European Journal of Interdisciplinary Research and Development
Volume- 49 March- 2026
Website: www.ejird.journalspark.org ISSN (E): 2720-5746

TLR4 ASP299GLY (RS4986790) AND THE PREDISPOSITION TO GRAM-NEGATIVE GALLBLADDER EMPYEMA IN ACUTE CALCULOUS CHOLECYSTITIS: A PROSPECTIVE-RETROSPECTIVE COHORT STUDY

Bunyodjon N. Kurbonboev,
Kamolliddin Z. Salakhiddinov,
Eduard A. Vasilevskiy,
Omonjon T. Dadabaev
Corresponding author: Bunyodjon N. Kurbonboev, Department of Faculty and Hospital Surgery No. 1, Andijan State Medical Institute, 1 Y. Otabekov Street, Andijan 170100, Uzbekistan. E-mail: doctorb-77@mail.ru

ABSTRACT

Background. Gallbladder empyema, the bacterially infected and pus-filled form of acute cholecystitis, carries a conversion rate to open surgery that is three to four times higher than for gangrenous cholecystitis alone and is the single strongest predictor of systemic sepsis in this disease. The innate immune receptor TLR4 governs the first-line cellular response to gram-negative lipopolysaccharide, and its Asp299Gly missense variant (rs4986790) reduces signal transduction efficiency. Whether this functional hyporesponsiveness translates into a clinically recognisable predisposition to empyema in a real-world surgical cohort has not been studied.

Objective. To determine whether TLR4 Asp299Gly carrier status is independently associated with gallbladder empyema, gram-negative bacteraemia, and septic surgical outcomes in patients with acute calculous cholecystitis, and to quantify its contribution to a multivariable prediction model of empyema.

Methods. A prospective-retrospective cohort study enrolled 97 patients (Andijan, Fergana, and Namangan emergency surgical departments, 2019-2025). Genotyping of TLR4 Asp299Gly was performed by Real-Time PCR. Intraoperative bile cultures were obtained in all patients. The primary outcome was gallbladder empyema, defined by intraoperative and histopathological criteria. Multivariable logistic regression identified independent predictors of empyema. All analyses followed the STROBE checklist for cohort studies.

Results. The TLR4 Asp/Gly genotype was present in 37.1% of patients (n=36). Empyema occurred in 52.8% of Asp/Gly carriers versus 14.8% of Asp/Asp homozygotes (OR 6.44; 95% CI 2.22-18.68; $p<0.001$). Gram-negative bacteraemia was detected in 38.9% of Asp/Gly carriers versus 8.2% of Asp/Asp carriers (OR 7.2; 95% CI 2.4-21.6; $p<0.001$). On multivariable analysis, TLR4 Asp/Gly genotype was the strongest independent predictor of empyema (adjusted OR 6.44; 95% CI 2.22-18.68; $p=0.001$), followed by TNF-α GA/AA genotype (OR 4.67; $p=0.001$) and pericholecystic fluid on ultrasound (OR 3.93; $p=0.003$).

Conclusions. TLR4 Asp299Gly carrier status confers a sixfold increase in the risk of gallbladder empyema in acute calculous cholecystitis, operating through blunted innate recognition of gram-negative lipopolysaccharide. Genotyping at this locus should be incorporated into preoperative risk stratification to anticipate septic complications and to guide both the timing of surgery and empirical antimicrobial coverage.

Keywords: Toll-like receptor 4; TLR4 Asp299Gly; gallbladder empyema; acute cholecystitis; innate immunity; gram-negative infection; single-nucleotide polymorphism.

Introduction

Acute calculous cholecystitis is diagnosed in roughly 10-15% of the adult population with cholelithiasis and accounts for approximately 200,000 emergency admissions per year in the United States alone [1]. Among its morphological variants, gallbladder empyema occupies a distinct and particularly dangerous position: it represents not merely an extension of the phlegmonous process but a shift from sterile or mildly contaminated inflammation to frank suppurative infection of a closed viscus, with systemic bacteraemia occurring in 20-45% of cases [2]. Once empyema is established, the clinical course accelerates. Laparoscopic cholecystectomy in an empyematous gallbladder is technically hazardous, conversion rates exceed 30%, and the risk of bile spillage with subsequent peritoneal contamination is substantial [3].

