Received: 13 March 2024 | Accepted: 10 May 2024

DOI: 10.1002/wjs.12219

ORIGINAL SCIENTIFIC REPORT

World Journal of Surgery

Immunohistochemical inflammation in histologically normal gallbladders containing gallstones

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Funding information

Medical Research Council, Grant/Award Number: MR/K00414X/1; Arthritis Research UK, Grant/Award Number: 19891; European Social Fund Plus, Grant/Award Number: 2013-ESPA-PE1-3680; NIHR Nottingham Biomedical Research Centre, Grant/Award Number: NIHR203310

Abstract

Background: The aim of this study was to establish features of inflammation in histologically normal gallbladders with gallstones and compare the expression of inflammatory markers in acutely and chronically inflamed gallbladders.

Methods: Immunohistochemistry was performed on formalin-fixed paraffin-embedded gallbladders for tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-2R, and substance *p* in three groups: Group I ($n = 60$) chronic cholecystitis, Group II ($n = 57$) acute cholecystitis and Group III ($n = 45$) histologically normal gallbladders with gallstones. Expression was quantified using the H-scoring system.

Results: Median, interquartile range expression of mucosal IL-2R in Groups I (2.65, 0.87–7.97) and II (12.30, 6.15–25.55) was significantly increased compared with group III (0.40, 0.10–1.35, $p < 0.05$). Submucosal IL-2R expression in Groups I (2.0, 1.12–4.95) and II (10.0, 5.95–14.30) was also significantly increased compared with Group III (0.50, 0.15–1.05, $p < 0.05$). There was no difference in the lymphoid cell IL-6 expression between Groups I (5.95, 1.60–18.15), II (6.10, 1.1–36.15) and III (8.30, 2.60–26.35, $p > 0.05$). Epithelial IL-6 expression of Group III (8.3, 2.6–26.3) was significantly increased compared with group I (0.5, 0–10.2, $p < 0.05$) as was epithelial TNF-α expression in Group III (85.0, 70.50–92.0) compared with Groups I (72.50, 45.25.0–85.50, $p < 0.05$) and II (61.0, 30.0–92.0, $p < 0.05$). Lymphoid cell Substance P expression in Groups I (1.90, 1.32–2.65) and II (5.62, 2.50–20.8) was significantly increased compared with Group III (1.0,1.0–1.30, $p < 0.05$). Epithelial cell expression of Substance P in Group III (121.7, 94.6–167.8) was significantly increased compared with Groups I (75.7, 50.6–105.3, $p < 0.05$) and II (78.9, 43.5–118.5, $p < 0.05$).

Conclusion: Histologically normal gallbladders with gallstones exhibited features of inflammation on immunohistochemistry.

KEYWORDS

cholecystitis, gallbladder, gallstones, immunohistochemistry, inflammation

1 | INTRODUCTION

Gallstone disease affects 10%–15% of the US population but 80% of people with gallstones are asymptomatic. Acute calculous cholecystitis is the first mode of presentation in 10%–15% of all patients with gallstones.[1] On the other hand, chronic cholecystitis is a lengthy, subacute condition caused by mechanical (gallstones) or functional dysfunction of gallbladder emptying.[2] When inflammation supervenes, signs such as fever, abdominal wall guarding, rebound tenderness, and raised blood inflammatory markers can be present but they are not consistent.[1,3] Confirmation of the presence of gallstones on ultrasound examination along with the other signs such as gallbladder wall thickening and pericholecystic fluid will contribute towards the diagnosis.[1] Nevertheless, some patients complain of non-specific abdominal symptoms similar to those in patients without gallstones.[4,5]

In a previous study of symptomatic appendicitis, histologically normal appendices resected from patients with right iliac fossa pain exhibited increased proinflammatory cytokine expression on immunohistochemistry suggesting the presence of an inflammatory process not detected on conventional microscopy with hematoxylin and eosin staining.[6] Kasprzak et al.[7] explored the expression of proinflammatory cytokines interleukin (IL)-1α, IL-6, and tumor necrosis factor (TNF)-α in gallbladder specimens with either acute or chronic inflammation in order to further understand the pathogenesis of cholecystitis. The expression of TNF-α was significantly greater in the chronic calculous cholecystitis group.[7] TNF-α expression also had a highly positive correlation with the total grading of gallbladder inflammation.[7] Administration of lipopolysaccharide (LPS) is known to promote the release of IL-1 and simultaneous administration of LPS and IL-1 into guinea pig gallbladders induced gallbladder inflammation manifested by increased prostaglandin and myeloperoxidase release.[8] IL-1 and TNF-α were also shown to directly affect the secretory and absorptive functions of gallbladder epithelial cells by decreasing mucosal-to-serosal sodium and chloride fluxes and increasing serosal-to-mucosal movement of sodium ions, which promoted the formation of gallstones.[9] IL-1α, IL-6 and TNF-α were detected in columnar epithelial cells of the gallbladder in both acute and chronic cholecystitis and were also detected in mononuclear inflammatory cells (primarily macrophages) scattered in the *lamina propria*.[7] The findings reported by Kasprzak et al.[7] were in keeping with those of studies that demonstrated cellular sources of IL-1, IL-6, and TNF-α.[10,11] Savard et al.[12] also demonstrated that gallbladder epithelial cells can adjust their cytokine RNA expression in response to LPS exposure in order to synthesize TNF-α. The presence of Substance P as well as other peptide transmitters is well documented in the gallbladder nervous system.[13,14] The findings reported by Prystowsky and Rege,[15] taken into consideration with previously reported data on the proinflammatory effects of Substance P and vasoactive intestinal polypeptide as well as their presence in gallbladder nervous system, could suggest that release of peptide transmitters by nociceptive neurons plays a crucial role in the development of cholecystitis.

