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Loss of gallbladder interstitial Cajal-like cells in patients with cholelithiasis

ARTUR PASTERNAK,*,†,[1] KRZYSZTOF GIL,‡,[1] ANDRZEJ MATYJA,* MARIUSZ GAJDA,§ KRYSTYNA SZTEFKO,¶ JERZY A. WALOCHA,† JAN KULIG* & PIOTR THOR‡

*First Department of General, Oncological and Gastrointestinal Surgery, Jagiellonian University Medical College, Krakow, Poland

†Department of Anatomy, Jagiellonian University Medical College, Krakow, Poland

‡ Department of Pathophysiology, Jagiellonian University Medical College, Krakow, Poland

§Department of Histology, Jagiellonian University Medical College, Krakow, Poland

¶Department of Clinical Biochemistry, Jagiellonian University Medical College, Krakow, Poland

Abstract

Background *Interstitial cells of Cajal (ICCs) play an important role in the regulation of gut motility. There is growing evidence that interstitial Cajal-like cells (ICLCs) are present in the gallbladder wall. We hypothesize that changes in the density of ICLCs in the gallbladder wall may lead to the development of cholelithiasis due to the impairment of the gallbladder motility. The purpose of this study was to identify ICLCs in the gallbladders of patients with gallstones and to assess their densities.* ***Methods*** *Data from 30 patients with gallstones and 25 individuals without gallstones were compared. Tissue samples were obtained during surgery, embedded in paraffin, and cut into sections. Following staining for CD117 and mast cell tryptase, the number of ICLCs and mast cells was determined using image analysis.* ***Key Results*** *Cells positive for the c-Kit receptor (CD117) were detected in the gallbladder wall in all cases examined. Interstitial Cajal-like cells were most frequently observed in the muscularis propria. The density of ICLCs in the muscularis propria was significantly lower in the patients with gallstones than the density observed in the controls (26.24 ± 10.89 vs 56.29 ± 13.35 cells/mm^2). In contrast, the number of mast cells in the gallbladder was increased in the patients with gallstones when compared with the controls (143 ± 24 vs 112 ± 19 cells/mm^2).* ***Conclusions & Inferences*** *The histopathological differences observed in this study may help elucidate the pathophysiology of gallstones. Gallbladder motility may be affected by the decreased number of ICLCs in patients with cholelithiasis.*

Keywords *c-kit, gallstones, interstitial Cajal like cells, mast cells.*

Abbreviations: ANOVA, analysis of variance; HIER, heat-induced epitope retrieval; ICC, interstitial cells of Cajal; ICLC, interstitial Cajal-like cells.

INTRODUCTION

Interstitial cells of Cajal (ICCs) were first described by Ramon y Cajal over 100 years ago as a specific gut neuron. Formerly called 'interstitial neurons', these cells were re-discovered approximately 40 years ago and have been successfully identified using contemporary methods, including electron microscopy and immunohistochemistry.[1–3] Interstitial cells of Cajal are found along the entire gastrointestinal tract and are localized mainly in the smooth muscle layers of the gut. Interstitial cells of Cajal are known to play a pivotal role in the control of gastrointestinal tract motility by providing electrical impulses for slow wave generation and regulating smooth muscle activity and neurotransmission.[4–7] A characteristic feature of ICCs is the expression of transmembrane tyrosine kinase receptor proteins, including the c-Kit receptor (CD117),

Address for Correspondence
Krzysztof Gil, Department of Pathophysiology, Jagiellonian University Medical College, Czysta 18 Street, 31-121 Krakow, Poland.
Tel: +4812 6333947; fax: +4812 6329056;
e-mail: mpgil@cyf-kr.edu.pl
[1]These authors contributed equally to this work.

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which enables the identification of ICCs using immunohistochemical and molecular methods.[8] Disturbances in gastrointestinal motility after the loss of or damage to ICCs have been widely reported in several clinical states, including gastroparesis, constipation, achalasia, Chagas disease, Hirschsprung's disease, congenital hypertrophic pyloric stenosis, intestinal pseudo-obstructions, and diverticular disease of the colon.[6,7]

Multiple research teams have investigated cells located in various tissues outside of the gut, including the pancreas, ureter, urethra, bladder, blood vessels, male and female reproductive organs, mammary glands, placenta, heart, and lungs that are similar to ICCs.[9–11] These cells are known as interstitial Cajal-like cells (ICLCs), a term that was proposed by Popescu and Faussone-Pellegrini in 2010 to be replaced by 'telocytes'.[10,11]

Interstitial Cajal-like cells were recently discovered in the wall of the human gallbladder by Hinescu *et al.*[12] and in the bile ducts by Ahmadi *et al.*[13] Interstitial Cajal-like cells in the gallbladder formed a cellular network that is likely involved in biliary tract motility. Previous studies[12,13] and research performed in animal models[14–16] have suggested the potential role of ICLCs in functional disturbances of the gallbladder. The presence of ICLCs in the gallbladder has been suspected in recent years because some gallbladder tumors have been reported to originate from cells with an ICC-like phenotype.[17–20] These tumors were previously considered to be gastrointestinal stromal tumors (GIST).