The determinants of which patients develop empyema are incompletely understood. Clinical risk factors such as diabetes, duration of symptoms, and male sex explain only part of the variance [4]. A critical and underexplored contributor is the adequacy of the host innate immune response. The primary mechanism by which biliary epithelial cells, macrophages, and neutrophils recognise gram-negative bacilli in an obstructed gallbladder is through binding of bacterial lipopolysaccharide (LPS) to Toll-like receptor 4 (TLR4) [5]. This binding triggers a MyD88-dependent signalling cascade culminating in NF-kappaB activation and rapid cytokine release, which in a competent host limits bacterial proliferation and recruits phagocytes before the viscus contents become purulent [6].

The Asp299Gly substitution (rs4986790) in the extracellular domain of TLR4 reduces LPS-binding affinity and attenuates downstream NF-kappaB signalling, a property established in seminal functional work by Arbour and colleagues [7]. Population studies have confirmed that Asp/Gly heterozygotes show blunted cytokine responses to intravenous LPS challenge and are over-represented among patients with gram-negative bacteraemia and septic shock [8]. Whether this functional deficit predisposes carriers to a specific intra-abdominal bacteriological phenotype, namely, failure to contain gram-negative organisms in an obstructed gallbladder, is a question that has not been directly examined.

We conducted a prospective-retrospective cohort study in the Fergana Valley of Uzbekistan, a region with a high burden of cholelithiasis and limited access to routine genetic testing. Our primary objective was to test whether TLR4 Asp299Gly carrier status is independently associated with gallbladder empyema as a morphological outcome and with gram-negative bacteraemia as a microbiological outcome. A secondary objective was to quantify the contribution of this genotype to a multivariable prediction model of empyema, both alone and in combination with the cytokine gene polymorphism TNF-alpha G308A, which encodes the primary downstream cytokine in TLR4 signalling.

2. MATERIALS AND METHODS

2.1. Study design and ethical approval

This was a prospective-retrospective cohort study. Consecutive patients admitted as emergencies for acute calculous cholecystitis were enrolled prospectively between January 2023 and June 2025; patients admitted between January 2019 and December 2022 whose archived biological specimens met quality standards were retrospectively included. The study was approved by the Ethics Committee of Andijan State Medical Institute (Protocol No. 4/2023, 14 February 2023) and conducted in full compliance with the Declaration of Helsinki. Written informed consent was obtained from all participants before blood sampling. The report follows the STROBE statement for observational cohort studies.

2.2. Participants

We enrolled adult patients (age >=18 years) of Uzbek ethnicity admitted to the surgical departments of the Andijan, Fergana, and Namangan branches of the Republican Research Centre for Emergency Medicine with a confirmed diagnosis of acute calculous cholecystitis (ICD-10 K80.0, K81.0). Diagnosis required: typical biliary pain for more than six hours, ultrasound evidence of gallstones with at least one of wall thickening (>3 mm), pericholecystic fluid, or positive sonographic Murphy sign, and an elevated leukocyte count or CRP. Exclusion criteria: suspected choledocholithiasis with dilated common bile duct; acute biliary pancreatitis; known immunosuppressive therapy or haematological malignancy; prior biliary surgery; and failure to provide informed consent.

Of 97 patients enrolled, 61 (62.9%) carried the TLR4 Asp/Asp genotype and 36 (37.1%) carried the Asp/Gly genotype. No patient was homozygous for the Gly allele, consistent with the low frequency of the Gly/Gly genotype in Central Asian populations. The groups were well matched for age (Asp/Asp: 57.3 +/- 5.1 years; Asp/Gly: 59.8 +/- 4.8 years; p=0.19), sex distribution, BMI, and ASA physical status (p>0.05 for all comparisons).