The aim of this study was to investigate tissue expression of TNF-α, IL-6, IL-2R and Substance P in formalin-fixed tissue of chronic and acute cholecystitis as well as histologically normal gallbladders resected from symptomatic patients with evidence of gallstones. We also described the differences in signs and symptoms, laboratory investigations and intraoperative findings between the three groups.

2 | METHODS

Formalin-fixed paraffin-embedded gallbladder specimens were selected retrospectively in patients over the age of 16 years who underwent cholecystectomy. Following a computerized search, the histology slides were re-examined by a senior histopathologist (AMZ) who was blinded to the initial diagnosis. The samples were included when the initial diagnosis matched with that of the histopathologist who re-examined them.

The specimens were allocated to three groups (Table 1). The tissue architecture of the histologically normal specimens on conventional histology (hematoxylin and eosin staining) was normal and did not exhibit any features of acute or chronic inflammation. Specimens were processed as described previously.[6] Immunohistochemical staining was performed using primary antibodies against TNF-α, IL-6, IL-2R, and Substance P as described previously.[6]

Manual immunohistochemistry assessment was chosen over computer-aided assessment as some of the inflammatory markers were detected in the membrane and some in the cytoplasm.[6] Ten randomly selected fields in full sections were scored by two observers (AMZ and EP) and their average formed the final score for each specimen. The expression of TNF-α

TABLE 1 Patient demographics.

Groups	N	Sex		Age (years)
		Male	Female	Median (IQR)
Chronic cholecystitis (Group I)	60	48	12	46.1 (34.4–58.6)
Acute cholecystitis (Group II)	58	35	23	60.4 (45.3–74.1)
Histologically "normal" gallbladders in patients with gallstones (Group III)	45	37	8	41.1 (31.9–53.6)

and IL-2R was quantified by counting the positively stained cells.[16,17] The expression of IL-6 and Substance P were semi-quantified using the H-scoring system. The H-score was calculated by multiplying the percentage of positive cells (0–100) by a number representing the intensity of immune-reactivity (1 for weak, 2 for moderate, and 3 for strong), giving a maximum score of 300.[18]

Clinical data relevant to the underlying pathology were collected by reviewing patient records from the perioperative period. We recorded data from patient history and examination on admission (anorexia, nausea and/or vomiting, severity of pain, duration of symptoms, previous episodes of RUQ pain) and blood results [while cell count, neutrophil and lymphocyte count, neutrophil:lymphocyte ratio (NLR), and C-reactive protein (CRP)]. We also recorded intra-operative findings such as adhesions in the right upper quadrant.

2.1 | Ethics and consent

The study protocol was approved by the Health Research Authority (18/HRA/0292) and the need to obtain informed consent was waived.

2.2 | Statistical analysis

Statistical analysis was performed on IBM® SPSS® statistics software v24 (IBM Corp.). Data were expressed as *n* (%) or median, interquartile range (IQR). The Mann–Whitney *U* test was used to compare two groups and the Kruskal-Wallis *H* test to compare three or more groups. Cross tabulation and the chi-square test were used for categorical variables. The results were considered as statistically significant at $p < 0.05$. The Bonferroni correction was applied when multiple comparisons were performed.

3 | RESULTS

We studied 161 patients who were admitted following a clinical diagnosis of biliary colic or cholecystitis (Table 1). Immunohistochemistry findings, and laboratory and clinical data are summarized in Table 2.

Anti-TNF-α antibody staining was demonstrated in epithelial cells and monocytes in the mucosa of the samples (Figure 1). Most of the monocytes stained by anti-TNF-α antibodies were macrophages.

Immunohistochemical staining with anti-IL-6 antibodies revealed cytoplasmic staining in the epithelial and inflammatory cells of the mucosa (Figure 2), with staining being observed in both mononuclear and polymorphonuclear cells. Anti-IL-2R antibody membrane staining was seen in the lymphocytes in the gallbladder mucosa and submucosa (Figure 3).

Immunohistochemical staining with anti-substance *p* antibody revealed cytoplasmic staining in the epithelial (Figure 4A, B) and inflammatory (Figure 4C, D) cells of the mucosa as well as the nerve ganglia (Figure 4E, F).

