Papers on Anthropology XXXIV/1–2, 2025, pp. 7–26

GALLBLADDER PRO-INFLAMMATORY MARKERS IN CHILDREN WITH CALCULOUS CHOLECYSTITIS

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ABSTRACT

Calculous cholecystitis is associated with inflammation and several problems. Although it is a frequent condition in children, its morphopathogenesis remains unclear partly due to the role that inflammatory variables play.

Tissue samples from eleven children undergoing surgery for calculous cholecystitis were acquired. Five unaffected gallbladders were used as control. Slides were examined under a light microscope after tissues were immunohistochemically stained for IL-1β, IL-12, IL-13, HSP 60, SHH, and NF-κβ p105.

The Mann-Whitney U test was employed to assess the statistical differences between the markers. Statistically significant differences were found between patient and control gallbladder epithelium for IL-13 and HSP60. However, in the connective tissue, statistically significant differences were found for IL-1β, IL-12, IL-13, HSP60, SHH, and NF-κB p105. These findings suggest a potential role of pro-inflammatory markers in the morphopathogenesis of calculous cholecystitis.

Keywords: *gallstones; cholelithiasis; paediatrics; children; interleukins; inflammation; calculous cholecystitis*

INTRODUCTION

Calculous cholecystitis has attracted a lot of interest due to its increasing prevalence in recent years. Currently, the pathogenesis of the disease remains relatively unknown. Some studies suggest that the possible pathogenesis of cholelithiasis could be influenced by different factors – such as genetic, environmental and metabolic – as well as stasis or impairment of bile flow [16]. Cholelithiasis,

https://doi.org/10.12697/poa.2025.34.1-2.01

although more common in adults, has become more common in children over recent decades. According to a study published in *BMC Gastroenterology*, the incidence of cholelithiasis in children and adolescents appears to be rising, although it remains a relatively rare disease in the paediatric population. In the same study, researchers looked at the prevalence of cholelithiasis in children, and the range was between 0.13% and 1.9%. Epidemiological trends highlight the need for specific paediatric guidelines for diagnosis and management [6]. Nonetheless, the exact reasons for this disease are not fully understood. This area remains under-researched, and there is a need for more evidence-based studies as well as consistent guidelines for paediatric cholelithiasis

Despite the established role of pro-inflammatory markers such as interleukin 1 beta (IL-1β), interleukin 12 (IL-12), interleukin 13 (IL-13), sonic hedgehog protein (SHH), HSP 60 and nuclear factor κB p105 (NFκβ p105) in various inflammatory and immune responses, their expression and significance in the context of paediatric gallbladder pathology remain underexplored. To date, there has been a lack of comprehensive studies examining these pro-inflammatory markers in the gallbladders of children affected by cholecystitis or other gallbladder diseases.

Recent evidence suggests that interleukins such as IL-1α, IL-4, IL-6, IL-7, IL-8, and IL-17A may contribute to the inflammatory response in gallbladder pathology [5]. These cytokines regulate immune cell recruitment, promote tissue remodelling, and exacerbate the inflammatory milieu, potentially influencing disease severity and progression in paediatric patients.

Given their potential to provide insights into the pathophysiological mechanisms underlying these conditions, it is imperative to investigate their expression profiles in paediatric patients. This study aims to address this gap by analysing the presence and role of these pro-inflammatory markers in the gallbladders of affected children, thereby contributing to a better understanding of the disease's molecular underpinnings.

Interleukin-1 Beta (IL-1β) is known as a pro-inflammatory cytokine protein [43]. In humans, it is encoded by the IL1B gene [4]. IL-1β is involved in initiating and amplifying immune response to infection, injury or cellular stress [4, 9]. IL-1β is primarily produced by macrophages, monocytes and dendritic cells in response to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [41, 21]. In the case of cholecystitis, IL-1β plays a crucial role by inducing the release of secondary inflammatory mediators, such as tumour necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) [35]. Although IL-1β secretion and release is not a single,

uniform process, there are reports on different possible mechanisms. The IL-1β mechanism is rather complex and not yet fully understood [21].

Interleukin-12 (IL-12) is known as a heterodimeric cytokine composed of two subunits, p35 and p40. Collectively, these subunits play a crucial role in immune responses [38]. It connects innate and adaptive immunity by promoting the differentiation of naive T cells into Th_1 cells [37]. This process is essential for driving cellular immunity, particularly in response to intracellular pathogens. In the context of gallbladder inflammation, higher levels of IL-12 have been observed in cases of cholecystitis. This implies its potential involvement in the pathogenesis of the disease [35]. By stimulating the production of interferon-gamma (IFN-γ), IL-12 increases macrophage activation, thereby promoting local inflammatory responses within the gallbladder wall [34]. Macrophages and dendritic cells regulate IL-12 and IL-10 in ways that are essential for immune modulation [23]. This sustained immune activation may play a role in the chronic inflammatory environment observed in calculous cholecystitis.

