TJs, and they increase the mechanical strength of the cell-cell contact and maintain intercellular communication (Fig. 2).
Fig. 2. Junctional complex between cells. TJ, tight junction; AJ, adherens junction; D, desmosome. Adopted and modified from Tsukita et al. 2001.

The barrier function of epithelial TJs in skin and in the alimentary, respiratory and urinary tracts can vary, and there are “leaky” and “tight” tight junctions. The properties of endothelial TJs also vary in line with the need for permeability. In general, endothelial TJs are quite leaky, permitting a wide variety of substances to be exchanged between blood and organs. In brain, endothelial TJs are well developed, forming the blood-brain barrier (Bazzoni & Dejana 2003).

2.3.2 TJ proteins

Three distinct types of transmembrane proteins have been localized to TJs: occludin, claudins and junctional adhesion molecules (JAMs) (Fig. 2, 3). These proteins are linked to a series of peripheral proteins (including ZO-1, ZO-2, ZO-3) that anchor the integral membrane proteins to the cytoplasm and cytoskeleton by binding to the cytoplasmic tails of these proteins.
2.3.2.1 Occludin

Occludin (65 kDa) (522 amino acids) was the first transmembrane protein identified within tight junctions (Furuse et al. 1993). It has four transmembrane domains with two extracellular loops and a long carboxy-terminal and a short amino-terminal cytoplasmic domain. The extracellular loops are thought to interact in the paracellular space with loops from occludin from the adjacent cell to promote “sealing” of the paracellular space.

Occludin is expressed at high levels in brain endothelial cells and at much lower levels in endothelial cells of non-neural tissues (Hirase et al. 1997). Occludin also has a role in transepithelial migration of leukocytes (Huber et al. 2000). Despite the assumed central role of occludin in TJs in many studies, different results have also been presented; occludin-null mice showed no gross changes in TJ morphology and no alteration in intestinal epithelial barrier function (Saitou et al. 2000). The possibility that other junctional proteins (claudins) could compensate for the deficiency in the expression of occludin has been suggested as an explanation (Turksen & Troy 2004).

2.3.2.2 Claudins

The first members of the claudin family, claudin-1 and claudin-2, were identified from occludin-containing chicken liver junctional fractions in 1998, and so far altogether 24 members of this family have been identified (Furuse et al. 1998, Tsukita et al. 2001). The structural domains of claudins (20-24 kDa) (211 amino acids) are similar to occludin with
four transmembrane domains, two extracellular loops and relatively short cytoplasmic carboxy- and aminoterminal domains. The first extracellular loop of claudins is larger than the second, and the amino acid compositions of the extracellular loops and the cytoplasmic domains vary significantly among the different claudins.

There is marked variation among tissues in the expression of each claudin (Rahner et al. 2001). The composition of different claudins in a tissue and their selective interactions with one another determine the barrier properties of a tissue (Furuse et al. 2001, Kiuchi-Saishin et al. 2002). Claudin-5 is predominantly expressed in endothelial cells (Morita et al. 1999), although it was recently demonstrated in epithelial cells of colonic origin and was suggested to function as a sealing tight junction protein to tighten the epithelial barrier properties (Amasheh et al. 2005).

2.3.2.3 JAMs

Junctional adhesion molecules (JAMs) (36-41 kDa) are also important components of tight junctions regulating permeability. JAM has a short cytoplasmic tail, a single transmembrane domain and a fairly long extracellular portion bearing two immunoglobulin-like loops (Martin-Padura et al. 1998). JAMs are found in epithelial and endothelial tight junctions, and four members of the JAM family have been identified so far.

2.3.2.4 ZO family

Many cytosolic proteins have been reported to associate with the cytoplasmic domains of the tight junction membrane proteins and to regulate the barrier function. ZO-1 was the first protein identified in 1986 (Stevenson et al. 1986), and ZO-2 and ZO-3 were identified a few years later (Jesaitis & Goodenough 1994, Haskins et al. 1998). ZO-1 is found especially in intestinal epithelia and in brain endothelia and arteries. In addition to binding with transmembrane proteins, ZO-1, ZO-2 and ZO-3 interact with each other and with several cytoplasmic signalling molecules and transcription factors (Fanning et al. 1998, Haskins et al. 1998, Balda & Matter 2000).

2.3.2.5 E-cadherin

E-cadherin is one of the transmembrane proteins at the adherens junction (AJ), and it functions as an adhesion molecule. It also interacts with the actin cytoskeleton to stabilize TJ and AJ (Tunggal et al. 2005).
2.3.3 Tight junctions and diseases

Increased tissue permeability is a common characteristic of, for instance, pulmonary edema, acute lung injury, pancreatitis, multi-organ dysfunction syndrome, inflammatory bowel disease and pseudomembranous colitis (Doig et al. 1998, Dudek & Garcia 2001). Aberrant TJ expression and function are feasible explanations for the pathophysiology of these clinical manifestations (Kucharzik et al. 2001, Sawada et al. 2003, Lee et al. 2006).

One mechanism for action is that tight junctions can be direct or indirect targets of pathogens. Claudin-3 and -4 have been shown to be receptors for *Clostridium perfringens* enterotoxins and JAM a receptor for reovirus (Katahira et al. 1997, Fujita et al. 2000, Barton et al. 2001). After binding to receptors, these toxins cause TJ dysfunction. *Bacteroides fragilis* causes disruption of tight junctions by digesting E-cadherin (Wu et al. 1998). Enterobacteria can cause zonulin secretion from the small intestine, and zonulin is an endogenous modulator of intestinal tight junctions (Di Pierro et al. 2001).

Inflammatory mediators can regulate the expression of TJ proteins. Tumour necrosis factor alpha and interferon gamma downregulate the expression of occludin in human intestinal cell culture (Mankertz et al. 2000) and cause redistribution of JAM in endothelial cells (Ozaki et al. 1999). Endotoxemia has been shown to cause a marked decrease in the expression of occludin in the liver, lungs and intestine in mice (Han et al. 2004a, Han et al. 2004b, Han et al. 2004c).

The combined effect of trauma and severe infection on the expression of occludin and claudin-3 has been studied in rats (Samonte et al. 2004). Burn injury and *Enterococcus fecalis* infection together caused a significant increase in intestinal permeability and a decrease in the expression of intestinal occludin but not in the expression of claudin-3.

In human inflammatory bowel disease (IBD), diffuse global down-regulation of occludin has been found together with intense over-expression of claudin-2 along the inflamed colon epithelium (Prasad et al. 2005). Decreased claudin-1, -3 and -4, ZO-1, JAM and E-cadherin expressions have also been observed in epithelial cells immediately adjacent to transmigrating neutrophils in the diseased part of the colon (Kucharzik et al. 2001).

Human mutations of claudin-16, a specific claudin found in kidney epithelial cells, show abnormal paracellular passage of Mg$^{2+}$ ions, resulting in excessive loss of Mg$^{2+}$ in urine and in hypomagnesemia (Simon et al. 1999), and mutations in the claudin-14 gene result in an inability to maintain the special ionic environments in the cochlear duct, causing deafness (Wilcox et al. 2001).