Comparison of the expression of the studied markers between the different populations of inflammatory cells was not performed as identification of these cell populations was very unreliable on immunohistochemical staining alone.

In the group of patients with histologically normal gallbladders (Group III), 81% of them complained of previous episodes of right upper quadrant pain whereas 53% and 50% of the patients in the groups of chronic (Group I) and acute cholecystitis (Group II) respectively complained of previous episodes of right upper quadrant pain. Moreover, the duration of symptoms to suggest the presence of gallstones in the patients with histologically normal gallbladders did not differ when compared with patients with chronic or acute cholecystitis.

There were no appreciable changes in the levels of statistical signifance for any of the studied parameters within each group of patients after applying the Bonferroni correction for multiple statistical testing.

4 | DISCUSSION

This study has elucidated experimental findings on the immunohistochemical expression of inflammatory markers combined with clinical data and laboratory investigations in the entire spectrum of cholecystitis as well as in gallbladders with gallstones but absence of inflammation on conventional histology.

The age of patients with acute cholecystitis was significantly higher compared with that of patients with chronic cholecystitis or histologically “normal” gallbladders. This could be explained by the fact that the slides were selected according to their histological appearance and, hence, age may not be a significant determinant. By re-examining the slides, we ensured the patients included in the study were accurately allocated to the appropriate experimental group. However, histological changes that could be interpreted as either chronic or acute inflammation or borderline between the two groups were excluded from the study.

This study provides new evidence regarding the group of histologically normal gallbladders resected from symptomatic patients with gallstones as we demonstrated that all the studied cytokine markers were significantly increased in this group. The increased expression of both TNF-α and IL-6 which are important pro-inflammatory cytokines could suggest the presence of bacterial toxins such as LPS in the gallbladder mucosa and the subsequent activation of the

TABLE 2 Summary of immunohistochemistry and clinical findings.

Variables		Patient groups			
		Chronic cholecystitis (group I)[a]	Acute cholecystitis (group II)[b]	Histologically "normal" gallbladders in patients with gallstones (group III)[c]	*p* value
TNF-α	Epithelial cells	72.5 (45.2–88.5)	61.0 (30.0–90.0)	85.0 (70.5–92.0)	**<0.05**
					a versus b = 0.543
					a versus c = 0.015
					b versus c = 0.012
	Inflammatory cells	0.2 (0–0.5)	0.2 (0–0.7)	0 (0-0)	**<0.05**
					a versus b = 0.288
					a versus c < 0.001
					b versus c < 0.001
IL-6	Epithelial cells	0.5 (0–10.2)	6.7 (0–62.5)	4.0 (0–74.5)	**<0.05**
					a versus b = 0.008
					a versus c = 0.018
					b versus c = 0. 992
	Inflammatory cells	5.9 (1.6–18.1)	6.1 (1.1–36.1)	8.3 (2.6–26.3)	>0.05
					a versus b = 0.810
					a versus c = 0.279
					b versus c = 0.542
IL-2R	Mucosa	2.6 (0.8–7.9)	12.3 (6.1–25.5)	0.4 (0.1–1.3)	**<0.05**
					a versus b < 0.001
					a versus c < 0.001
					b versus c < 0.001
	Submucosa	2.0 (1.1–4.9)	10.0 (5.9–14.3)	0.5 (0.1–1.0)	**<0.05**
					a versus b < 0.001
					a versus c < 0.001
					b versus c < 0.001
Substance P	Epithelial cells	75.7 (50.6–105.3)	78.9 (43.5–118.5)	121.7 (94.6–167.8)	**<0.05**
					a versus b = 0.781
					a versus c < 0.001
					b versus c < 0.001
	Inflammatory cells	1.9 (1.3–2.6)	5.6 (2.5–20.8)	1.0 (1.0–1.3)	**<0.05**
					a versus b < 0.001
					a versus c < 0.001
					b versus c < 0.001
	Nerve ganglia	90.0 (0–125.0)	0 (0–12.5)	80.0 (0–150.0)	**<0.05**
					a versus b < 0.001
					a versus c = 0.891
					b versus c = 0.002

TABLE 2 (Continued)

Variables	Patient groups			*p* value
	Chronic cholecystitis (group I)[a]	Acute cholecystitis (group II)[b]	Histologically "normal" gallbladders in patients with gallstones (group III)[c]	
White cell count ($\times 10^9$/L)	7.7 (6.3–9.9)	9.8 (7.1–13.5)	6.9 (5.8–8.3)	**<0.05**
				a versus b = 0.002
				a versus c = 0.050
				b versus c < 0.001
Neutrophil count ($\times 10^9$/L)	4.8 (3.3–6.5)	6.4 (4.5–10.5)	4.0 (3.2–5.2)	**<0.05**
				a versus b < 0.001
				a versus c = 0.073
				b versus c < 0.001
Lymphocyte count ($\times 10^9$/L)	2.3 (1.8–2.5)	1.6 (1.0–2.2)	1.9 (1.5–2.4)	**<0.05**
				a versus b < 0.001
				a versus c = 0.121
				b versus c = 0.012
Anorexia [*n*(%)]	17 (44%)	20 (71%)	15 (48%)	0.42
Duration of pain (days)	150.0 (82.5–365.0)	35.0 (3.0–365.0)	165.0 (90.0–555.0)	**<0.05**
Nausea/vomiting [*n* (%)]	25 (42%)	26 (45%)	24 (53%)	**<0.05**
Previous episodes of RUQ pain [*n* (%)]	19 (53%)	14 (50%)	25 (81%)	**<0.05**
Adhesions in RUQ [*n* (%)]	13 (32%)	11 (35%)	9 (30%)	0.48