Additionally, IL-12 does not act alone but rather functions as part of a broader immunological response. It not only facilitates Th_1 polarization but also plays a role in reinforcing this lineage through the regulation of transcription factors, such as STAT4 [11]. Recently published studies emphasize the importance of IL-12 signalling in maintaining Th_1cell-driven immunity and its contribution to tissue-specific inflammation [27]. Furthermore, studies have also shown that IL-12 influences inflammatory progression through sequential polarization mechanisms, thus potentially shaping the immunopathological features of affected organs, including the gallbladder [12, 30]. Collectively, these findings highlight IL-12 role as a key immunoregulatory cytokine that may play a role in both the initiation and maintenance of inflammation in paediatric calculous cholecystitis.

Interleukin-13 (IL-13) is a protein that in humans is encoded by the IL13 gene [14]. IL-13 is associated with Th2-mediated immune responses. It is more commonly associated and known for its involvement with allergic tissues [1]. However, it is also known for its role in regulating inflammation and tissue remodelling [29]. A study has shown that IL-13 stimulates eosinophilic and lymphocytic inflammation, thus inducing alveolar remodelling in the lungs through the induction of various matrix metalloproteinases (MMPs) [19]. Some evidence suggests that IL-13 also plays a role in tissue fibrosis and extracellular matrix remodelling. That could potentially be relevant in pathogenesis of cholecystitis [8, 29]. Additionally, IL-13 has been identified as a stimulator of

inflammation and tissue remodelling at sites of Th_2 inflammation. Furthermore, it contributes to the pathogenesis of disorders like asthma [3]. To conclude, these studies prove the involvement of IL-13 beyond allergic responses. While its role in cholecystitis is not yet fully understood, it may contribute to tissue fibrosis and expression of extracellular matrix components.

Sonic Hedgehog Protein (SHH) is part of the Hedgehog protein family, which also includes Desert Hedgehog (DHH) and Indian Hedgehog (IHH), encoded by the SHH gene [15]. Furthermore, SHH is a morphogenic protein integral that is known due to its involvement in embryonic development and tissue homeostasis [15]. In a recent study, SHH has been highlighted for its role in gastrointestinal inflammation together with IL-1β [40]. The study found that IL-1β promotes gastric atrophy by suppressing the expression of SHH, which leads to reduced gastric acid secretion, thus facilitating atrophic changes [40]. Another study indicates that the suppression of SHH by IL-1β can drive gastric and gallbladder inflammation [32]. This evidence suggests that SHH might not only be important for tissue remodelling but also in modulating immune responses in inflammatory conditions and possible cancer development [22, 32].

Heat Shock Protein 60 (HSP60) is known as a molecular chaperone. HSP60 is involved in protein folding and cellular stress responses [7, 13]. Its expression is upregulated by inflammatory conditions, further acting as a danger signal for the human body and activating its immune response [39]. Its altered expression has been linked to tumorigenesis, influencing cancer cell survival and proliferation. For example, decreased HSP60 expression in bladder carcinomas has been associated with poor prognosis – increased tumour infiltration. However, increased expression in ovarian tumours correlates with potentially better outcomes [2, 38]. These findings emphasize the multifaceted roles of HSP60 and the possible development of calculous cholecystitis.

Nuclear Factor Kappa B p105 (NF-κB p105) subunit is a protein that in humans is encoded by the NFKB1 gene [24]. NF-κB p105 serves as a precursor for the p50 subunit of the NF-κB transcription factor. It regulates genes that are involved in inflammation and immune responses-[33]. In the case of cholecystitis, activation of NF-κB pathways may lead to the transcription of pro-inflammatory cytokines like TNF-α and IL-6 as well as chemokines, such as IL-8 and adhesion molecules like intercellular adhesion molecule-1 (ICAM-1) [28, 10]. Thus, it sustains a chronic inflammatory reaction. As previously established, NF-κB p105 is a key transcription factor that regulates pro-inflammatory gene expression and contributes to chronic inflammation [20]. However, in

another study, dysregulation has been implicated in multiple inflammatory conditions, including gallbladder diseases, through pathways that sustain immune activation [25, 26].

To conclude, there is a pressing need for further evidence-based research related to cholelithiasis, specifically in the paediatric population and specific markers.

MATERIALS AND METHODS

Characteristics of Subjects

A total of 11 tissue samples were obtained from 11 female patients aged 12–17 years who underwent surgery for calculous cholecystitis. The inclusion criteria comprised a confirmed diagnosis of calculous cholecystitis, the presence of the chronic pain syndrome, confirmation by a gastroenterologist that the pain was gallbladder-related and that further pharmacological treatment was deemed inappropriate, along with the provision of informed consent by both the patients and their parents for surgical intervention. The exclusion criteria included the absence of a confirmed diagnosis of calculous cholecystitis, the lack of the chronic pain syndrome, or cases where the pain was not confirmed by a gastroenterologist to be gallbladder-related.