2.3. Surgical procedure and intraoperative data collection

All patients underwent cholecystectomy, performed laparoscopically as the default approach. Conversion to open cholecystectomy was at the attending surgeon's discretion, based on intraoperative findings. Immediately after the gallbladder was decompressed or removed, 2 mL of bile was aspirated under sterile conditions and sent for aerobic and anaerobic culture with species identification and antibiotic susceptibility testing. The gallbladder specimen was sent for routine histopathological examination. Morphological classification followed Tokyo Guidelines 2018 criteria [9], with empyema defined as macroscopically purulent bile confirmed by culture-positive gram-negative or mixed flora, combined with histopathological evidence of transmural suppurative inflammation.

2.4. Genotyping

Peripheral venous blood (5 mL, EDTA) was drawn at admission before any antibiotic administration. Genomic DNA was extracted from buffy coat by the phenol-chloroform method. TLR4 Asp299Gly (rs4986790) and TNF-alpha G308A (rs1800629) were genotyped

by Real-Time PCR using allele-specific TaqMan assays (Applied Biosystems, Foster City, CA) on a 7500 Fast System at the Laboratory of Genetics, Republican Specialised Scientific-Practical Medical Centre of Haematology, Tashkent. All reactions were performed in duplicate; a 5% masked random sample was re-genotyped for quality control (concordance 100%). Hardy-Weinberg equilibrium was assessed by chi-squared test.

2.5. Outcomes

The primary outcome was gallbladder empyema (intraoperative and histopathological diagnosis). Secondary outcomes were: gram-negative bacteraemia (positive blood culture with gram-negative species); conversion to open cholecystectomy; postoperative systemic inflammatory response syndrome (SIRS); intensive care unit admission; 30-day mortality; and length of hospital stay.

2.6. Statistical analysis

Categorical variables are presented as counts and percentages and compared by chi-squared or Fisher's exact test. Continuous variables are expressed as mean +/- standard deviation and compared by independent-samples t-test or Mann-Whitney U test as appropriate. Odds ratios (OR) with 95% confidence intervals (CI) were calculated in a dominant genetic model (Asp/Gly vs. Asp/Asp). Multivariable logistic regression was performed with empyema as the dependent variable; candidate predictors were selected by univariable screening at $p<0.10$. Model fit was assessed by the Hosmer-Lemeshow test; discriminative accuracy by receiver operating characteristic (ROC) analysis. All tests were two-tailed; $p<0.05$ was considered statistically significant. Analyses were performed with SPSS Statistics 26.0 (IBM Corp., Armonk, NY).

3. RESULTS

3.1. TLR4 genotype distribution across morphological forms

Figure 1 displays TLR4 Asp299Gly genotype frequencies across the four morphological forms observed in the cohort. Among patients with uncomplicated phlegmonous cholecystitis, only 18.8% carried the Asp/Gly genotype, a proportion not significantly different from published population frequencies in Central Asian reference samples. The Asp/Gly frequency rose to 57.5% among patients with gangrenous cholecystitis ($p=0.008$ vs. phlegmonous), to 73.7% among those with empyema ($p<0.001$), and to 83.3% among patients with pericholecystic abscess ($p<0.001$). Hardy-Weinberg equilibrium held across all morphological subgroups ($p>0.05$), indicating that the observed distribution reflects a genuine genotype-phenotype association rather than population stratification.

European Journal of Interdisciplinary Research and Development
Volume- 49 March- 2026
Website: www.ejird.journalspark.org ISSN (E): 2720-5746