Note: Data are presented at median (interquartile range) or *n* (%). Statistical significance for differences between three or more groups was calculated using the Kruskal-Wallis *H* test. Cross tabulation and the chi-square test were used for categorical variables. Statistically significant differences are shown in bold font.

host defense response as part of the innate immunity. This finding along with the increased expression of IL-2R could suggest a fully activated immune system. Our observations could reflect the presence of an inflammatory response at such an early stage where there was no evidence of inflammatory cell infiltrate on conventional microscopy. The finding of increased TNF-α expression by mononuclear inflammatory cells, IL-6 expression by inflammatory cells and IL-2R in the histologically inflamed gallbladders, as well as the increased expression of Substance P, could be easily explained as it is known that these markers play a crucial role in the acute inflammatory response.[12,19–25] Injury to the gallbladder epithelium by gallstones could damage the epithelial barrier allowing increased bacterial translocation and, therefore, LPS forms a primary target for the immune system.[12,20,26] Bacterial endotoxins trigger the innate immune response through communication with antigen presenting cells (macrophages and dendritic cells).[27] Activated macrophages produce IL-1β, a potent proinflammatory mediator, which is able to upregulate the expression of TNF-α and IL-6, further promoting gastrointestinal inflammation.[28–31] Antigen presenting cells of the gastrointestinal *lamina propria* present antigens to B and T lymphocytes leading to activation of the adaptive immunity.[27] Once T lymphocyte activation is facilitated, IL-2 is rapidly synthesized.[27,32] This is succeeded by expression of the IL-2 receptor, hence the IL-2/IL-2R interaction promotes proliferation of the activated T lymphocytes.[33–37] IL-2 also stimulates expression of IL-2R in B lymphocytes, further enhancing their sensitivity to IL-2, promoting antibody secretion from the activated B lymphocytes.[38,39]

The expression of TNF-α, either in epithelium or monocytes, between chronically and acutely inflamed specimens was very similar. The IL-6 expression of inflammatory cells between the chronically and acutely inflamed gallbladder specimens did not differ either. This could be attributed to the fact that most acute cholecystitis samples have some evidence of underlying chronic inflammation due to the long-standing presence of gallstones.

Our finding of increased epithelial IL-6 expression in the acute cholecystitis samples compared with the chronic cholecystitis samples might reflect a more advanced inflammatory status. This is in keeping with previously reported studies that have linked IL-6 expression with the severity of inflammation.[40–44] It has been shown that IL-6 is a potent pro-coagulant