Five healthy tissue samples were used for controls, the control group contained only females that were 9–17 years old. Control samples were obtained during post-mortem autopsies with no known gallbladder disease and no signs of chronic inflammation in routine histology.

The obtained tissue samples were 2–4 mm in size, covering the full wall of the gallbladder in the region between the fundus and the neck. The Department of Children Surgery of the Children's University Hospital referred all the tissue samples for investigation to the Institute of Anatomy and Anthropology at Riga Stradiņš University. The research was done in accordance with the Helsinki declaration. The study was approved by the Ethical Committee at Riga Stradiņš University; the permit was issued on 10 May 2007. Written informed consent was obtained from parents in each case.

Histopathological analysis with hematoxylin and eosin

For the general morphological evaluation of the wall of the gallbladder, routine staining with hematoxylin and eosin was performed. The samples were fixed in a solution of 2% formaldehyde and 0.2% picric acid in 0.1 M phosphate buffer with a pH of 7.2. Then, the tissues were rinsed in Tyrode's solution containing 10% sucrose for 12 hours. Thereafter, the samples were embedded in paraffin and sectioned into thin slices measuring 6–7 μm. Histological staining with hematoxylin and eosin was performed to all samples. Each specimen was subsequently examined by bright-field microscopy to assess the morphological structure of the gallbladder wall in patients and controls.

Immunohistochemical Analysis

For the staining of specimens for specific markers, IL-1β (ab2105, rabbit polyclonal to anti-IL-1β, working dilution 1:100, Abcam Limited, Cambridge, United Kingdom), anti-IL-12 (ab106270, rabbit polyclonal to IL-12 p40 antibody, working dilution 1:200, Abcam Limited, Cambridge, United Kingdom), anti-IL-13 (orb10895, rabbit polyclonal to IL-13, working dilution 1:100, Biorbyt Ltd., Cambridge, United Kingdom), anti-HSP60(sc-1052, goat polyclonal to HSP 60, working dilution 1:100, Santa Cruz Biotechnology Inc., Dallas, United States of America), anti-SHH (ab53281, rabbit monoclonal to Sonic Hedgehog, working dilution 1:100, Abcam Limited, Cambridge, United Kingdom), anti-NF-κB p105 (ab32360, rabbit monoclonal to NF-κB p105/p50, working dilution 1:100, Abcam Limited, Cambridge, United Kingdom) were used.

The sample slides were analysed by light microscopy using non-parametric evaluation which includes grading of positively stained cells in connective tissues and epithelium of the gallbladder in the visual field. The results were labelled as follows: 0 – no positive cells, 0/+ – scant number of positive cells, 0/+ – occasional positive cells, + – few positive cells, +/++ – few to moderate number of positive cells, ++ – moderate number of positive cells, ++/+++ – moderate to numerous positive cells, +++ – numerous positive cells in the visual field [5].

For the examination of the samples, a Leica DC 300F camera microscope was used applying brightfield microscopy. Histological images were taken and analysed with the Image-Pro Plus 7.0 image visualization software.

Statistical analysis

The obtained data were processed using IBM SPSS Statistics software, version 29.0.0.0 (241) (IBM company, North Castle, Armonk, NY, USA). To analyse the difference between the patient and control groups, the Mann-Whitney U test was performed, and the level of significance was 5%; thus the p-value was <0.05. The data were ranked as ordinal values, with 0 representing no epithelium, 1 representing no tissue sample, 2 corresponding to no positive structures observed in the visual field, 3 a scant number of positive cells (00/+), 4 indicating occasional positive cells (0/+), 5 corresponding to few positive cells (+), 6 representing few to moderate number of positive cells (+/++), 7 being moderate number of positive cells (++), 8 corresponding to moderate to numerous positive cells (++/+++), 9 being numerous positive cells in the visual field (+++).

RESULTS

Routine histopathological analysis

The tissue samples obtained contained the whole gallbladder wall. Staining with hematoxylin and eosin revealed numerous inflammatory cells (predominantly lymphocytes, plasma cells, and neutrophils) in the connective tissue layers, indicating an inflammatory process. The smooth muscle layer appeared disorganized and possibly thickened. Epithelial lining hyperplasia could also be observed in some samples (Figure 1). Moreover, tissue samples showed muscular vacuolization and vascular obliteration.