Gallstone disease is likely to be a heterogeneous entity that involves several coexisting defects, including cholesterol hypersecretion with non-physiological oversaturation of the bile, gallbladder hypomotility (i.e., biliary stasis), accelerated cholesterol crystallization in the bile and mucus hypersecretion with gel formation in the gallbladder.[21,22] Gallbladder stasis may be a key event in the pathogenesis of cholelithiasis by allowing time for cholesterol microcrystals (nucleation) to precipitate from lithogenic bile that is supersaturated with cholesterol as well as allowing for their subsequent growth into macroscopic stones.[23–25] A subpopulation of cholesterol gallstone patients ('bad contractors') demonstrate severely decreased or even absent postprandial gallbladder emptying.[22] To date, there is no direct evidence that the impairment of gallbladder motility is essential for gallstone formation in all patients. However, a decrease in the density of ICLCs in the muscular layer of the gallbladder could induce bile stasis and lead to gallstone formation. The purpose of this study was to identify ICLCs within the human gallbladder and to assess ICLC density in patients with gallstones.

MATERIALS AND METHODS

SUBJECTS

A total of 55 patients surgically treated in the First Department of General, Oncological and Gastrointestinal Surgery at the Jagiellonian University Medical College in 2010 were enrolled in the study. Thirty consecutive patients operated for symptomatic gallstone disease were qualified to participate in the study group (8 males, mean age 51.9 ± 10.7 years; 22 females, mean age 52.9 ± 15.1 years). The presence of gallstones in the gallbladder (cholecystolithiasis) was confirmed with an abdominal ultrasound scan performed 2 weeks prior to surgery. Patients with gallstones had detectable calculi in the gallbladder on ultrasound examination. They presented with recurrent episodes of biliary colic. None of these patients had associated choledocholithiasis or acute cholecystitis. Elective laparoscopic cholecystectomies were performed on the patients in the study group.

The control group consisted of 25 consecutive patients (11 males, mean age 62.0 ± 7.6 years; 14 females, mean age 61.0 ± 9.8 years) who were electively treated for pancreatic head tumors and had no pre- or intraoperative signs of cholelithiasis or jaundice. A pancreaticoduodenectomy was performed according to the Whipple or Traverso-Longmire techniques in patients with a resectable pancreatic head tumor. In cases of non-resectable lesions, a bypass (gastroenterostomy) was generally performed for palliative purposes. Gallbladders that were not affected with tumors and did not have any gallstones were removed. The serum bilirubin levels, which were measured preoperatively, were normal in both groups.

Ethical approval

The study was conducted in accordance with the moral, ethical, regulatory, and scientific principles governing clinical research. All surgical samples were retrieved with the approval of the Jagiellonian University Bioethical Committee conforming to the Declaration of Helsinki guidelines (protocol number – KBET/30/B/2010).

Tissue specimens

Fresh cholecystectomy specimens were cut longitudinally from the fundus to the neck and rinsed thoroughly with PBS (phosphate-buffered saline, 0.01 mol L^{-1}, pH = 7.4). Tissue samples were fixed in 4% phosphate-buffered paraformaldehyde, routinely processed and embedded in paraffin. Serial sections were cut and mounted on poly-L-lysine-coated glass slides. The sections were deparaffinized in xylene, rehydrated through a graded series of alcohol, and transferred to PBS prior to staining.

Histopathological examination and count of toluidine blue-labeled mast cells

Sections from each gallbladder were examined after routine staining with hematoxylin and eosin (H&E). A microscopic examination was performed to assess the type and intensity of

Table 1 Inflammation and mast cell count in the gallbladder wall in patients with gallstones and the control group

	Inflammation (% of patients)			Number of mast cells (cells/per 1 mm^2)	
Group	Mild	Moderate	Severe	TB	IHC
Cholelithiasis	17	50	33	143 ± 24	205 ± 33
Controls	79	21	–	112 ± 19	161 ± 37

TB, toluidine blue staining; IHC, immunohistochemistry/mast cell tryptase staining.

the inflammatory infiltration, and to detect the presence of fibrosis, cholesterolosis or other lesions. Slides were also stained with toluidine blue to identify and count mast cells (Table 1).