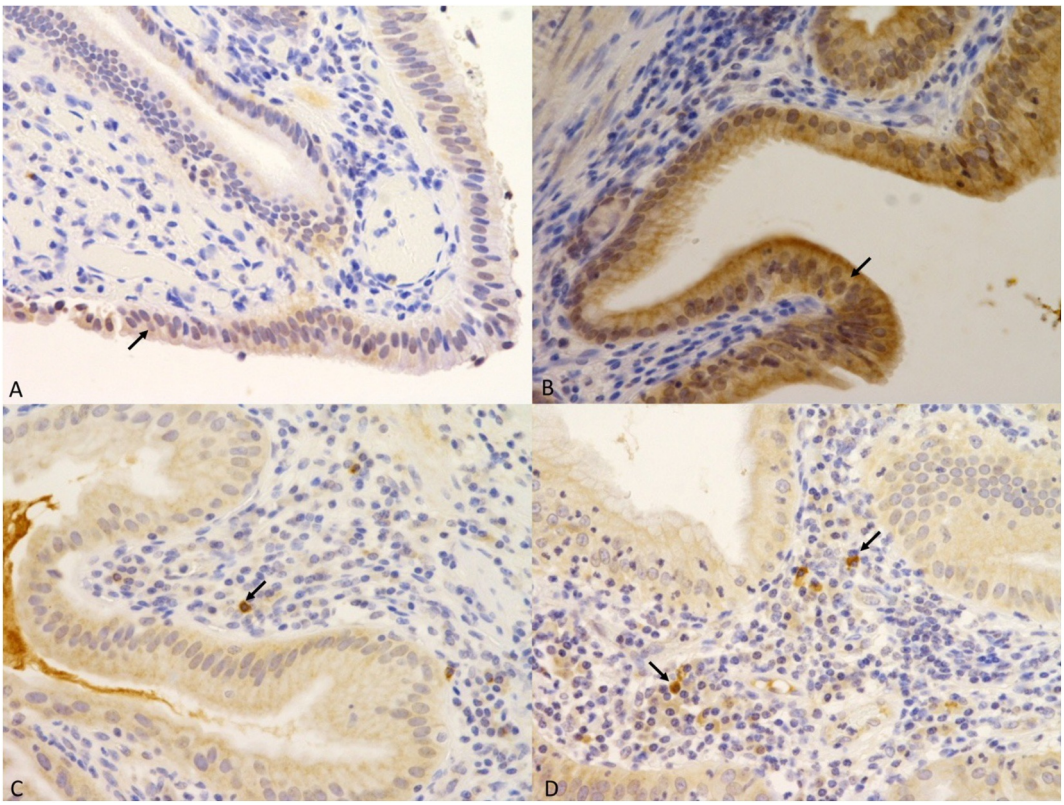

FIGURE 1 Expression of TNF-α using immunohistochemical staining at ×400 magnification. Brown cytoplasmic staining (A and B, black arrows) in epithelial cells (A) Low expression; (B) High expression of TNF-α. Expression of TNF-α in monocytes (C and D, black arrows) (C) Low expression; (D) High expression of TNF-α.

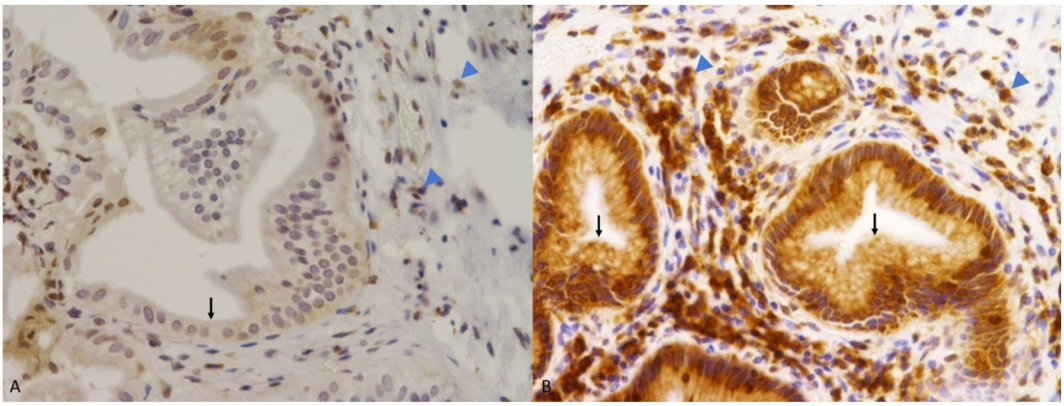

FIGURE 2 Expression of IL-6 (brown cytoplasmic staining) in epithelial (black arrows) and inflammatory (blue arrowheads) cells using immunohistochemical staining at ×400 magnification. (A) Low expression; (B) High expression of IL-6.

cytokine stimulating tissue factor mRNA expression.[24] Therefore, increased IL-6 expression could promote local thrombosis and subsequently gangrene and perforation; features of an acute inflammatory process. Although IL-2R expression was increased in the histologically "normal" group with gallstones, it did not reach the very elevated levels seen in the samples with gallbladder inflammation as IL-2R is involved in regulating the immune response by promoting lymphocyte proliferation. This could explain the interesting finding of significantly increased epithelial cell TNF-α and IL-6 expression in histologically "normal" gallbladders compared with the inflamed samples while the expression of IL-6 from the inflammatory cells did not differ between the three groups. It could be speculated that the epithelial TNF-α and IL-6 expression was at higher levels during the very first stages of the inflammatory process when the antigen presentation took place. The theory that histologically "normal" gallbladders removed from symptomatic patients could