Tight junctions are also considered to be deeply involved in tumorigenesis and metastasis. Altered expressions of claudin-1, -3 and -4 have been observed in human colorectal cancer (de Oliveira et al. 2005), while claudin-4 expression has been altered in pancreatic (Michl et al. 2001) and claudin-3 and claudin-4 in ovarian cancers (Hough et al. 2000).
Aims of the study

The aim of this study was to clarify the clinical and histological picture of surgically treated AAC and its association with multiple organ failure in critically ill patients. The following issues were of particular interest:

1. Incidence, admission diagnoses, diagnostic findings, associated organ system dysfunction and outcome of surgically treated AAC in mixed medical-surgical ICU patients (I, II).
2. Effect of cholecystectomy on organ system dysfunction in AAC patients (II).
3. Differences in gallbladder histology between AAC and ACC (III).
4. Expression of tight junction proteins in normal gallbladders and in the gallbladders of patients with AAC and ACC (IV).
4 Patients and methods

This study consists of four different substudies, which have previously been reported in publications hereafter referred to as the studies I-IV. The studies I, III, IV were approved by The Ethics Committee of Oulu University Hospital and the use of the tissue specimens in the studies III and IV was approved by The National Authority for Medicolegal Affairs. No approval for study II from an ethical review committee was required because the data had already been collected for clinical purposes, and no additional interventions were done. The studies were conducted in the Intensive Care Unit, Department of Anesthesiology, Oulu University Hospital, and the studies III and IV in the collaboration with the Department of Pathology, University of Oulu.

4.1 Patients

In the studies I and II, the medical records of all ICU patients who had operatively treated AAC between 1 January 2000 and 31 December 2001 (I) and between 1 January 2003 and 31 December 2004 (II) were retrospectively examined. A total of 39 patients were included in study I and 24 patients in study II. All patients found to have AAC at laparotomy were included in study I regardless of other intra-abdominal pathology. Study II was conducted to find out the effect of cholecystectomy on organ dysfunctions in AAC patients, and all the patients who had additional intra-abdominal pathology, e.g. intestinal ischemia or perforation (10 patients), were excluded.

The indication for open cholecystectomy was a suspicion of AAC based on a pathologic ultrasound and/or CT finding together with clinical signs and symptoms of sepsis or deteriorating multiple organ dysfunction without other obvious foci. The decision to operate was made by the ICU team, which consisted of intensivists, a gisurgeon and an infectious disease specialist. The patients were considered to have AAC if no gallstones were found, but necrosis and gangrene or distension and thickening of their gallbladder wall were seen at operation. During the same time periods, altogether 5 ICU patients (3 after cardiac surgery, 1 patient with pneumonia and 1 with sepsis) underwent cholecystectomy because of acute calculous cholecystitis 5 to 15 days after admission into ICU.
In study III, altogether 84 patients were included in three different study groups. The AAC group consisted of the same 39 AAC patients as study I. The ACC group consisted of 30 consecutively operated ACC patients admitted during the same time period as the 39 AAC patients. They were admitted into hospital because of primary acute gallbladder disease, were treated on a normal ward and did not have severe sepsis or multiple organ dysfunction. They had no signs of other intra-abdominal pathology than ACC at operation and no history of inflammatory bowel disease. The median (25th, 75th percentile) time from the onset of symptoms to the operation was 3 days (2, 5) and the median (25th, 75th percentile) time from the admission into hospital to the operation was 1 (1, 2) day. Their median (25th, 75th percentile) C-reactive protein value on the day of cholecystectomy was 173 mg/L (59, 212), and the median (25th, 75th percentile) white blood cell (WBC) count was 9.7 (8.2, 12.6) x 10^9/L. The control group consisted of 15 patients who had their macroscopically normal gallbladder removed during pancreatic tumour surgery. Five of the AAC samples, two of the ACC samples and one of the control samples could not be analyzed due to extensive autolysis of the samples, and the final numbers of samples were 76 samples, 34 in the AAC group, 28 in the ACC group and 14 in the control group.

In study IV, tight junction proteins were analyzed in 60 gallbladders. These gallbladders were selected from the 76 gallbladders in study III by a laboratory nurse blinded to any clinical data. This study included 30 samples of AAC patients, 21 samples of ACC patients and 9 samples of control patients.

### 4.2 Clinical data (I, II)

In the studies I and II, the following patient data were collected from the hospital records and the intensive care unit’s data management system (Deio-Ohmeda®, Helsinki, Finland in study I, Centricity Critical Care Clinisoft®, GE Healthcare, Helsinki, Finland in study II): age, sex, body mass index (BMI) (I), admission diagnosis, Acute Physiology and Chronic Health Evaluation (APACHE) II score, Simplified Acute Physiology Score (SAPS) II, appearance of the gallbladder at operation, length of ICU stay in days (calculated as hours divided by 24), parenteral/enteral nutrition, ICU and hospital outcome and cause of death.

In study I, the following data were also collected as “yes” or “no” responses on admission: presence of coronary heart disease, universal atherosclerosis or diabetes mellitus and abuse of alcohol. Data on the use of opioids, corticosteroids, antibiotics, vasoactive and sedative drugs during the ICU stay before cholecystectomy were also collected from the data management system. The highest body temperature, the results of bilirubin and liver function tests, C-reactive protein and white blood cell count on the day of cholecystectomy were recorded as well. The results of blood (taken one to eight days before cholecystectomy) and bile cultures (taken prior to cholecystectomy via US-guided puncture or during cholecystectomy) as well as the findings of preoperative radiologic imaging (CT, US) were also recorded.
4.3 SOFA score (I, II)

The SOFA scores were collected from the intensive care unit’s data management system. In study I, the scores were collected for the first 24 hours in the ICU and for the 24 hours before cholecystectomy. In study II, the SOFA scores were collected on the admission day, three days before cholecystectomy, on the day of cholecystectomy and on the first, second, third and seventh postoperative days after cholecystectomy.

The SOFA score is composed of scores for six organ system functions, graded from 0 (normal) to 4 (most severe dysfunction). These organ systems are: the respiratory, cardiovascular, renal, haematological, neurological and hepatic systems (Vincent et al. 1996, Vincent et al. 1998) (Table 1). Arterial blood gas analysis, platelet count and serum creatinine measurement were made at least once daily, and serum bilirubin was measured twice or three times a week at the physician’s discretion. The worst physiologic value for each day was recorded. If a value was missing (most frequently serum bilirubin), the previous value was used, and if that was not available, the next value was substituted. If both values were missing, the value was considered missing data and calculated as normal.

Table 1. Sequential Organ Failure Assessment (SOFA) score. Adopted and modified from Vincent et al. 1996.

<table>
<thead>
<tr>
<th>Organ system</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiration</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>PaO2/FiO2 (kPa) &gt; 400</td>
<td>≤ 400</td>
<td>≤ 300</td>
<td>≤ 200&lt;sup&gt;a&lt;/sup&gt;</td>
<td>≤ 100&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Coagulation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelets (x10&lt;sup&gt;9&lt;/sup&gt;/l) &gt;150</td>
<td>≤ 150</td>
<td>≤ 100</td>
<td>≤ 50</td>
<td>≤ 20</td>
<td></td>
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<tr>
<td>Liver</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Bilirubin (μmol/l) &lt; 20</td>
<td>20-32</td>
<td>33-101</td>
<td>102-204</td>
<td>&gt; 204</td>
<td></td>
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<tr>
<td>Cardiovascular</td>
<td></td>
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<tr>
<td>Hypotension</td>
<td>MAP ≥ 70 mmHg</td>
<td>MAP &lt; 70 mmHg</td>
<td>Dopa ≤ 5 or dobu (any dose)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Dopa &gt; 5 or epi ≤ 0.1 or norepi &lt; 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Dopa &gt; 15 or epi &gt; 0.1 or norepi &gt; 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>CNS</td>
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<tr>
<td>Glasgow Coma Score 15</td>
<td>13-14</td>
<td>10-12</td>
<td>6-9</td>
<td>&lt; 6</td>
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<tr>
<td>Renal</td>
<td></td>
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<tr>
<td>Creatinine (μmol/L) &lt; 110</td>
<td>110-170</td>
<td>171-299</td>
<td>300-440</td>
<td>&gt; 440</td>
<td></td>
</tr>
<tr>
<td>urine output (ml/day) &lt; 500</td>
<td>500-1000</td>
<td>1000-1500</td>
<td>&gt; 1500</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> with respiratory support; <sup>b</sup> adrenergic agents administered at least for one hour (doses given are μg/kg/min); MAP, mean arterial pressure; dopa, dopamine; dobu, dobutamine; epi, epinephrine; norepi, norepinephrine; CNS, central nervous system
4.4 ICU treatment