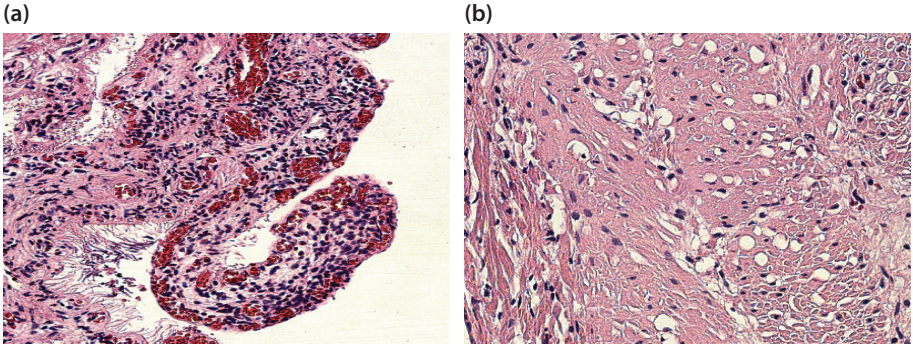


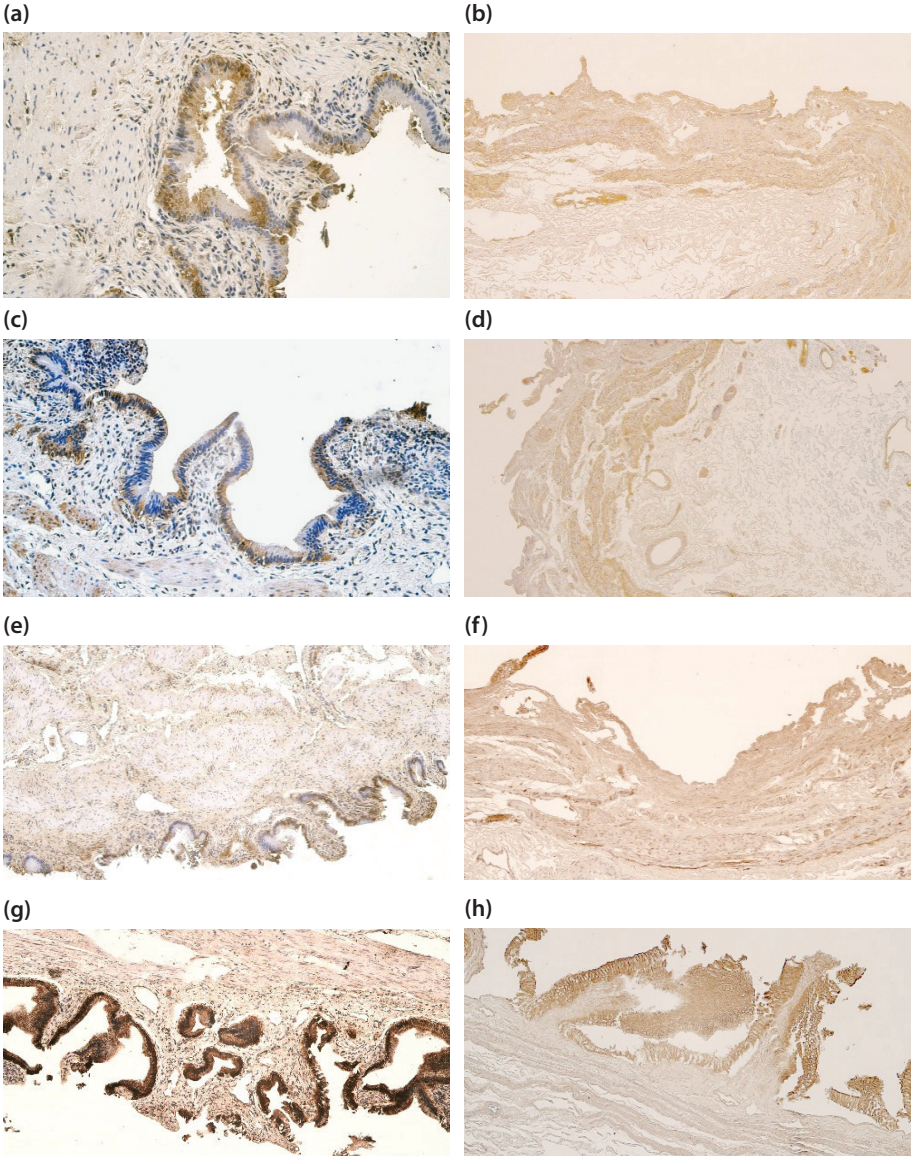
Figure 1. (a–b) Micrographs of gallbladder structures in children with calculous cholecystitis. Note fibrotic and inflammatory changes. Hematoxylin and eosin, ×250 and ×400.