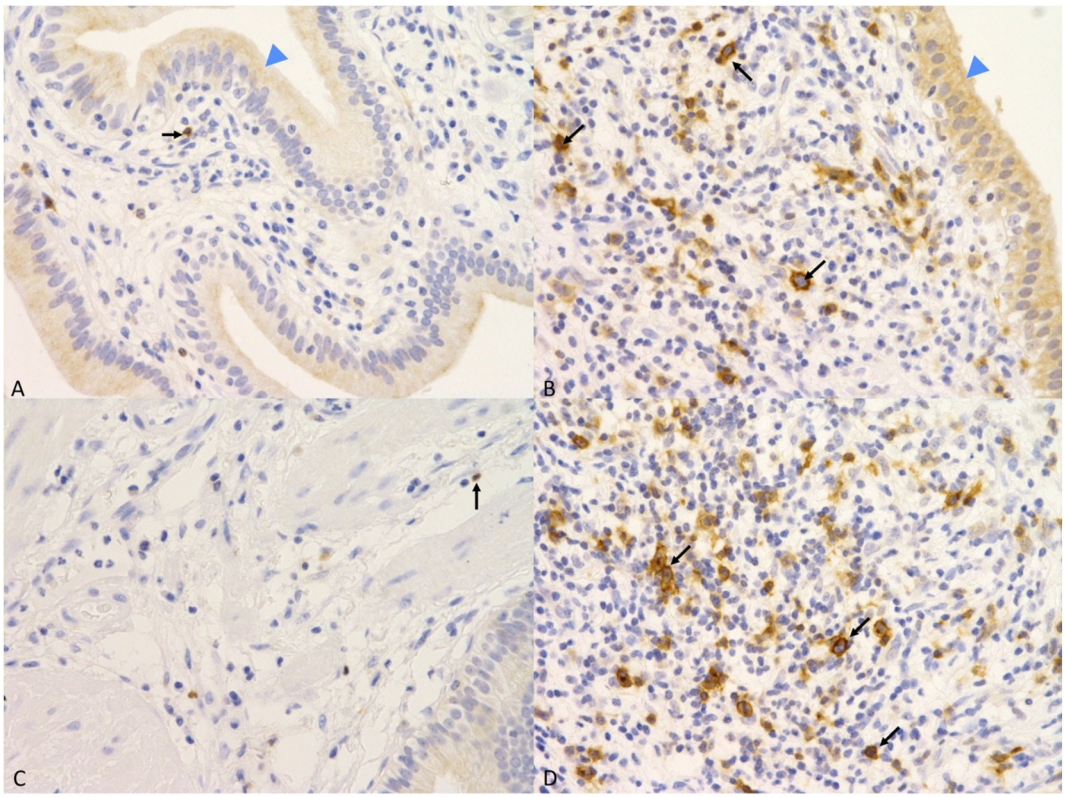

FIGURE 3 (A, B) – Expression of IL-2R (brown membrane staining) in the mucosa (blue arrowheads) and lamina propria (black arrows) using immunohistochemical staining at ×400 magnification. (C, D) – Expression of IL-2R (brown membrane staining) in the submucosa (black arrows) (A and C) Low expression; (B and D) High expression of IL-2R.

represent an inflammatory response at a very early stage is further supported by the findings of Murphy et al.[23] who reported changes in inflammatory gene expression before cellular infiltration was present in the mucosa.

Contrary to the widely accepted perception that the initial response to local infection is proinflammatory, it has been shown that many features of the acute phase response are anti-inflammatory suggesting that suppression of the inflammatory response could be beneficial for the host.[41] It is also known that IL-6 is not only an important proinflammatory mediator but it can also exhibit anti-inflammatory properties in both local and systemic anti-inflammatory responses.[45] This is further supported by the finding that IL-6 can restrict neutrophil recruitment and promote their replacement by mononuclear cells steering the inflammatory response towards chronicity.[21] This transition is directed by IL-6 *trans* signaling.[19,20]

There is evidence to suggest that TNF-α can also exhibit anti-inflammatory properties.[25,26] During acute intestinal inflammation, TNF-α promotes the release of intestinal glucocorticoids which in turn control the activation of intestinal T lymphocytes.[25] Furthermore, TNF-α can induce apoptosis of immune and non-immune cells.[46,47] However, although TNF-α-induced apoptosis of T cells helps to terminate or control intestinal inflammation, apoptosis of epithelial cells further compromises the integrity of the intestinal barrier allowing constant activation of intestinal immune cells with luminal antigens.[46,48] This could be supported by our finding of elevated IL-2R expression in the histologically "normal" gallbladder specimens, as IL-2 expression is transient and depends on sustained antigenic stimulation.[49] Therefore, the markedly increased expression of IL-6 and TNF-α in the histologically "normal" gallbladders could reflect an aim towards resolution of an inflammatory response the duration of which is abnormally long. IL-2 could act synergistically with IL-6 and TNF-α as it plays a crucial role in the maintenance of immune homeostasis by downregulating the immune response.[50–52]

Moreover, the increased levels of IL-6 and TNF-α could imply an abnormally regulated expression which is responsible for a chronic proinflammatory state.[49] Finally, it has been demonstrated that Substance P is also able to promote mucosal healing. This is achieved through stem cell proliferation by *trans* activation of epithelial growth factor receptor which exerts anti-apoptotic effects through Akt phosphorylation both in vivo and in vitro.[53–55] Substance P has also been found to promote gallbladder contraction.[56] The finding of raised Substance P expression in histologically "normal" gallbladders with gallstones, could imply a