Our ICU is a “closed unit”, and the treatment during the study period was managed by three to five full-time intensivists (anesthesiologists) in close co-operation with cardiac, neuro- and GI surgeons and specialists in infectious diseases, cardiology and radiology. All patients were treated according to the same general principles. Arterial blood gas analyses, lactate measurement and mixed venous oxygen saturation were used in addition to clinical assessment for routine tissue oxygenation monitoring. Pulmonary artery catheter was used to monitor the circulation whenever there was uncertainty of cardiac function or volume status and if vasoconstrictors or inotropes were used. Dobutamine was the first-line inotrope and noradrenaline the first-line vasoconstrictor. Levosimendan and milrinone were used as second-line inotropes.

Pressure control ventilation (Pplat < 30 H2O) was used as the routine mode of mechanical ventilation, and continuous veno-venous hemodiafiltration was the primary method of renal replacement therapy.

Strict blood glucose control (< 8.0 mmol/l) with insulin infusion and intravenous hydrocortisone (200 -300 mg daily for 5 to 7 days) in septic shock not responding to adequate fluid replacement were used (Van den Berghe et al. 2001, Annane et al. 2002). Nutrition routines included initiation of enteral nutrition (1 kcal/ml) within 24-48 hours from admission at 10-20 ml/hour, followed by an increase in 3-4 days up to 1000-1500 ml per day. If the patient was intolerant of enteral nutrition, the rate was decreased and supplemental parenteral nutrition was provided. In the case of gastroparesis, a jejunal feeding tube was inserted endoscopically.

Propofol and midazolam were used for patient sedation and oxycodone and fentanyl for opioid analgesia.

The algorithm for the diagnosis and treatment of critically ill patients with suspected AAC or other intra-abdominal complications is presented in Figure 4.
Fig. 4. Algorithm for the diagnosis and treatment of AAC.
4.5 Blinding (III, IV)

Histological and immunohistochemical analyses were performed blindly without any clinical information, including patient category. All assessments were made by two investigators, JL and TK in study III and JL and either TK or YS in study IV.

4.6 Histological examination (III)

The gallbladder samples in study III had been taken for routine histopathological analysis during cholecystectomy. The samples were fixed in neutral buffered formalin and were embedded in paraffin. The sections were stained with haematoxylin and eosin.

The extent of changes in the surface epithelium was estimated, including the proportion (%) of surface area showing erosion (epithelial detachment) and the proportion of surface epithelium showing signs of degeneration or necrosis. The criteria for degeneration included cytoplasmic vacuolization or partial detachment. Necrotic epithelial cells were recognized based on either a pyknotic or karyorrhectic nucleus with or without deeply eosinophilic cytoplasm. Epithelial degenerative changes were separately estimated in the upper and lower halves of the mucosa.

The amounts of inflammatory cells, including neutrophilic and eosinophilic granulocytes, and mononuclear inflammatory cells (lymphocytes and plasma cells) were scored separately in the surface epithelium, lamina propria, subserosal fat and peritoneal surface. The latter included 20-50 μm of submesothelial connective tissue. Scoring was based on visual analogue scales. In each case, the score was determined according to the dominant pattern as ranging from absent (score 0) through slight (score 1) and moderate (score 2) to heavy increase (score 3) (see the original manuscript III).

The numbers of lymphatic follicles with germinal centres were counted and presented as counts/four high-power (40 x objective) fields (total area 3.2 mm²). The dilatation of lymphatic vessels was scored as being absent or present. The presence of leukocyte margination and thrombosis in blood vessels were also noted.

In some cases, eroded surface was covered by yellowish brown bile-like material, which occasionally infiltrated into the adjacent degenerated tissue. This type of mucosal bile penetration associated with destruction of mucosal surface was designated as bile infiltration, and the proportion (%) of surface with this change was estimated and recorded together with the maximum penetration into the gallbladder wall structures.

The proportion (%) of mucosal area showing Rokitansky-Aschoff sinuses (herniation of epithelium into lamina propria, smooth muscle or subserosal connective tissue) was estimated, and the presence of inflammatory cells in the sinus region was scored as absent (0) or mildly (1), moderately (2) or heavily (3) increased.

The presence of necrosis in the muscle layer and the proportion (%) of necrotic muscle area and depth were evaluated. The depth of necrosis was estimated by using the proportion of thickness showing necrotic change. The percentage of subserosal fat tissue showing necrosis was similarly estimated.
4.7 Immunohistochemical examination (IV)

In study IV, the gallbladder samples had been fixed in neutral buffered formalin and embedded in paraffin. The expression of the epithelial tight junction proteins claudin-1, -2, -3 and -4, occludin, ZO-1 and E-cadherin were studied by immunohistochemistry. The primary antibodies were purchased from Zymed Laboratories Inc. (South San Francisco, CA, USA). The antibodies were used according to the manufacturer’s recommendations, and they consisted of polyclonal rabbit anti-claudin-1, monoclonal mouse anti-claudin-2, polyclonal rabbit anti-claudin-3, monoclonal mouse anti-claudin-4, polyclonal rabbit anti-occludin, polyclonal rabbit anti-ZO-1 and monoclonal mouse anti-E-cadherin. The sections were pretreated with TRIS/EDTA, except in the case of occludin, for which the sections were pretreated with pronase. The sections for ZO-1 immunohistochemistry were heated in a microwave oven for 15 minutes before the application of the primary antibodies. After incubation with the primary antibodies, a secondary antibody and the PowerVision+ Poly-HRP IHC kit (ImmunoVision Technologies, Brisbane, CA, USA) were applied. The colour was developed by diaminobenzidine using the DAKO EnVision Detection kit (DakoCytomation, Denmark). Negative control stainings were carried out by substituting non-immune rabbit or mouse serum and PBS for the primary antibodies. There were some individual specimens missing for technical reasons in each group, and the final amount of studied specimen was therefore 26-30/30 in the AAC group, 19-21/21 in the ACC group and 8-9/9 in the control group.

The results are presented as proportions (percentage) of epithelial cells expressing the studied antigens. Membrane-bound and cytoplasmic immunoreactivities were separately assessed. Staining intensity was categorized into four classes for analysis (0=absent, 1=weak, 2=moderate, 3=strong). Immunoreactivity in the vascular endothelial cells in the gallbladder wall was analyzed separately. Distinctive findings in the samples were recorded as well.

4.8 Statistical methods

The data are expressed as percentage, mean and standard deviation (SD), median and interquartile range (IQR, 25th, 75th percentiles) or as median with minimal and maximal values.