Immunohistochemistry

Tissue antigens were retrieved using the heat-induced epitope retrieval (HIER) method. Deparaffinized sections were incubated in a citrate buffer solution (pH 6.0) for 15 min in a microwave oven (250 W, temperature 96 °C), followed by cooling at room temperature. A preincubation step was performed with 5% normal goat serum and 0.5% Triton X-100 for 20 min to reduce non-specific binding and to increase penetration of the antibodies. For the simultaneous visualization of two antigens, an indirect double immunofluorescence procedure was used. The sections were incubated for 17 h at room temperature in humidified chambers with a mixture of either a rabbit polyclonal anti-c-Kit antibody (anti-CD117; A4502; Dako, Glostrup, Denmark; diluted 1 : 150) and a mouse monoclonal anti-mast cell tryptase antibody (M7052; Dako; 1 : 800) or the anti-CD117 antibody and a mouse monoclonal anti-CD34 antibody (NCL-ENDO; Novocastra, Newcastle, UK; 1 : 50). The sections were rinsed in PBS and incubated for 1 h at room temperature with a mixture of a Cy3-conjugated goat anti-rabbit antibody (111-165-144; Jackson ImmunoResearch, West Grove, PA, USA; 165-144; 1 : 600) and a biotinylated goat anti-mouse antibody (115-065-146; Jackson IR; 1 : 600). The primary and secondary antibodies were diluted in the same solution used in the preincubation step. After washing in PBS, the slides were incubated with DTAF-conjugated streptavidin (016-010-084; Jackson IR; 1 : 500 in PBS) for 1 h. After a final rinse in PBS, the nuclei were counterstained with DAPI (D9542; Sigma, St. Louis, MO, USA; 1 : 30 000) for 30 s. The sections were mounted in Vectashield medium (H-1000; Vector Laboratories, Burlingame, CA, USA) to minimize photobleaching of fluorophores.

Microscopic examination and quantification of immunohistochemically labeled ICLCs and mast cells

An Olympus BX50 epifluorescence microscope (Olympus, Tokyo, Japan) equipped with a mercury arc lamp and U-MNG, U-MNIBA, and U-MWA filter sets was used to evaluate Cy3 (red), DTAF (green), and DAPI (blue) fluorescence, respectively. The same microscope was used for histopathological evaluation using a bright field configuration. Images were collected using Olympus DP71 digital CCD camera controlled by Olympus AnalySIS FIVE software and stored as TIFF files.

The quantity and distribution in the lamina propria and muscularis propria, respectively, of c-Kit-positive ICLCs and tryptase-positive cells (Table 2) were assessed using image analysis software (Multiscan v.18; Computers Scanning System, Warsaw, Poland). The use of mast cell tryptase staining to identify and distinguish c-Kit-positive mast cells from ICLCs highlighted the morphological variability in the mast cells and the potential for misidentification of these two distinct cell populations when using c-Kit immunostaining alone (Fig. 1).

The distribution of ICLCs in the gallbladder corpus fragment, which was primarily localized 1–2 cm from the gallbladder fundus, was quantitatively assessed by applying the criteria for ICLCs (i.e., c-Kit positive and tryptase-negative; Fig. 1). These cells were counted in 10 consecutive high-power fields (400×). The data are expressed as the mean number of cells per 1 mm^2 of gallbladder muscle tissue. The width of the muscularis propria in the same region of the gallbladder was measured and the number of CD34-positive cells was also evaluated.

Statistical analysis

The data are expressed as the mean and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA), followed by a *post hoc* LSD test. All statistical analyses were performed using the STATISTICA 9.0 software package (StatSoft, Tulsa, OK, USA). *P* values less than 0.05 were considered statistically significant.