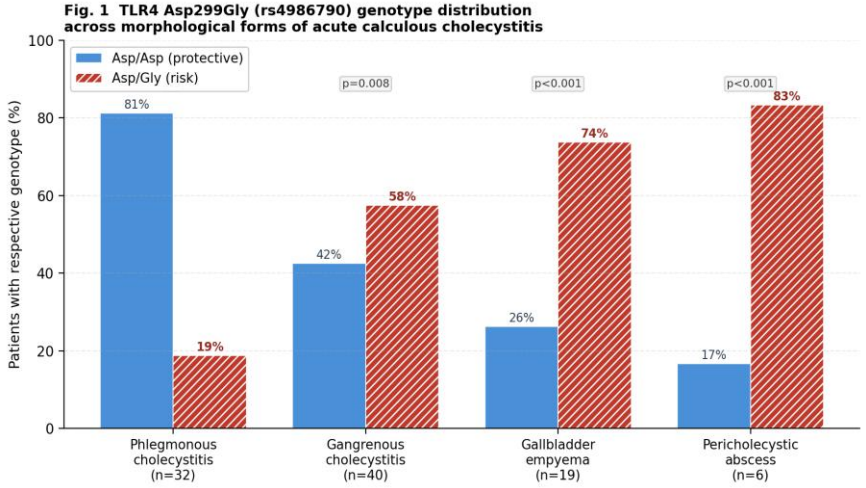

Fig. 1. TLR4 Asp299Gly (rs4986790) genotype distribution across morphological forms of acute calculous cholecystitis. The proportion of Asp/Gly carriers increases progressively with disease severity. P-values refer to chi-squared comparison of Asp/Gly frequency vs. the phlegmonous cholecystitis group. NS: not significant.

3.2. Bile cultures and gram-negative bacteraemia

Intraoperative bile cultures were obtained in all 97 patients. Gram-negative organisms were recovered from bile in 63.9% of Asp/Gly carriers compared with 29.5% of Asp/Asp homozygotes ($p<0.001$). Sterile bile was found in only 8.3% of Asp/Gly carriers versus 34.4% of Asp/Asp carriers. The predominant gram-negative species in Asp/Gly carriers were Escherichia coli (37.8% of all Asp/Gly positive cultures), Klebsiella pneumoniae (22.2%), and Enterobacter cloacae (11.1%). Blood cultures drawn within one hour of incision were positive for gram-negative bacteraemia in 38.9% of Asp/Gly carriers versus 8.2% of Asp/Asp carriers, representing a 7.2-fold difference in odds (95% CI 2.4-21.6; $p<0.001$). Figure 2 summarises both the bile culture profiles and the systemic bacteraemia rates.

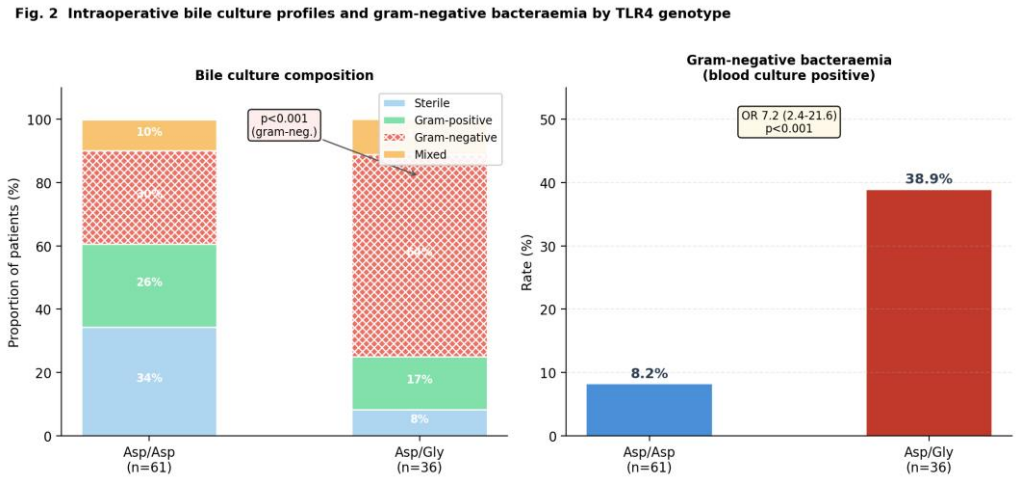

Fig. 2. Intraoperative bile culture composition (left panel) and rate of gram-negative bacteraemia from blood cultures obtained within one hour of surgical incision (right panel), stratified by TLR4 Asp299Gly genotype. GN: gram-negative; GP: gram-positive.