Marker analysis

A statistically significant epithelial expression of HSP60 and IL-13 was observed, as shown in Table 3. On average, IL-1β expression in the epithelium of patient samples was classified as few to moderate (++/+++), as seen in Table 1. By comparison, the control group exhibited only occasional to few positive epithelial cells (Table 2). IL-12 showed few to moderate epithelial positivity (++/+++) on average in patients, while the control group demonstrated only occasional to few positive epithelial cells (Table 2), confirming an upregulated pro-inflammatory profile in the gallbladder epithelium (Figure 2c, 2d). IL-13 was observed in the epithelial layer at a few to moderate level (++/+++) on average in patient tissue, with some samples approaching moderate to numerous positivity (Table 1, Figure 2e). In contrast, the control samples displayed few to moderate positive cells on average (Table 2, Figure 2f), indicating a general upregulation in the patient group but with some overlap. For HSP60 the epithelial expression in patient samples was generally few to moderate (++/+++) (Table 1), with one patient displaying a numerous (++++) expression pattern in the visual field (Figure 2g). By contrast, the control group showed a lower range of expression, mostly in the few to moderate category or lower (Figure 2h, Table 2), emphasizing an elevated stress response in the diseased epithelium. On average, few (++) SHH-positive cells occasionally extending into the connective tissue were detected in patient epithelium (Table 1), (Figure 2i). However, the control group exhibited no detectable SHH expression (0) in either compartment (Figure 2j, Table 2), highlighting a potential disease-specific upregulation. Lastly, NF-κB p105 was expressed at a few to moderate level (++/+++) in the epithelium of patient samples on average, with one sample showing numerous positive cells (Table 1, Figure 2k). In contrast, control epithelial tissues showed few to moderate expression (Table 2, Figure 2l), reflecting a broader trend of increased activation in patient tissue.

In the connective tissue, statistically significant increases were observed for IL-1β, IL-12, IL-13, HSP60, SHH, and NF-κB p105 (Table 3). Patient samples showed IL-1β expression at a few to moderate level on average (++/+++), whereas control samples portrayed only few (++) positive cells (Table 1 and 2; Figure 2a, 2b). IL-12 was similarly elevated in patients averaging moderate (+++) positive cells, compared to occasional (+) positivity in control samples (Figure 2c, 2d; Tables 1 and 2). IL-13 expression in the connective tissue of patient samples averaged at moderate (+++) (Figure 2e), whereas the control group exhibited no detectable IL-13 positivity (0) (Figure 2f; Table 1 and 2). Likewise, HSP60 was observed at a few to moderate levels (++/+++) in patient

connective tissue (Table 1), while control samples showed no positive cells (0) (Table 2). SHH also showed a statistically significant increase, with few positive cells (++) seen in patient connective tissue, and no positivity (0) in controls (Figure 2i, 2j; Table 1 and 2). Finally, NF-κB p105 levels in the connective tissue averaged from few to moderate (++/+++) positive cells in patient tissues (Table 1), compared to only few (++) positive cells in the control group (Table 2), further supporting enhanced inflammatory activity in gallbladder disease.