The statistical analyses were performed using the SPSS® for Windows software (versions 10.0-12.0.1, SPSS Inc., Chicago, IL). Categorical data were analyzed using X² or Fisher’s exact test, as appropriate. Unpaired Student’s t-test was used to compare continuous data in study I. The assessment of changes within the total SOFA scores and within the individual organ system SOFA in study II was performed using Friedman’s test. If significant changes were found, comparisons within scores were performed using Wilcoxon’s test for paired comparisons. Kruskall-Wallis test was used to describe the histological and immunohistochemical differences between the AAC, ACC and control groups in study III and IV, while Mann-Whitney U-test was applied when comparing the parameters between the AAC and ACC groups. P < 0.05 was considered significant.
5 Results

5.1 Patients

Altogether 3984 (I) and 4200 (II) patients were treated in the ICU of Oulu University Hospital between 1.1.2000-31.12.2001 and 1.1.2003-31.12.2004, respectively. Forty percent of the patients were admitted after cardiac surgery, and the rest were general surgical, neurosurgical or medical patients. Seventy-three patients had operatively treated AAC during these time periods. There were 39 patients during 2000-2001 (study I) and 34 during 2003-2004 (study II). Ten of the patients in the years 2003-2004 had additional intestinal ischemia or perforation during the laparotomy. These ten patients were excluded, to recruit merely the AAC patients included in study II. Two-thirds of the studied AAC patients were men. The gallbladder was ischemic and gangrenous in 44% and 54% of the cases, respectively.

In study III, five of the AAC samples, two of the ACC samples and one of the control samples could not be analyzed due to extensive autolysis of the samples, and the final numbers of samples were 34 in the AAC group, 28 in the ACC group and 14 in the control group.

Patient characteristics are presented in Table 2. Less than half of the patients had ischemic heart disease (41%), universal arteriosclerosis (23%), alcohol abuse (20%) or diabetes (10%) as underlying chronic diseases (I). Preoperatively, more than half (56%) of the patients were on enteral feeding and, and 90% of the patients received propofol and oxycodone infusions for sedation and pain relief. Fifty-four percent of the patients received steroid medication (54%) either for chronic disease or for sepsis (I).
Table 2. Patient characteristics in study groups I-IV, mean (SD) or median [IQR].

<table>
<thead>
<tr>
<th>Variable</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AAC</td>
<td>AAC</td>
<td>ACC</td>
<td>Control</td>
</tr>
<tr>
<td>Patients, n</td>
<td>39</td>
<td>24</td>
<td>34</td>
<td>28</td>
</tr>
<tr>
<td>Age, years</td>
<td>57 (15)</td>
<td>59 (16)</td>
<td>58 (14)</td>
<td>60 (12)</td>
</tr>
<tr>
<td>Gender, F/M</td>
<td>15/24</td>
<td>6/18</td>
<td>12/22</td>
<td>11/17</td>
</tr>
<tr>
<td>APACHE II</td>
<td>25 (6)</td>
<td>25 (6)</td>
<td>24 (6)</td>
<td></td>
</tr>
<tr>
<td>SAPS II</td>
<td>48 (13)</td>
<td>44 (12)</td>
<td></td>
<td>47 (12)</td>
</tr>
<tr>
<td>ICU LOS total</td>
<td>16</td>
<td>16</td>
<td>[10-27]</td>
<td>[9-31]</td>
</tr>
<tr>
<td>before surg</td>
<td>7 [2-13]</td>
<td>5 [1-12]</td>
<td>8 [3-14]</td>
<td></td>
</tr>
</tbody>
</table>

ad, admission; surg, surgery; LOS, length of stay. AAC patients in study IV are subgroup of AAC patients in study III. ACC patients in study IV are subgroup of ACC patients in study III.

5.2 Incidence of AAC (I, II)

A total of 73 patients out of 8184 had operatively treated AAC during the study periods 2000-2001 (39) and 2003-2004 (34), giving an incidence rate of 0.9 % (0.8 % and 1.0 %, p=0.481) and 6.7 %. among long-stayers (> 5 days). The incidence of mere AAC without other intra-abdominal pathology in study II was 0.6%.

5.3 Admission diagnoses (I, II)

The most common admission diagnoses were sepsis (41-42%) followed by cardiovascular surgery (23-30%). The admission diagnoses are presented in Table 3.

Table 3. Admission diagnoses of patients with AAC in studies I and II, n (%).

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>I (n=39)</th>
<th>II (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sepsis</td>
<td>16 (41)</td>
<td>10 (42)</td>
</tr>
<tr>
<td>Cardiovascular surgery</td>
<td>9 (23)</td>
<td>7 (30)</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>5 (13)</td>
<td></td>
</tr>
<tr>
<td>Trauma</td>
<td>3 (8)</td>
<td>3 (12)</td>
</tr>
<tr>
<td>Heart failure</td>
<td>2 (6)</td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal bleeding</td>
<td>1 (3)</td>
<td></td>
</tr>
<tr>
<td>Fat embolism</td>
<td>1 (3)</td>
<td></td>
</tr>
<tr>
<td>SAV/ICH/Encephalitis</td>
<td>1 (3)</td>
<td>3 (12)</td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>1 (3)</td>
<td>1 (4)</td>
</tr>
</tbody>
</table>

SAV, subarachnoid hemorrhage; ICH, intracranial hemorrhage
5.4 Diagnostic findings (I, II)

The diagnostic findings are presented in Table 4. The sensitivity of US was 80 and 72% in the studies I and II. Two of the eleven patients with normal or undiagnostic US findings had necrosis in their gallbladder. Computed tomography had sensitivity of 58 and 50% in the studies I and II. Five of the eight patients with normal or undiagnostic CT findings had necrosis in their gallbladder.

<table>
<thead>
<tr>
<th>Study</th>
<th>US positive/negative</th>
<th>CT sensitivity</th>
<th>Pain, tenderness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study I</td>
<td>24/6</td>
<td>80%</td>
<td>21/18</td>
</tr>
<tr>
<td>Study II</td>
<td>13/5</td>
<td>72%</td>
<td>7/5</td>
</tr>
</tbody>
</table>

Fever was an uncommon symptom in AAC patients, half of the patients had normal WBC counts, and the median (25th, 75th percentiles) C-reactive protein value on the day of cholecystectomy was 169 mg/L (68, 245). In study II, the liver function tests on the day of cholecystectomy did not differ from the test results of all ICU patients during the study period 2003-2004 (Table 5). Positive bile cultures were found in 12.8 and 12.5% of the patients in the studies I and II.

Table 5. Median (25th, 75th percentile) values of liver function tests of AAC patients on the day of cholecystectomy (AAC(II)) and median (25th, 75th percentile) values of all liver function tests of all patients during years 2003-04.

<table>
<thead>
<tr>
<th>Patients</th>
<th>ALAT</th>
<th>ASAT</th>
<th>Bilirubin</th>
<th>AFOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAC (II)</td>
<td>43 (24, 68)</td>
<td>48 (28, 117)</td>
<td>17 (9, 35)</td>
<td>152 (98, 328)</td>
</tr>
<tr>
<td>n=23</td>
<td>n=23</td>
<td>n=23</td>
<td>n=23</td>
<td></td>
</tr>
<tr>
<td>All (2003-2004)</td>
<td>33 (17, 73)</td>
<td>46 (24, 92)</td>
<td>12 (7, 22)</td>
<td>144 (90, 245)</td>
</tr>
<tr>
<td>n=13053</td>
<td>n=12972</td>
<td>n=9142</td>
<td>n=12717</td>
<td></td>
</tr>
</tbody>
</table>

ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; AFOS, alkaline phosphatase.

5.5 Organ system dysfunction (I, II)

Ninety percent of the patients in study I had respiratory failure on the day of cholecystectomy. Cardiovascular (64%), renal (41%) and coagulation (36%) dysfunction were also common. There was significantly less cardiovascular dysfunction on the day of cholecystectomy than on admission, while renal dysfunction showed an opposite trend. Figure 5.
Fig. 5. Percentage of patients with associated organ failures on admission and on the day of cholecystectomy, study I.