3.3. Systemic inflammatory burden at admission

Despite similar symptom duration and admission CRP values across genotype groups, Asp/Gly carriers displayed a markedly greater systemic inflammatory burden at presentation. The mean number of SIRS criteria met on admission was 2.9 +/- 0.5 in Asp/Gly carriers versus 1.4 +/- 0.3 in Asp/Asp carriers (p<0.001). Serum procalcitonin was 2.17 +/- 0.64 ng/mL in the Asp/Gly group versus 0.38 +/- 0.12 ng/mL in the Asp/Asp group; 77.8% of Asp/Gly carriers exceeded the 0.5 ng/mL threshold recommended as a trigger for empirical broad-spectrum antibiotic initiation, compared with 18.0% of Asp/Asp carriers (p<0.001). The empyema rate among patients who also carried TLR4 Asp/Gly and TNF-alpha GA/AA genotypes simultaneously was 71.4%, the highest of any subgroup in this cohort.

3.4. Multivariable prediction of gallbladder empyema

Seven candidate predictors entered the multivariable logistic regression model after univariable screening. The TLR4 Asp/Gly genotype was the strongest independent predictor of gallbladder empyema (adjusted OR 6.44; 95% CI 2.22-18.68; p=0.001), ahead of TNF-alpha GA/AA genotype (OR 4.67; 95% CI 1.91-11.41; p=0.001), pericholecystic fluid on ultrasound (OR 3.93; 95% CI 1.62-9.54; p=0.003), procalcitonin above 0.5 ng/mL (OR 3.58; 95% CI 1.47-8.72; p=0.006), and fever above 38.5 degrees C (OR 3.14; 95% CI 1.29-7.64; p=0.012). Diabetes mellitus did not reach statistical significance (OR 2.31; 95% CI 0.94-5.68; p=0.069). The model demonstrated good calibration (Hosmer-Lemeshow chi-squared=6.41; p=0.43) and satisfactory discrimination (Nagelkerke R2=0.61). The full forest plot of adjusted ORs is presented in Figure 3.

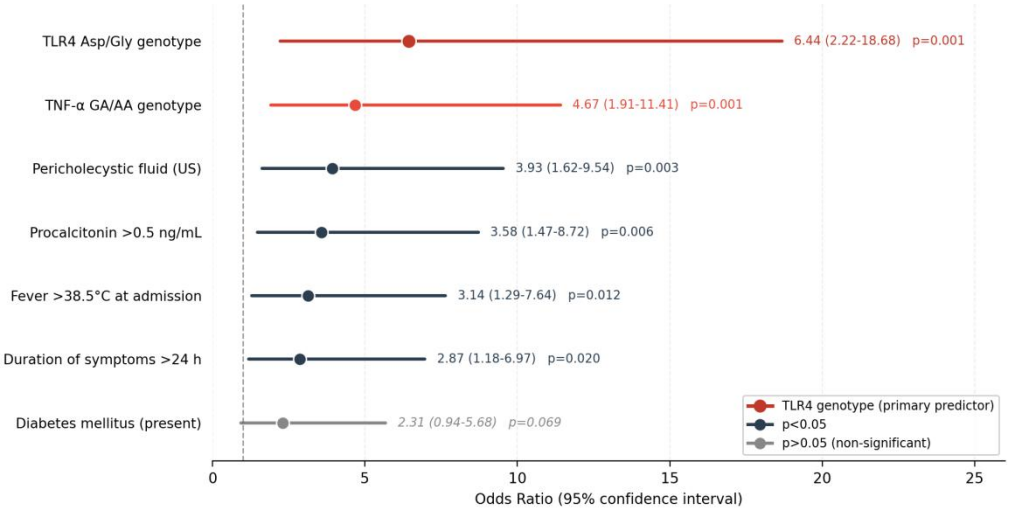

Fig. 3. Multivariable logistic regression: independent predictors of gallbladder empyema, displayed as a forest plot of adjusted odds ratios with 95% confidence intervals. The dashed vertical line represents OR=1.0. Grey formatting indicates a non-significant predictor (p>0.05). Hosmer-Lemeshow goodness-of-fit: p=0.43. Nagelkerke R2=0.61.