RESULTS

Histopathological examination of the gallbladders revealed chronic inflammation of varying intensity in both groups of patients. The inflammation was assessed by a pathologist and designated as mild, moderate, or severe. In contrast with the control group, where only mild to intermediate inflammation was

Table 2 Densities of mast cells and interstitial Cajal-like cells in immunostained sections of the gallbladder wall

Group	Control		Cholelithiasis		
Grade of inflammation	Minor	Medium	Minor	Medium	Major
Mast cells – lamina propria	74.0 ± 42.0	101.6 ± 47.9	122.40 ± 66.9	104.8 ± 33.0	160.3 ± 52.7
Mast cells – muscularis propria	69.2 ± 21.9	76.5 ± 21.7	71.6 ± 20.3	82.1 ± 30.8	80.8 ± 27.5
Mast cells – cumulative	143.3 ± 45.8	178.2 ± 54.4	194.0 ± 71.2	186.8 ± 44.8	241.1 ± 53.5
Mast cells – cumulative in group	160.7 ± 36.6			204.6 ± 33.2	
Interstitial Cajal-like cells	52.8 ± 25.4	48.6 ± 19.8	21.2 ± 18.4	24.9 ± 17.0	25.0 ± 14.1
Interstitial Cajal-like cells – cumulative in group	56.29 ± 13.35			26.24 ± 10.89	

Data expressed as mean number and standard deviation of cells per 1 mm^2.

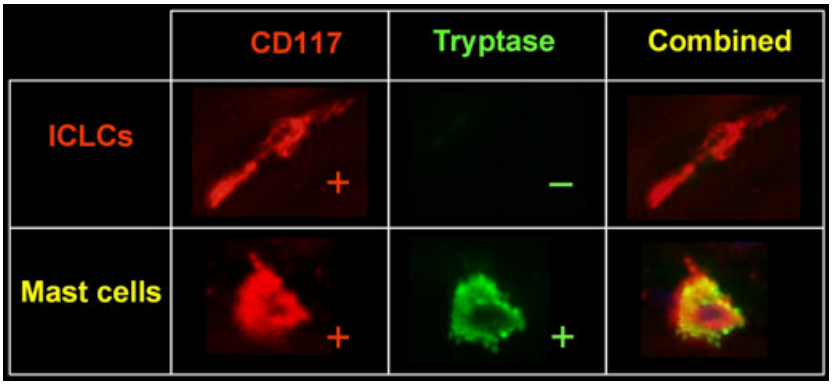

Figure 1 Identification of interstitial Cajal-like cells (ICLCs) in cross-sections stained for CD117 (red) and tryptase (green). Only CD117-positive and tryptase-negative cells were considered to be ICLCs.

observed, severe inflammation was predominantly present in the study group (Table 1).

The number of mast cells in the gallbladder wall was counted in specimens stained with toluidine blue and immunostained for tryptase. Tryptase immunolabeling allowed to identify more mast cells than toluidine blue staining as even the partially degranulated cells remained tryptase-immunopositive. While mast cells were present in all layers of the gallbladder wall, they were predominantly localized in the lamina propria, than in the muscularis propria (Table 2, Fig. 2).

The total number of mast cells, as well as the mast cell infiltrates located in the lamina propria were significantly increased ($P < 0.05$) in patients with gallstones when compared with control subjects (Table 1) and were correlated with the severity of the inflammation. The mast cells number in muscularis propria did not differ between controls and cholelithiasis group (Table 2).

In immunostained slides, the c-Kit and tryptase double-positive mast cells were generally round or oval shaped and had a centrally located nucleus. Spindle-shaped, elongated profiles were occasionally observed (Figs 3 and 4). Interstitial Cajal-like cells were defined as c-Kit-positive nucleated cells that lacked mast cell tryptase expression (Fig. 1). Interstitial Cajal-like cells were predominantly fusiform in shape with branches that were visible in some sections. Interstitial Cajal-like cells were observed throughout the gallbladder, including the fundus, body (corpus), and neck, although they were predominantly located in the corpus. The ICLCs were detected almost exclusively within the muscularis propria and were primarily parallel to the smooth muscle cells. However, in a few cases, the ICLCs were localized within the connective tissue separating the smooth muscle bundles. The ICLCs typically appeared individually or in small clusters of two to three cells. There were no differences in the shape, size, and morphology of the ICLCs in the study and control groups.

The number of ICLCs in the gallbladder wall between the first and second centimeter of the corpus was significantly lower in the study group when compared with the control group (26.24 ± 10.89 cells/

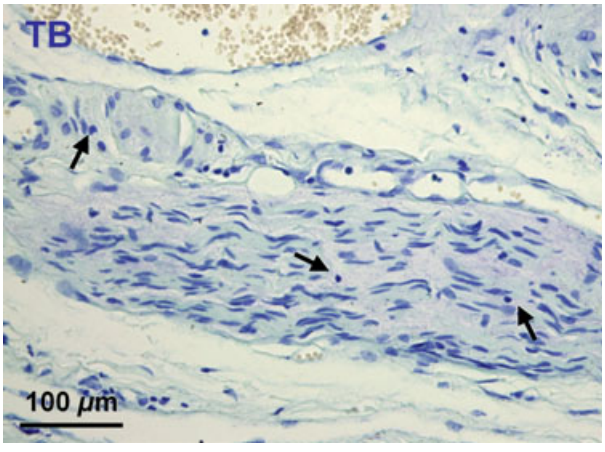

Figure 2 Cross-sections of the gallbladder wall from the control group stained with toluidine blue. Mast cells (arrows) within the muscularis propria.