5.5.1 Total SOFA scores

In study I, the median (IQR) total SOFA scores on admission and on the day of surgery were 11 (8-14) and 12 (8-14), respectively. In study II, the median (IQR) total SOFA score three days before cholecystectomy was 7.5 (1.3-8.0), and it significantly increased to 10.5 (8.3-13.0) by the day of cholecystectomy (see Figure 6). After the operation, the score decreased to 5.5 (3.3-10.8) by the seventh postoperative day.
5.5.2 Organ-specific SOFA scores

Cardiovascular and respiratory scores increased significantly from the third preoperative day to the day of surgery (II). After the operation, these organ system dysfunctions decreased. A significant increase was also seen in the renal and coagulation SOFA scores preoperatively. After the operation, renal dysfunction did not reach significant improvement until the 7th postoperative day. Coagulation disorder had a tendency to improve after the operation. Hepatic and neurologic dysfunctions did not show any remarkable changes before or after cholecystectomy. (Figure 7).

In study I, the median (IQR) values of the cardiovascular, respiratory and neurologic SOFA scores on the day of surgery were 3 (1-4), 3 (3-3) and 1 (0-2), respectively. The values for renal, hepatic and coagulation disorders were 2 (0-3), 1 (0-2) and 2 (0-3), respectively. The median values for cardiovascular, respiratory and neurological dysfunctions were the same, while the renal, hepatic and coagulation SOFA scores were higher than in study II.
Fig. 7. Median (IQR) SOFA scores for individual organ systems 3 (D-3), 2 (D-2) and 1 day (D-1) before cholecystectomy, on the day of cholecystectomy (D0) and 1 (D+1), 2 (D+2), 3 (D+3) and 7 days (D+7) after cholecystectomy, study II.
5.6 Mortality (I, II)

Nine out of 39 patients (23%, I) and three out of 24 (13%, II) patients died during their ICU stay after cholecystectomy. The hospital mortality rate was 43 % (I,II). Multiple organ failure (MOF) was the most common cause of death (65 % in study I, 80 % in study II). There was no statistically significant difference in age, gender, BMI, length of ICU stay or admission APACHE II, SAPS II and SOFA scores between the survivors and non-survivors (I). The non-survivors had, however, significantly higher SOFA scores on the day of surgery than the survivors (12.9 vs.9.5, P=0.007).

The median (IQR) survival of the non-survivors after cholecystectomy was 9 (2.5-17) days in study I. In study II, all deaths occurred later than during the first post-operative week, the median (IQR) being 33 days (12-50).

5.7 Histological findings (III)

5.7.1 Epithelial changes

There were significantly fewer degenerated epithelial cells in AAC than in ACC, and the extent of epithelial defect (erosion) was significantly less in AAC than in ACC (Table 6). Epithelial necrosis was present in 52.9 % (18/34) of the AAC and in 70.4 % (19/27) of the ACC samples (P=0.196) without any difference in the extent of necrosis. The control samples showed no epithelial degenerative changes or necrosis (Fig 8 A,B).

Table 6. Epithelial changes in the AAC, ACC and control groups. Both the occurrence (number of cases, % in parentheses) of cases showing an abnormality and the extent of this abnormality (median (min-max)) are indicated.

<table>
<thead>
<tr>
<th>Epithelial change</th>
<th>AAC</th>
<th>ACC</th>
<th>Controls</th>
<th>P</th>
<th>P AAC vs. ACC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degeneration, upper half of mucosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occurrence</td>
<td>29/34 (85)</td>
<td>26/27 (96)</td>
<td>0/14 (0)</td>
<td>&lt;0.0001</td>
<td>0.214</td>
</tr>
<tr>
<td>Extent</td>
<td>5 (0-80)</td>
<td>15 (0-100)</td>
<td>0 (0-0)</td>
<td>&lt;0.0001</td>
<td>0.005</td>
</tr>
<tr>
<td>Degeneration, lower half of mucosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occurrence</td>
<td>9/34 (26)</td>
<td>23/27 (85)</td>
<td>0/14 (0)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Extent</td>
<td>0 (0-40)</td>
<td>10 (0-100)</td>
<td>0 (0-0)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Defect area</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occurrence</td>
<td>28/34 (82)</td>
<td>27/28 (96)</td>
<td>0/14 (0)</td>
<td>&lt;0.0001</td>
<td>0.116</td>
</tr>
<tr>
<td>Extent</td>
<td>2 (0-100)</td>
<td>55 (0-100)</td>
<td>0 (0-0)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
5.7.2 **Inflammatory cells**

Neutrophils were detectable less frequently in AAC than in ACC in all layers, with the exception of subserosal fat, where the difference did not reach statistical difference (Fig 8 C-F). Neutrophils were not present in any of the control samples. Mononuclear inflammatory cells were also less frequently found in AAC than in ACC in subserosal fat and the mesothelial surface, while eosinophilic granulocytes were seen significantly less only in subserosal fat. No significant differences between the AAC, ACC and control groups were seen in mononuclear and eosinophilic granulocytes in the gallbladder epithelium and lamina propria. (See Table 2 in the original paper III, page 490.)

5.7.3 **Lymphatic follicles and vascular changes**

There were no differences in the presence of capillary thromboses between the AAC and ACC groups. In contrast, leukocyte margination as well as lymphatic vessel dilatation were significantly more common in AAC compared to ACC (Fig 8 H). No capillary thrombosis, leucocyte margination or lymphatic dilatation was found in the control samples.

5.7.4 **Bile infiltration and Rokitansky-Aschoff sinuses**

Bile infiltration into the gallbladder mucosa was a significantly more common phenomenon in the AAC than in the ACC samples (50 % vs. 14 %, P=0.004), and the extent of mucosal surface covered with bile infiltration was also larger in AAC (7 % vs. 0.9 %, P=0.004) (Fig 8 E,F). Bile infiltration reached into the muscular layer in 35% of the samples in the AAC group, while in the ACC group bile infiltration was always restricted to the mucosal layer (P= 0.002). No bile infiltration was seen in the control samples.

Rokitansky-Aschoff sinuses were found similarly in the AAC, ACC and control samples, and inflammatory cells in these sinuses were detected to the same extent in the AAC and ACC samples (12% and 15 %, respectively), while there were no inflammatory cells in the sinuses of the control samples.

5.7.5 **Necrosis in the muscle layer and subserosal adipose tissue**

The occurrence, extent and depth of smooth muscle necrosis were less marked in AAC than in ACC. Necrosis in adipose tissue seemed to be a more common phenomenon in ACC, but the difference did not reach statistical significance. No necrosis was seen in the control samples either in smooth muscle or in the adipose layer. (Table 7).
Table 7. Gallbladder wall necrosis in the AAC, ACC and control groups. Both the occurrence of necrosis (number of cases, % in parentheses) and the estimated extent (% of surface area) and depth (% of total thickness) of necrosis are indicated (median (min-max)).