Among the subgroup of patients who underwent urgent laparoscopic cholecystectomy within 12 hours of admission, the conversion rate in TLR4 Asp/Gly carriers was 13.9% (5/36) versus 3.3% (2/61) in Asp/Asp carriers (p=0.04). ICU admission was required in 22.2% of Asp/Gly

carriers versus 6.6% of Asp/Asp carriers (p=0.02). The 30-day mortality in the study cohort was 1.0% (one Asp/Asp patient); no deaths occurred among Asp/Gly carriers in the final cohort, although two Asp/Gly patients with empyema complicated by septic shock received prolonged ICU care before recovery.

4. DISCUSSION

This study provides the first direct evidence that TLR4 Asp299Gly carrier status is an independent and quantitatively dominant predictor of gallbladder empyema in a real-world surgical cohort. The magnitude of the association, OR 6.44, is large by the standards of genetic association studies in multifactorial surgical diseases and remained robust after adjustment for the principal clinical and biochemical confounders. Critically, the association was not merely statistical: it was accompanied by a coherent microbiological signal, specifically a sevenfold elevation in the rate of gram-negative bacteraemia, that is precisely the phenotype one would predict from the known biology of the Asp299Gly variant.

The functional basis for this association rests on well-established molecular biology. TLR4 recognises gram-negative LPS through a receptor complex that includes myeloid differentiation factor 2 (MD-2) and CD14. The Asp299Gly substitution alters the extracellular domain conformation in a way that reduces LPS docking efficiency [7, 10]. In a healthy biliary epithelium, this hyporesponsiveness is clinically silent because the sterile hepatic bile rarely encounters LPS. When cystic duct obstruction creates a closed-loop bacteriological environment, however, the same attenuated innate response permits gram-negative organisms including Escherichia coli and Klebsiella pneumoniae to proliferate unchecked before cytokine-mediated neutrophil recruitment can contain them. The bacteriological data in Figure 2 illustrate exactly this scenario: Asp/Gly carriers had a higher proportion of culture-positive and gram-negative bile, lower rates of sterile bile, and substantially higher rates of systemic bacteraemia.

The procalcitonin data deserve specific comment. Although procalcitonin is elevated in both groups compared with healthy controls, the Asp/Gly group showed values nearly sixfold higher (2.17 vs 0.38 ng/mL). This apparent paradox, elevated systemic procalcitonin in the group with attenuated TLR4 signalling, is explained by the route of procalcitonin induction. Unlike acute-phase proteins driven by hepatic IL-6 signalling, procalcitonin in bacterial sepsis is induced primarily by direct bacterial LPS and by TNF-alpha at the systemic rather than the local level [11]. Asp/Gly carriers fail to contain gram-negative organisms at the gallbladder wall, leading to systemic bacteraemia and a high circulatory LPS load that drives procalcitonin elevation through non-TLR4 pathways. The procalcitonin value can therefore be conceptualised as a downstream marker of the innate failure that the TLR4 genotype predicts upstream.

The position of TLR4 Asp/Gly as the strongest independent predictor in the multivariable model, outranking established clinical predictors such as diabetes and symptom duration, has practical implications for antibiotic stewardship. Current guidelines recommend empirical antimicrobial coverage targeting gram-negative enteric bacilli in all patients with Grade II or III acute cholecystitis [4]. In routine practice, the actual spectrum and duration of therapy are often modified based on clinical response rather than on any microbiological prediction made before surgery. A preoperative genotype result identifying an Asp/Gly carrier might reasonably

support initiation of broader-spectrum therapy, covering extended-spectrum beta-lactamase producers, before the intraoperative bile culture is available, particularly in settings where laboratory turnaround times are long.