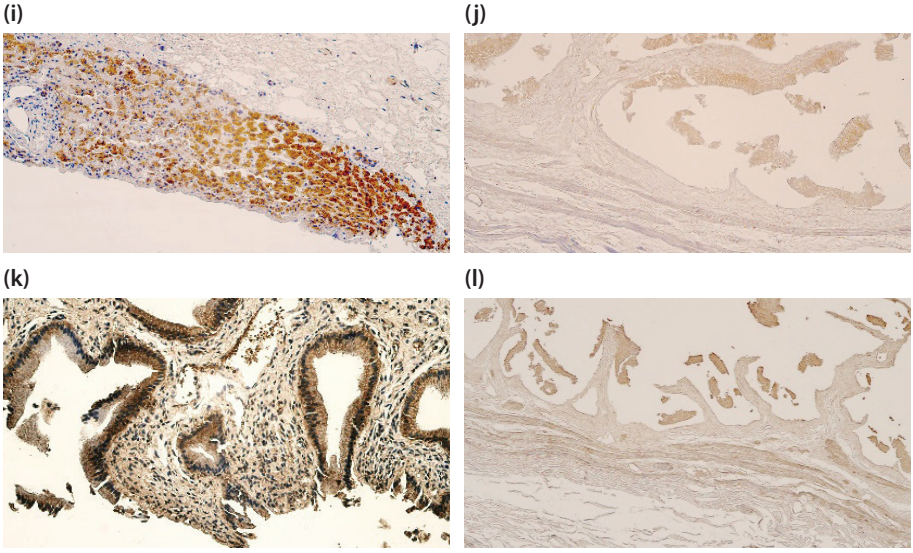


Figure 2. (a–l) Micrographs of gallbladder tissue in children with calculous cholecystitis and controls. (a) IL-1β expression in patient sample showing few to moderate (++/+++) positive epithelial and connective tissue cells, ×200. (b) IL-1β expression in control tissue with few (++) positive cells in the connective tissue, ×100. (c) IL-12 expression in patient sample showing moderate (+++) positive cells in both layers, ×200. (d) IL-12 expression in control tissue with occasional (+) positive cells in the connective tissue, ×100. (e) IL-13 expression in patient sample showing moderate to numerous (+++/++++) positive cells, ×100. (f) IL-13 expression in control tissue with few to moderate (++/+++) positive cells in the epithelium, ×100. (g) HSP60 expression in patient epithelium with numerous (++++) positive cells, ×200. (h) HSP60 expression in control tissue showing few to moderate (++/+++) positive cells, ×100. (i) SHH expression in patient tissue showing few (++) positive cells in the epithelium and connective tissue, ×200. (j) SHH expression in control tissue showing no detectable (0) positive cells, ×100. (k) NF-κB p105 in patient sample showing numerous (++++) positive cells in the epithelium, ×200. (l) NF-κB p105 expression in control tissue with few to moderate (++/+++) positive cells, ×100.

Tables 1 and 2 provide an overview of the immunohistochemical expression of pro-inflammatory markers in the epithelium and connective tissue of paediatric patients with calculous cholecystitis and control subjects. Following the data in Tables 1 and 2, statistical analysis using the Mann-Whitney U test revealed several significant differences between the patient and control groups. Across pro-inflammatory markers, patient samples demonstrated visibly increased staining intensity and broader distribution, particularly within the connective tissue.

Tables 1 and 2 illustrate the semi-quantitative distribution of marker positive cells in gallbladder tissues of paediatric patients and controls. In the connective tissue of patient samples, the average expression levels were observed for

IL-12 (average: ++), IL-13 (average: ++), and NF-κB p105 (average: ++), indicating moderate presence of positively stained cells (Table 1). For the patient samples of connective tissue, IL-1β, HSP60, and SHH showed slightly lower averages, each grading at +/++, reflecting few to moderate numbers of positive cells (Table 1). In contrast, control samples showed minimal expression for all markers in the connective tissue, with average scores not exceeding +, and several markers, including SHH, remaining at 0, indicating the absence of staining (Table 2).

In the epithelial layer, patient samples showed elevated expression of IL-13 (average: ++), while IL-1β, IL-12, and HSP60 averaged +/++, suggesting low-to-moderate inflammatory activity (Table 1). Meanwhile, in the same tissue, SHH and NF-κB p105 remained low in the epithelial layer, with a mean value of + (Table 1). Control samples in the epithelium exhibited overall lower expression across all the markers, with average values ranging between 0 and +/++, and SHH remaining completely negative (average: 0) (Table 2).

Statistical evaluation

To determine whether there were statistically significant differences in marker expression between patient and control groups, quantitative analysis was performed for IL-1β, IL-12, IL-13, HSP60, SHH, and NF-κB p105 in both the epithelium and connective tissue. Independent sample t-tests revealed significant increases in the number of positive cells in patient tissues compared to controls. Statistically significant differences were observed for the following markers: IL-1β ($p = 0.003$) and IL-12 ($p < 0.001$) in the connective tissue, IL-13 in both the epithelium ($p = 0.009$) and connective tissue ($p < 0.001$), HSP60 in both layers ($p = 0.013$ and $p < 0.001$), SHH ($p = 0.019$), and NF-κB p105 in the connective tissue ($p < 0.001$). These findings indicate a consistent upregulation of inflammatory and stress markers in diseased gallbladder tissues. The mean values and standard deviations for each marker are summarized in Table 3.

Table 1. Number of pro-inflammatory marker-positive cells in the gallbladder epithelium and connective tissue of patient samples.

Marker/ Patient	IL -1β		IL-12		IL-13		HSP 60		SHH		NF-κB p105	
	E	CT	E	CT	E	CT	E	CT	E	CT	E	CT
1.	++/+++	++	+++	++	++/+++	++/+++	+++	++	++/+++	++	++/+++	++
2.	+++	++	+++	++	+++	++/+++	++	++	++	++	++	++
3.	++/+++	++	+++	+++	+++	+++	++	++	++	+/++	++	++
4.	N	++	0/+	++/+++	+++	++	N	++	+	0	++	++
5.	N	++	N	++	+++	++/+++	N	+/++	N	+	++/+++	++
6.	++	++	++/+++	++	++/+++	+/++	++	++	0	+	++	++
7.	++	+	+++	++	+++	++	+/++	+/++	0/+	0	+/++	+++
8.	++	++	+++	++	++++	++/+++	++	++	+++	++	++	++
9.	++	++	+++	++/+++	+++	++	++	++	0	++	++	++
10.	+++	++	+++	++	++/+++	++/+++	++	++	++	+	++	+
11.	N	++	N	++	N	+/++	N	++	0	0	+	++
Avg.	+/++	+/++	+/++	++	++	++	+/++	+/++	+	+	+/++	++