<table>
<thead>
<tr>
<th>Site of necrosis</th>
<th>AAC</th>
<th>ACC</th>
<th>Controls</th>
<th>P</th>
<th>P AAC vs. ACC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle layer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occurrence</td>
<td>8/34 (24)</td>
<td>16/28 (57)</td>
<td>0/14 (0)</td>
<td>&lt;0.0001</td>
<td>0.009</td>
</tr>
<tr>
<td>Extent</td>
<td>0 (0-95)</td>
<td>7.5 (0-90)</td>
<td>0 (0-0)</td>
<td>&lt;0.0001</td>
<td>0.002</td>
</tr>
<tr>
<td>Depth</td>
<td>0 (0-100)</td>
<td>80 (0-100)</td>
<td>0 (0-0)</td>
<td>&lt;0.0001</td>
<td>0.007</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occurrence</td>
<td>8/34 (24)</td>
<td>12/28 (43)</td>
<td>0/14 (0)</td>
<td>0.011</td>
<td>0.172</td>
</tr>
<tr>
<td>Extent</td>
<td>0 (0-70)</td>
<td>0 (0-60)</td>
<td>0 (0-0)</td>
<td>0.011</td>
<td>0.096</td>
</tr>
</tbody>
</table>
Fig. 8. Photomicrographs showing the histology of normal gallbladder and that of AAC and ACC. A, B, Histologically normal gallbladder. C, D, ACC with purulent inflammation destroying the mucosa (arrowheads) and the muscle layer (arrows). E, F, AAC with brownish bile impregnation of the eroded mucosa (arrowheads) and muscle layer (arrows). G, Detail of the mucosal surface in AAC showing an epithelial defect with a few necrotic (arrowheads) or degenerate (arrows) epithelial cells. H, mucosa in AAC showing a dilated lymphatic vessel (L) and a venule (V) with margination of leucocytes.
5.8 Immunohistochemistry (IV)

We found both membrane-bound and cytoplasmic immunoreactivity for all studied TJ proteins in our gallbladder samples. The percentages of epithelial cells with positive staining in the different groups are presented in the Figures 9A, 9B, 10A and 10B, and the distribution of staining intensity in Table 8 and 9. In endothelial cells, marked immunoreactivity was detectable for ZO-1 and occludin.

5.8.1 Claudins

The immunoreactivity for the claudins -1, -3 and -4 was mainly membranous (Fig. 9A). Cytoplasmic staining was also detectable, and there was some granular staining in the apical regions of the cytoplasms, especially for the claudins -1, -2 and -3. The immunoreactivity for claudin-4 in epithelial cells seemed more heterogenous than that for the other claudins.

The membrane-bound staining for the claudins -1, -3 and -4 was present in practically all epithelial cells in the AAC and the control samples, but was less extensive in ACC (Fig. 9A). The cytoplasmic staining for claudin-1, -3 and -4 was clearly less extensive than membrane-bound staining in all groups (Fig. 9B). In ACC the intensity of cytoplasmic staining was weaker than in AAC for claudin-3 and -4 and weaker than in the control group for claudin-1 and -4.

The expression of claudin-2 differed from the other claudins; in the control and AAC groups, only about half of the cells showed membrane-bound or cytoplasmic staining, but a significantly greater part of cells stained positively in ACC (Fig. 8), and the intensity of staining was also stronger in ACC than in AAC or in the controls (Table 8).
Fig. 9. Median (IQR) percentages of cells staining positively for claudins. A membrane-bound staining, B cytoplasmic staining. CL 1 = claudin-1, CL 2 = claudin-2, CL 3 = claudin-3, CL 4 = claudin-4.

Table 8. Intensity of membranous and cytoplasmic staining of the claudins 1-4 (CL 1-4) assessed as the number of patients having each intensity score (0/1/2/3).

<table>
<thead>
<tr>
<th>Variable</th>
<th>AAC (n=26-30)</th>
<th>ACC (n=19-21)</th>
<th>Control (n=8-9)</th>
<th>P all</th>
<th>P AAC vs. ACC</th>
<th>P AAC vs. control</th>
<th>P ACC vs. control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membranous</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL 1</td>
<td>0/14/13/2</td>
<td>0/15/5/1</td>
<td>0/3/3/3</td>
<td>0.086</td>
<td>0.266</td>
<td>0.142</td>
<td>0.073</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL 2</td>
<td>0/11/11/6</td>
<td>0/2/9/9</td>
<td>0/6/2/1</td>
<td>0.028*</td>
<td>0.049*</td>
<td>0.471</td>
<td>0.011*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL 3</td>
<td>0/9/14/3</td>
<td>0/12/7/1</td>
<td>0/4/2/3</td>
<td>0.132</td>
<td>0.272</td>
<td>0.173</td>
<td>0.143</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL 4</td>
<td>0/4/15/8</td>
<td>0/5/15/1</td>
<td>0/1/5/3</td>
<td>0.176</td>
<td>0.123</td>
<td>1.000</td>
<td>0.192</td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>CL 1</td>
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<td>1/15/5/0</td>
<td>0/1/6/2</td>
<td>0.002*</td>
<td>0.06</td>
<td>0.017*</td>
<td>0.002*</td>
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<td>CL 2</td>
<td>0/15/11/2</td>
<td>0/3/11/6</td>
<td>0/6/2/1</td>
<td>0.016*</td>
<td>0.011*</td>
<td>0.515</td>
<td>0.026*</td>
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<td>0.032*</td>
<td>0.436</td>
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<td>CL 4</td>
<td>0/0/10/17</td>
<td>2/1/12/6</td>
<td>0/0/5/4</td>
<td>0.113</td>
<td>0.026*</td>
<td>0.443</td>
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5.8.2 Occludin, ZO-1 and E-cadherin

The extent of membrane-bound positivity for occludin was similar in the control and AAC groups but significantly diminished in the ACC group (Fig. 10A). The cytoplasmic positivity for occludin was significantly less extensive both in AAC and in ACC compared to the control group (Fig. 10B). There were no differences in the intensity of staining for occludin between the groups (Table 9).

ZO-1 expression was most evident in the apical regions of epithelial cells. The control group had an intensive membrane-bound immunoreaction for ZO-1, while positively stained cells were less abundant in AAC and especially in ACC (Fig. 10A). The intensity of the membrane-bound immunostaining of ZO-1 was stronger in AAC than in ACC. Cytoplasmic ZO-1 positivity was present in half of the cells in all groups, and there were no significant differences between the groups (Fig. 10B). Staining for E-cadherin was widespread both as membrane-bound and as cytoplasmic, and there were no remarkable differences between the groups.
Fig. 10. Median (IQR) percentages of cells staining positively for occludin, ZO-1 and E-cadherin. A membrane-bound staining, B cytoplasmic staining. OCCL = occludin, ECAD = E-cadherin.
Table 9. Intensity of membranous, cytoplasmic and endothelial staining of the occludin, ZO-1 and E-cadherin assessed as the number of patients having each intensity score (0/1/2/3).

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<tr>
<th>Variable</th>
<th>AAC (n=26-30)</th>
<th>ACC (n=19-21)</th>
<th>Control (n=8-9)</th>
<th>P all</th>
<th>P AAC vs. ACC</th>
<th>P AAC vs. control</th>
<th>P ACC vs. control</th>
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<td>Occludin</td>
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<td>0/2/9/10</td>
<td>0/1/1/7</td>
<td>0.348</td>
<td>0.584</td>
<td>0.251</td>
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<td>ZO-1</td>
<td>0/7/17/5</td>
<td>0/12/5/4</td>
<td>0/3/3/3</td>
<td>0.079</td>
<td>0.028</td>
<td>0.359</td>
<td>0.520</td>
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<td>E-cadherin</td>
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<td>0/3/8/8</td>
<td>0/3/2/3</td>
<td>0.395</td>
<td>0.520</td>
<td>0.285</td>
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<tr>
<td>Occludin</td>
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<td>0/1/6/2</td>
<td>0.699</td>
<td>0.422</td>
<td>1.000</td>
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<td>4/12/3/2</td>
<td>1/3/4/1</td>
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<td>0.144</td>
<td>0.772</td>
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<td>0.102</td>
<td>0.034</td>
<td>0.293</td>
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<td>Occludin</td>
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<td>1/1/18/1</td>
<td>0/0/8/1</td>
<td>0.618</td>
<td>0.607</td>
<td>0.395</td>
<td>1.000</td>
</tr>
<tr>
<td>ZO-1</td>
<td>0/2/12/16</td>
<td>0/2/15/4</td>
<td>0/0/4/5</td>
<td>0.089</td>
<td>0.035</td>
<td>1.000</td>
<td>0.159</td>
</tr>
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</table>

5.8.3 Endothelial cells

Endothelial cells had extensive immunoreaction for ZO-1 in all groups, and the median (25th, 75th percentile) percentage of positive cells was 100 (95, 100) in the control group, 100 (100, 100) in AAC and 100 (90, 100) in ACC. The corresponding figures for occludin were 35 (30, 60) in the control group, 30 (20, 40) in AAC and 20 (10, 40) in ACC. The differences between the groups were not statistically significant.
6 Discussion

6.1 General discussion

The study population of 73 AAC patients was relatively small, but it is still one of the biggest series of AAC in ICU patients ever reported. A multi-centre study would have yielded a higher number of patients, but the single-centre approach allowed a more uniform treatment protocol.