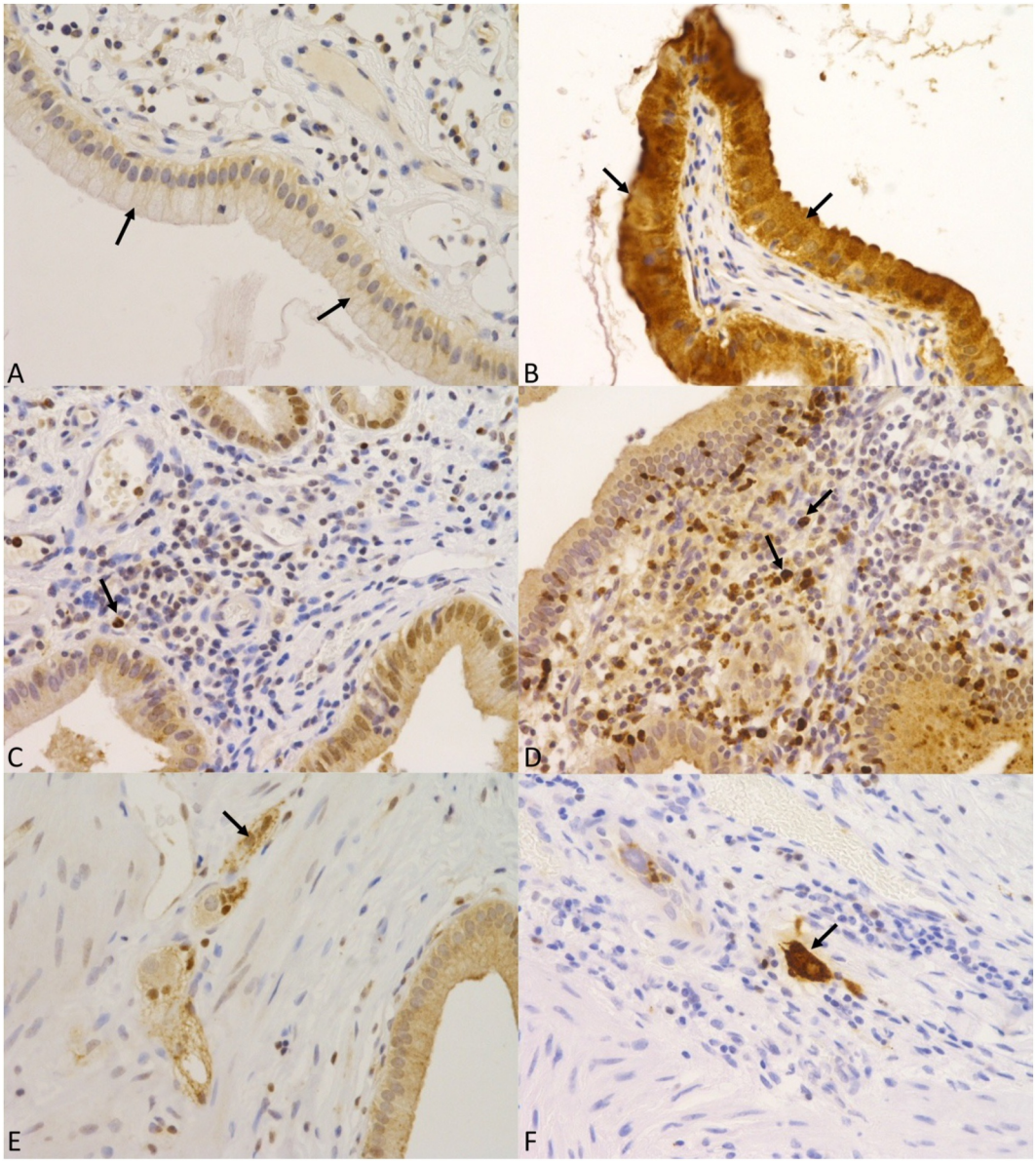

FIGURE 4 (A, B) – Expression of Substance P (brown cytoplasmic staining) in epithelial cells (black arrows) using immunohistochemical staining at ×400 magnification. (C, D) – Expression of Substance P in inflammatory cells (black arrows). (E, F) – Expression of Substance P in nerve ganglia (black arrows) (A, C and E) Low expression; (B, D and F) High expression of Substance P.

mechanism of intermittent pain due to gallbladder spasm. Although this could be easily treated with cholecystectomy further research on this theory is merited.

As the pathogenesis of cholecystitis is not fully understood, Prystowsky and Rege explored the concept of neurogenic inflammation in cholecystitis.[15] The presence of Substance P and other peptide transmitters is well documented in both guinea pig and human gallbladder nervous systems.[13,14] It was shown that stimulation of afferent, C-fiber nociceptors by capsaicin induced gallbladder inflammation by significant secretion of fluid into the lumen as well as release of myeloperoxidase, IL-1 and prostaglandin E2. Moreover, administration of lidocaine inhibited the inflammatory effects of capsaicin. Thus, the findings reported by Prystowsky and Rege,[15] taken into consideration with the known proinflammatory effects of Substance P and its presence in gallbladder nervous system, could suggest that release of peptide neurotransmitters by nociceptive neurons could play a role in the development of cholecystitis.