Abbreviations for Table 1: IL's – Interleukins; IL-1β – interleukin 1β; IL-12 – interleukin 12; IL-13 – interleukin 13; HSP-60 – heat shock protein 60; SHH – Sonic hedgehog protein; NF-κB p105 – Nuclear Factor Kappa B (NF-κB) p105 subunit; E – epithelium; CT – connective tissue; N – no epithelium; No – no tissue sample; Avg – average; 0 – no positive cells, 00/+ – a scant number of positive cells, 0/+ – occasional positive cells, + – few positive cells, +/++ – few to moderate number of positive cells, ++ – moderate number of positive cells, ++/+++ – moderate to numerous positive cells, +++ – numerous positive cells in the visual field.

Table 2. Number of pro-inflammatory marker-positive cells in the gallbladder epithelium and connective tissue of control samples.

Marker/ Controls	IL -1β		IL-12		IL-13		HSP 60		SHH		NF-κB p105	
	E	CT	E	CT	E	CT	E	CT	E	CT	E	CT
1.	+/+ +	+	+ + +	+	0/+	0	+ +	0	0	0	+ +	0
2.	0	+	+ +	0/+	+ +	0	+ + +	0	0	0	+ + +	0
3.	0	+	N	0	+ +	0	+ + +	0	0	0	N	0
4.	+	+/++	+ + +	+	+ +	0	+ + +	0	0	0	+ + +	0
5.	0	+	+ +	+	+ +	0	+ + +	0	0	0	+ +	0
Avg.	0/+	+	+/+ +	0/+	+/+ +	0	+ +/+ + +	0	0	0	+/+ +	0

Abbreviations for Table 2: IL's – Interleukins; IL-1β – interleukin 1β; IL-12 – interleukin 12; IL-13 – interleukin 13; HSP-60 – heat shock protein 60; SHH – Sonic hedgehog protein; NF-κB p105 – Nuclear Factor Kappa B (NF-κB) p105 subunit; E – epithelium; CT – connective tissue; N – no epithelium; No – no tissue sample; Avg – average; 0 – no positive cells, 00/+ – a scant number of positive cells, 0/+ – occasional positive cells, + – few positive cells, +/++ – few to moderate number of positive cells, ++ – moderate number of positive cells, ++/+++ – moderate to numerous positive cells, +++ – numerous positive cells in the visual field.

Table 3. Significant differences in pro-inflammatory markers of epithelium and connective tissue between patient and control tissue samples.

Marker	IL -1β E	IL -1β CT	IL-12 E	IL-12 CT	IL-13 E	IL-13 CT	HSP60 E	HSP60 CT	SHH E	SHH CT	NF-κB p105 E	NF-κB p105 CT
***p*-value**	0.052	0.003	0.583	<0.001	0.009	<0.001	0.013	<0.001	0.090	0.019	0.583	<0.001
Mean	4.56	6.31	6.69	6.38	7.50	5.75	6.25	5.31	3.94	4.06	6.75	5.44
STD	3.777	0.946	3.57	1.746	2.449	2.720	3.256	2.330	2.792	2.235	2.049	2.502

Abbreviations for Table 3: IL's – Interleukins; IL-1β – interleukin 1β; IL-12 – interleukin 12; IL-13 – interleukin 13; HSP-60 – heat shock protein 60; SHH – Sonic hedgehog protein; NF-κB p105 – Nuclear Factor Kappa B (NF-κB) p105 subunit; E – epithelium; CT – connective tissue; STD – standard deviation

DISCUSSION

In the past, gallbladder disease was perceived as a condition affecting mostly adults. It was also frequently associated with dietary habits, a sedentary lifestyle, or metabolic syndrome. In paediatric populations, particularly among adolescent girls, the occurrence of calculous cholecystitis has often been underestimated or misattributed to functional gastrointestinal disorders [21]. This underrecognition has contributed to delayed diagnoses and inconsistent management approaches.

Recent epidemiological data, however, reveal a rising incidence of cholelithiasis and related inflammatory gallbladder disorders in children and adolescents [17, 6]. This trend has led to increased awareness within the medical community regarding the need to investigate paediatric-specific mechanisms, beyond the traditional views of disease models. Notably, several recent studies highlight the involvement of proinflammatory cytokines, stress-response proteins, and gene-regulated pathways in the morphopathogenesis of paediatric calculous cholecystitis [5, 33, 42].

This study provided valuable insight into and knowledge of the morphopathogenesis and inflammatory marker profile associated with paediatric calculous cholecystitis by analysing the expressions of IL-1β, IL-12, IL-13, HSP60, SHH, and NF-κB p105 markers in gallbladder tissues of affected and unaffected individuals. Thus, the study emphasized their potential roles in the inflammatory processes that contribute to tissue damage and chronic inflammation within the gallbladder epithelium and connective tissue and pathogenesis of calculous cholecystitis.