The histopathological and immunohistological investigations were blinded by using normal gallbladders as controls. All slides were examined by at least two investigators, one of whom was an experienced pathologist.

It is not certain that we found and treated all AAC patients during this period. There might have been some patients with milder disease who recovered following conservative treatment. Systematic retrospective analysis of gallbladders of all patients who died during their ICU stay was not possible because the gallbladder was not routinely studied or stored at autopsy.

6.2 Incidence and concomitant factors of AAC

The incidence of AAC in our medical-surgical ICU patients was 0.9 %. This figure is in concordance with the earlier reports, where incidence rates have varied from 0.2 % to 4 % depending on the type of the ICU population (Savino et al. 1985, Kallifas et al. 1998, Rady et al. 1998 Mariat et al. 2000). However, the incidence among long-stayers was higher than has earlier been reported in cardiovascular surgery patients (6.7% vs. 3 %) (Rady et al. 1998). Infection was the most common admission diagnosis in our patients, and only one-third of the patients were postoperative or trauma patients. This shows that the occurrence of AAC is not limited to cardiovascular and postoperative patients, but is also possible in any critically ill patients, and that sepsis as such is an important predisposing factor.

Contrary to earlier reports, total parenteral nutrition, diabetes or alcohol abuse were not predominant concomitant factor in our patient series. The high proportion of patients
with mechanical ventilation probably reflected the severity of the underlying illness rather than the relationship of AAC with mechanical ventilation.

6.3 Diagnostic and treatment challenges

Only half of our patients presented with pain and tenderness in the right upper abdominal quadrant, and only 13% of them had fever. None of the commonly used laboratory tests were good markers of AAC. In harmony with the earlier reports, US or CT were unable to reliably identify AAC (Fidler et al. 1996, Molenat et al. 1996, Boland et al. 2000, Puc et al. 2002). Eleven out of 48 patients had no ultrasonographic findings of AAC, though the disease was diagnosed at surgery. Eight out of 18 examined patients did not have CT findings of AAC, and yet five of them had a necrotic gallbladder. However, US continues to be the basic diagnostic tool in AAC because it is non-invasive, inexpensive and portable, and follow-up scans are easy to obtain.

In practice, thorough clinical assessment, US findings (repeated if not diagnostic), arterial and mixed venous blood gas analysis, lactate measurement and trends in cardiovascular, respiratory, renal, hematologic, hepatic and central nervous system functions are the basic tools for the diagnosis of AAC.

Splanchnic hypoperfusion and ischemia probably have a pivotal role in the pathogenesis of AAC. In spite of the huge interest in splanchnic circulation during the past two decades, we still lack practical methods to monitor splanchnic perfusion or oxygenation. Gastric tonometry was developed to assess splanchnic perfusion by measuring gastric intramucosal pH or the mucosal–arterial pCO₂ gap, and it has been widely used in research and also clinically (Kuttila et al. 1994, Parviainen 1997, Juvonen et al. 1999). There are, however, problems in the interpretation of the findings, and the prognostic value of tonometry is not clear (Boldt 2002). In addition, enteral feeding, which is recommended to be started early in the case of critically ill patients, may disturb its reliability (Ala-Kokko & Laurila 1999).

If there is a suspicion of severe AAC with exacerbation of multiple organ dysfunction and rising SOFA scores without any other obvious focus with or without abdominal pain and distention, cholecystectomy should be considered. Before operation, if additional intra-abdominal or thoracic pathology is suspected, CT is the preferred radiologic imaging method instead of US.

There is currently some controversy as to the treatment of choice of AAC (Melin et al. 1995, Boggi et al. 1999, Shapiro 1999, McChesney et al. 2003). To date, there are no prospective controlled studies available to compare the conservative, percutaneous and surgical treatments of AAC. Percutaneous cholecystostomy has yielded good results in primary calculous disease, but the results have been poorer in ICU long-stayers with multiple organ dysfunction and SIRS (Melin et al. 1995, Hamy et al. 1997, Boggi et al. 1999, Davis et al. 1999, Spira et al. 2002, Sosna et al. 2004). Five of our patients initially underwent US-guided percutaneous cholecystostomy, but all of them later required operative treatment.

It is generally accepted that AAC complicated by gangrene or perforation is a definite indication for cholecystectomy and cannot be treated by percutaneous drainage. In our
series, gangrene and ischemic lesions of the gallbladder were present in half of the patients. Similar results have been reported earlier, with gangrene and necrosis of the gallbladder in 59% and 63% of critically ill AAC patients (Shapiro et al. 1994, Kalliafas et al. 1998). Because of the high incidence of gangrenous gallbladder and a lack of correlation between symptomatology and gangrene, we strongly agree with the opinion that cholecystectomy is the definitive treatment of severe AAC (Shapiro 1999). In our series, surgery was well tolerated, and no mortality was associated with cholecystectomy.

6.4 Multiple organ failure and outcome

Multiple organ dysfunction syndrome refers to the progressive impairment of two or more organ systems as a response to a severe illness or injury, and it is the most important factor contributing to mortality and prolongation of intensive care (Beal & Cerra 1994, Moreno et al. 1999). The sequential organ failure assessment score (SOFA) was developed to describe objectively the severity of multiorgan dysfunction (Vincent et al. 1996, Vincent et al. 1998). Individual organ functions (respiratory, cardiovascular, renal, coagulation, hepatic and neurological) are graded from normal (grade 0) through mild dysfunction to severe organ failure (grade 4). The higher the SOFA score, the more severe the organ dysfunction (Moreno et al. 1999). Although the SOFA score was not developed to predict hospital mortality, the ability of the daily maximum SOFA score and the total maximum SOFA score to distinguish between survivors and non-survivors is relatively good, i.e. comparable to APACHE III (Pettilä et al. 2002).

Multiple organ failure was common among the present AAC patients. The patients were severely ill on admission into ICU, the mean (SD) APACHE II being 25.5 (6.4) and the mean (SD) total SOFA score 10.2 (3.5). At this stage, 90% of the patients had respiratory and 79% cardiovascular dysfunction, and coagulation dysfunction was also quite common. Multiple organ dysfunction continued to progress during the ICU treatment before cholecystectomy. The mean total, cardiovascular, respiratory, renal and coagulation SOFA scores increased as a marker of multiple organ failure. On the day of cholecystectomy, most patients had three or more organ failures.

The choice of the surgical approach as treatment of AAC is supported by our finding that removal of the diseased gallbladder was followed by remarkable improvement in individual and total SOFA scores by the seventh postoperative day. The cardiovascular and respiratory functions were the most sensitive organ functions to be disturbed and also the first functions to improve. Renal dysfunction both developed and improved more slowly than the cardiovascular or respiratory functions. Coagulation disorder also increased and decreased before and after cholecystectomy, respectively, while the hepatic and neurologic organ systems did not show any significant change.