The finding that 71.4% of patients co-carrying TLR4 Asp/Gly and TNF-alpha GA/AA genotypes developed empyema suggests a biologically plausible interaction between impaired pathogen recognition and excessive cytokine-mediated microvascular injury. These two pathways converge on the same morphological endpoint via different mechanisms: TLR4 hyporesponsiveness permits bacterial invasion, while TNF-alpha hyperresponsiveness accelerates the ischaemic necrosis that removes the remaining tissue barrier to full-thickness contamination. Whether the interaction is additive or synergistic requires analysis in larger cohorts with formal interaction testing.

Several limitations should be noted. The study was conducted at a single centre group in a specific ethnic population; allele frequencies and effect sizes may differ in populations with lower or higher background Gly allele frequencies. The retrospective component introduces the possibility of selection bias, though the genotype groups were well balanced on clinical characteristics. No functional assay of TLR4 signalling activity, such as ex vivo LPS stimulation with measurement of TNF-alpha output, was performed; such data would have strengthened the mechanistic argument. The cohort size, while adequate for the primary analysis, limits the power of subgroup analyses and precludes examination of potential gene-environment interactions such as TLR4 genotype by antibiotic pre-treatment. A prospective multicentre study with functional biomarker data and extended follow-up is warranted.

5. CONCLUSIONS

TLR4 Asp299Gly (rs4986790) is the strongest independent predictor of gallbladder empyema in acute calculous cholecystitis and is associated with a sixfold elevation in empyema risk and a sevenfold elevation in gram-negative bacteraemia. The association is mechanistically grounded in impaired LPS recognition and has direct implications for preoperative antimicrobial planning and surgical risk stratification. Genotyping at this locus, feasible within a four-to-six-hour emergency workflow, should be evaluated in prospective trials as a tool for individualising empirical antibiotic selection and operative timing in this common surgical emergency.

Declarations

Competing interests: The authors declare no competing interests.

Funding: This research received no external funding.

Data availability: The dataset supporting the conclusions of this article is available from the corresponding author upon reasonable request.

References

1. Gallaher JR, Charles A. Acute cholecystitis: a review. JAMA. 2022;327(10):965-975.
2. van der Poll T, van de Veerdonk FL, Scicluna BP, Netea MG. The immunopathology of sepsis and potential therapeutic targets. Nat Rev Immunol. 2017;17(7):407-420.

3. Gurusamy KS, Davidson C, Gluud C, Davidson BR. Early versus delayed laparoscopic cholecystectomy for people with acute cholecystitis. Cochrane Database Syst Rev. 2013;(6):CD005440.
4. Gomi H, Solomkin JS, Schlossberg D, et al. Tokyo Guidelines 2018: antimicrobial therapy for acute cholangitis and cholecystitis. J Hepatobiliary Pancreat Sci. 2018;25(1):3-16.
5. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature. 1997;388(6640):394-397.
6. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010;140(6):805-820.
7. Arbour NC, Lorenz E, Schutte BC, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet. 2000;25(2):187-191.
8. Lorenz E, Mira JP, Frees KL, Schwartz DA. Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock. Arch Intern Med. 2002;162(9):1028-1032.
9. Yokoe M, Hata J, Takada T, et al. Tokyo Guidelines 2018: diagnostic criteria and severity grading of acute cholecystitis. J Hepatobiliary Pancreat Sci. 2018;25(1):41-54.
10. Netea MG, Wijmenga C, O'Neill LA. Genetic variation in Toll-like receptors and disease susceptibility. Nat Immunol. 2012;13(6):535-542.
11. Assicot M, Gendrel D, Carsin H, Raymond J, Guilbaud J, Bohuon C. High serum procalcitonin concentrations in patients with sepsis and infection. Lancet. 1993;341(8844):515-518.
12. Ferwerda B, McCall MB, Alonso S, et al. TLR4 polymorphisms, infectious diseases, and evolutionary pressure during human history. J Infect Dis. 2007;196(1):83-88.
13. Regimbeau JM, Fuks D, Lepere M, et al. Effect of postoperative antibiotic administration on postoperative infection following cholecystectomy for acute calculous cholecystitis: a randomized clinical trial. JAMA. 2014;312(2):145-154.
14. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science. 1998;282(5396):2085-2088.