Our results revealed that the highest statistically significant differences were observed for IL-12 and NF-κB p105 in the connective tissue, both markers indicating p-values of < 0.001. Likewise, IL-13 and HSP60 in the connective tissue also exhibited highly significant differences ($p < 0.001$). The role of HSP60 in immune activation has been well-documented. One of the roles of HSP60 is to function as a damage-associated molecular pattern (DAMP). This means that it acts as a danger signal to the immune system when released extracellularly [36, 7]. One study found that extracellular HSP60 induces inflammation by activating and upregulating Toll-like receptors (TLRs), particularly TLR2 and TLR4, leading to an elevated immune response. This mechanism could potentially explain why HSP60 levels were significantly higher in the connective tissue of patients with calculous cholecystitis rather than in the control group, indicating a prolonged immune response and potential tissue damage [36].

We observed that IL-13 in the epithelium was associated with a *p*-value of 0.009, and HSP60 in the epithelium with a *p*-value of 0.013. This demonstrates statistically significant differences, but they are of higher variability, as indicated respectively by their standard deviations of 2.449 and 3.256. This variability can also be explained by differences in disease severity among patients, heterogeneity in the extent of fibrosis and tissue damage, as well as age-related or genetic factors that can influence cytokine expression. Furthermore, research has shown that IL-13 can alter the epithelial cell function. Thus, it leads to increased epithelial permeability and enhanced immune cell infiltration. This is important in the context of chronic inflammatory conditions like cholecystitis where a disrupted epithelial barrier may contribute to persistent inflammation. A study by Bhardwaj and Ghaffari has highlighted the role of IL-13 in modulating mucosal immunity and epithelial responses in other inflammatory conditions, which could provide insights into IL-13 effects in the gallbladder [1].

Furthermore, SHH in the connective tissue showed a moderate but statistically significant difference ($p = 0.019$), proving its potential involvement in tissue-specific inflammatory or regulatory processes. However, the mean value of 4.06 suggests that SHH is present in the connective tissue but at a lower level compared to other markers, for example IL-13 and HSP60. The standard deviation of 2.235 implies substantial differences in SHH expression between patients, which could also be explained by differences in disease severity among patients, heterogeneity in the extent of fibrosis and tissue damage. In addition, age-related or genetic factors can influence the marker expression. Furthermore, the relatively high variability can indicate that SHH expression is not consistent across all affected individuals. The SHH signalling pathway has been implicated in tissue regeneration and inflammatory processes, particularly within the gastrointestinal tract [18]. The increase in SHH expression could potentially suggest its potential role in gallbladder epithelial repair mechanisms. However, further research is needed to determine whether SHH correlates with disease severity or a specific morphopathological process and to understand the precise function of SHH in paediatric cholecystitis.

Interestingly, in this study, we found no statistically significant difference in the epithelial layer expression of IL-1β, IL-12, SHH, and NF-κB p105 between affected and unaffected individuals. At the same time, all the studied markers notably exhibited statistical significance in connective tissue layers of affected and unaffected individuals. Therefore, this unexpected discrepancy highlights the complexity of pro-inflammatory and signalling molecule distribution across tissue and suggests that further research is necessary to clarify the underlying mechanisms and their pathological relevance.

This study has some limitations that should be acknowledged. One significant limitation is the small sample size, which consists solely of the female paediatric population. This restricts the generalizability of our findings and highlights the need for larger-scale studies to confirm these results. Additionally, while immunohistochemical analysis offers valuable insights into the distribution of pro-inflammatory markers, future research that includes genetic analysis could provide a deeper understanding of the molecular mechanisms driving disease progression. Specifically, identifying endodermal genes that influence disease susceptibility and specific inflammatory gene profiles could help to correlate with the development of pathology.

CONCLUSIONS

An increased expression of IL-1β, IL-12, IL-13, HSP60, SHH, and NF-κB p105 in paediatric calculous cholecystitis-affected gallbladder wall displays their potential role in the morphopathogenesis of the disease. The elevated levels of these markers in epithelial and connective tissue layers suggest their potential involvement in immune system response, tissue remodelling, and chronic inflammation. The significant correlations that were observed between these markers in this study indicate a complex process with the potential involvement of other cytokines and markers.

FUNDING

This research received no external funding.

INSTITUTIONAL REVIEW BOARD STATEMENT

The study was approved by the research Ethics Committee of Riga Stradiņš University with wide approval on 10 May 2007. The protocol was designed in accordance with the Declaration of Helsinki guidelines.

ACKNOWLEDGEMENT

Dr. Arnis Engelis is greatly acknowledged for obtaining the material.

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