The studied cytokines are also considered to be important hyperalgesic mediators and, therefore, a major cause of abdominal pain in patients with intra-abdominal inflammation.[33,57,58] Ibeakanma and Vanner[57] reported that supernatant from biopsies taken from patients with ulcerative colitis induced hyperexcitability of nociceptive colonic neurons which was

mediated by TNF-α. In a study by Humes et al.,[58] patients with symptomatic diverticular disease were shown to have increased expression of TNF-α and IL-6 which was associated with visceral hypersensitivity to rectal balloon distension. The aforementioned studies provide strong evidence that TNF-α, IL-6 and Substance P are able to induce hyperalgesia when their expression is upregulated in gastrointestinal inflammation. However, despite our effort we have not found studies to directly link the expression of TNF-α, IL-6 and Substance P in gallbladder nociception. Therefore, the symptom of right upper quadrant pain suggesting cholecystitis in patients with subsequent histologically "normal" gallbladders could potentially be attributed to increased levels of TNF-α, IL-6 and Substance P.

Due to the presence of gallstones repeated episodes of injury to the gallbladder epithelium could lead to recurrent episodes of inflammation episodes that could compromise the function as well the integrity of the mucosa leading to a vicious cycle of inflammation and tissue repair. This could give rise to signs and symptoms suggesting cholecystitis and it is in agreement with our findings of recurrent episodes of abdominal pain in a large proportion of patients with histologically "normal" gallbladder specimens. This is also in keeping with our findings of increased lymphocyte count in patients with histologically "normal" gallbladders.

We recognize that this study had some limitations. First, this study was retrospective in nature and was conducted in a single center. Moreover, we examined the expression of only four inflammatory markers and a bigger study is needed to examine the expression of several other markers such as IL-1, IL-12, IL-15. The use of immunohistochemistry alone could also be considered a limitation of this study. Immunohistochemistry is a semi-quantitative method compared with other techniques such as quantitative polymerase chain reaction or fluorescence *in-situ* hybridization analysis of mRNA expression. However, the mRNA expression does not always correlate with functional protein expression owing to post-translational modifications. On the other hand, immunohistochemistry can assess the expression of protein which is the final product of gene expression. Nevertheless, better understanding of the gene expression in the entire spectrum of cholecystitis is crucial and more studies are needed to investigate the differences in gene expression between different types of cholecystitis. In particular, it would be worthwhile to understand why some patients with gallstones have gallbladders with abundant inflammatory infiltrates whereas others appear normal. Our findings suggest a potential role for the neurotransmitter substance *p* in modulating interplay between epithelium, leucocytes, and neurons in cholecystitis, which would be worthwhile to investigate further. Finally, we recognize that we have performed multiple comparisons between variables risking type I errors. However, when we applied the Bonferroni correction, the level of statistical significance did not change appreciably.

Gallstones are frequently found during ultrasonography for other conditions such as pregnancy. Therefore, an incidental finding of asymptomatic gallstones taken together with the risks associated with gallstones including malignancy [59] could challenge surgeons' decision-making skills on whether to offer prophylactic cholecystectomy or not. Thus, the surgeon's advice to the patient could be based on the rate of conversion from asymptomatic to symptomatic gallstones which has been reported to vary significantly from 10% to 26%.[60–62] Finally, given our findings that even the histologically "normal" gallbladders exhibit abnormal levels of inflammatory markers on immunohistochemistry, the significant conversion rate from asymptomatic to symptomatic gallstones taken together with the risks associated with gallstones and the low morbidity and mortality rates of cholecystectomy [63] a prophylactic cholecystectomy in a patient with asymptomatic gallstones might not be unjustifiable. However, further research is required to establish if patients with histologically "normal" gallbladders become asymptomatic post cholecystectomy or continue to have symptoms to suggest a non-biliary origin of the initial symptomatology.

AUTHOR CONTRIBUTIONS

Emmanouil Psaltis: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; writing – original draft; writing – review & editing. **Abed M. Zaitoun**: Conceptualization; data curation; formal analysis; investigation; methodology; resources; supervision; writing – original draft; writing – review & editing. **Keith R. Neal**: Conceptualization; investigation; methodology; writing – review & editing. **Dileep N. Lobo**: Conceptualization; data curation; funding acquisition; investigation; methodology; project administration; supervision; writing – original draft; writing – review & editing.

ACKNOWLEDGMENTS

This work was supported by the Medical Research Council [grant number MR/K00414X/1], Arthritis Research UK [grant number 19891] and the National Institute for Health Research Nottingham Biomedical Research Centre [grant number NIHR203310]. EP was funded by "State Scholarships Foundation" from the resources of EC "Education and Lifelong Learning Program", European Social Fund (ESF) and the NSRF 2007–2013 (grant number: 2013-ESPA-PE1-3680). This paper represents independent research. The funders had no role in the design or conduct of the work, or in the decision to publish. The views expressed are those of the authors and not necessarily those of the funders, NHS or Department of Health.

CONFLICT OF INTEREST STATEMENT

None of the authors has a direct conflict of interest to report. DNL has received an unrestricted educational grant from B. Braun for unrelated work. He has also received speaker's honoraria for unrelated work from Abbott, Nestlé and Corza.

DATA AVAILABILITY STATEMENT

Data will be available upon reasonable request from EP (gmanolisps@googlemail.com).

ETHICS STATEMENT

The study protocol was approved by the Health Research Authority (18/HRA/0292) and the need to obtain informed consent was waived.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

None used.

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