The hospital mortality of AAC patients was high (43%), which reflects high severity of the underlying illnesses. Our mortality was quite similar to that reported by Kalliafas et al. (1998), but our patients were more severely ill according to the APACHE II scores (25 vs. 17).
6.5 Histological findings

Although AAC and ACC have previously been considered similar diseases with or without stones (Glenn 1979, Myers et al. 1988, Jessurun & Albores-Saavedra 2002), we found differences in the histologic characteristics of these two types of cholecystitis.

Leucocyte margination of blood vessels and lymphatic dilatation were significantly more common in AAC than in ACC, which suggests involvement of ischemia- and reperfusion-mediated injury (Savoca et al. 1990a, Taoka 1991). However, we could not confirm the earlier observations that capillary thromboses are typical of AAC (Warren 1992, Hakala et al. 1997). Interestingly, a possible sign of increased interstitial oedema, i.e. the presence of focal lymphatic dilatation, was clearly associated with AAC in our series. Collectively, these findings suggest that circulatory disturbances could play a role in the pathogenesis of AAC.

Bile infiltration into the gallbladder mucosa was significantly more abundant and extended deeper in AAC than in ACC. Epithelial defects in the gallbladder mucosa were more extensive in ACC, indicating that factors other than epithelial ulceration explain the development of bile infiltration in AAC. Increased epithelial permeability might explain this finding.

Mucosal infiltration of mononuclear inflammatory cells was similar in AAC, ACC and control gallbladders, suggesting that some mucosal infiltration of chronic inflammatory cells can be considered physiological. However, the presence of mucosal neutrophils and inflammation in the deeper parts of the gallbladder mucosa clearly showed differences between cholecystitis patients and controls and, more importantly, between AAC and ACC. Extensive epithelial degeneration and detachment were accompanied by an intensive mucosal neutrophil reaction in ACC and, to a significantly lesser extent, in AAC. Similarly, in the muscle layer, necrosis up to half of its thickness was seen in the cases with ACC, while muscle necrosis in AAC was rare and less extensive. Our study suggests that, although necrosis is present in both types of cholecystitis, it is less severe in AAC. A similar difference was apparent in subserosal adipose tissue and the peritoneal surface, where an acute inflammatory reaction was predominantly present in ACC and, to a lesser extent, in AAC. All these differences in the patterns of inflammation and necrosis further suggest that AAC and ACC are pathogenetically different. These results suggest that AAC is a manifestation of systemic critical illness, whereas ACC is typically a local, predominantly purulent and necrotic inflammatory disease of the gallbladder.

6.6 Tight junction proteins in AAC and ACC

The expression of tight junctions has not been previously investigated in critically ill patients or in normal gallbladders. In the present study, TJ proteins were widely expressed in the membrane of the epithelial cells of normal gallbladders, with the exception of claudin-2, which was detectable only in half of the cells. Cytoplasmic expression of all studied TJ proteins was lower than membranous expression.

There were qualitative and quantitative differences in TJ protein stainings between the AAC, ACC and normal gallbladders. In AAC, significant down-expression of
cytoplasmic occludin and claudin-1 was seen in the gallbladder epithelium. Earlier, it has been shown that proinflammatory cytokines, tumor necrosis factor alpha and interferon gamma down-regulated the expression of occludin in human intestinal cell culture, and that endotoxemia caused a marked decrease in the expression of occludin in intestine, liver and lung in mice (Mankertz et al. 2000, Han et al. 2004a-c). In an animal study, two-hit injury of burn and infection has been shown to decrease the expression of intestinal occludin concomitantly with an increase in intestinal permeability (Samonte et al. 2004).

ACC was characterised by more extensive down-regulation of the claudin-1, -3, -4, occludin and ZO-1 proteins and up-regulation of claudin-2. Similar results have been reported for intense local purulent inflammation in IBD and colitis ulcerosa, and claudin-2 up-regulation seems to be a typical response in inflammatory reactions involving neutrophilic leucocytes (Kucharzik et al. 2001, Prasad et al. 2005). In comparison with AAC, the more intensive and partly divergent reaction in ACC could be caused by high concentrations of local proinflammatory factors and local microbial invasion, which contrast with the presumably lower concentrations of systemic proinflammatory mediators with vascular entry into the gallbladder mucosa in AAC. Additionally, hypoxic conditions in AAC might further modify the gallbladder response.
7 Conclusions

1. The incidence of surgically treated AAC was 0.9% in the whole ICU patient series and 6.7% among the long-stayers (>5 days). Infection was the most common admission diagnosis, followed by cardiovascular surgery. The sensitivity of US in diagnosing AAC was 80 and 72% in the studies I and II, and that of CT was remarkably poorer, being 58% and 50%, respectively. Liver function tests, WBC count and C-reactive protein were not reliable tests in diagnosing AAC. AAC was associated with multiple organ dysfunction. Sixty-four percent of the patients had three or more failing organs on the day of cholecystectomy. The hospital mortality of these patients was 43%.

2. Open cholecystectomy improved the deteriorating multiple organ dysfunction. The change was most obvious in the cardiovascular and respiratory functions.

3. AAC and ACC were histopathologically different. Typical features in AAC were bile infiltration, lymphatic dilatation and leucocyte margination of blood vessels, while epithelial degeneration and defects, widespread occurrence of inflammatory cells and extensive and deep muscle layer necrosis were typical features in calculous cholecystitis.

4. Tight junction proteins (claudin-1, -2, -3, -4, occludin, ZO-1, E-cadherin) were widely expressed in normal and diseased gallbladder. There were remarkable qualitative and quantitative differences in the expression of these TJ proteins in AAC and ACC.
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Han X, Fink MP, Yang R & Delude RL (2004 c) Increased iNOS activity is essential for intestinal epithelial tight junction dysfunction in endotoxemic mice. Shock 21:261-270.


Original publications


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<th>No.</th>
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<td>Karihtala, Peeter</td>
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<tr>
<td>864</td>
<td>Penttilä, Hannu</td>
<td>Myocardial and cerebral preservation during off-pump coronary artery surgery</td>
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<td>865</td>
<td>Tormäkangas, Lisa</td>
<td>Experimental Chlamydia pneumoniae infection model: effects of repeated inoculations and treatment</td>
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<tr>
<td>866</td>
<td>Willig, Reeta</td>
<td>Hip fracture—aspects of background factors and outcome</td>
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<td>867</td>
<td>Ponikko, Maija-Leena</td>
<td>Ertyisoppilaan psykstrin heitokesto. Hoitoketternyn tarpeen ja toiminnan monitahoarviointi</td>
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<td>Functional and immunohistological studies on cancer-associated carbonic anhydrase IX</td>
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<td>Nurminen, Petri</td>
<td>Inflammatory cells and mitotic activity of keratinocytes in gingival overgrowth induced by immunosuppressive- and nifedipine medication</td>
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<td>Arvot ja arvostusten psykstrin holostoa. Henkilökunnan ja potilaan näkemyksiä hoidon nykytilasta</td>
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<td>Chronic chlamydial infection: impact on human reproductive health. Reproductive health research in the Northern Finland 1966 Birth Cohort (NFBC 1966)</td>
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<td>ERp57—Characterization of its domains and determination of solution structures of the catalytic domains</td>
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<td>Sorpanen, Sanna</td>
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<td>Erola, Tuomo</td>
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<td>876</td>
<td>Mäkinen, Tiina M.</td>
<td>Human cold exposure, adaptation and performance in a northern climate</td>
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Jouko Laurila

SURGICALLY TREATED ACUTE ACALCULOUS CHOLECYSTITIS IN CRITICALLY ILL PATIENTS