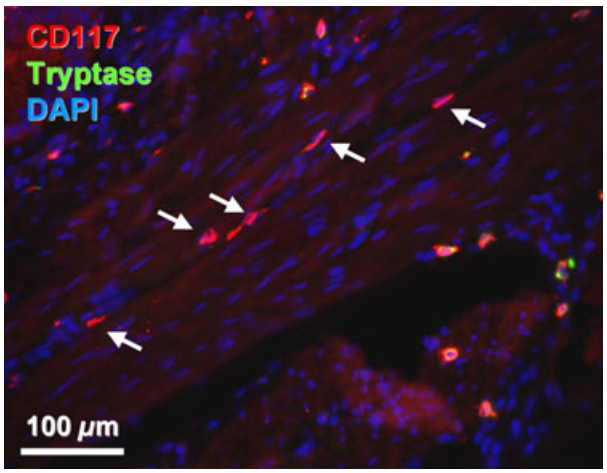

Figure 3 Cross-sections of the gallbladder wall from the control group stained for CD117 (red) and tryptase (green). The nuclei are counter-stained with DAPI (blue). Numerous CD117-positive and tryptase-negative ICLCs are present (arrows).

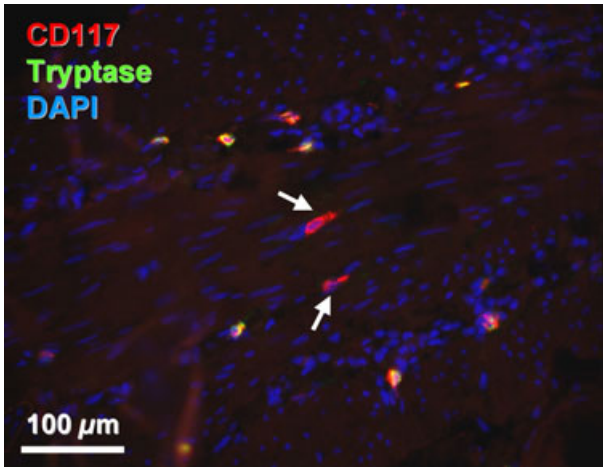

Figure 4 Cross-sections of the gallbladder wall from the study group stained for CD117 (red) and tryptase (green). The nuclei are counter-stained with DAPI (blue). Very few CD117-positive and tryptase-negative ICLCs are present (arrows).

mm^2 *vs* 56.29 ± 13.35 cells/mm^2 in the muscularis propria, $P < 0.001$) (Figs 3 and 4). Furthermore, ICLCs in the mild, moderate, and severe cases were evaluated separately to ascertain, whether the number of these cells was similar or not in patients with gallstones. Interestingly, the decrease in ICLCs density was present in all subgroups of patients with cholelithiasis and was unrelated to the inflammatory grade (Table 2).

The thickness of gallbladder wall was measured using image analysis. In particular, the thickness of the muscularis propria was significantly increased in the study group when compared with the control group (4267 ± 567 μm *vs* 3610 ± 346 μm; $P < 0.01$) (Fig. 5).

CD34-positive cells were identified in the gallbladder wall; however, they appeared to be predominantly of vascular origin. CD34-positive cells were rarely observed in the muscularis propria (Fig. 6). Based on the CD34 and CD117 staining, these CD34-positive cells were c-Kit negative, and no prolongations were observed. Unfortunately, reliable quantitative analysis was not possible due to the limited number of cells.

DISCUSSION

Interstitial Cajal-like cells in the human gallbladder were originally described in GISTs that originated from cells with a phenotype similar to ICCs in the gallbladder wall expressing CD117.[17–20] Interstitial Cajal-like cells were subsequently identified in animals. Lavoie *et al.*[14] provided morphological and physiological evidence for the existence of ICLCs in the guinea pig gallbladder and proved their function in electrical coupling in smooth muscle cells. Balemba *et al.*[26] described the important role of the mitochondrial Ca^{2+} handling in spontaneous rhythmic activity of the smooth muscle cells and ICLCs, underscored the role of ICLCs in regulating the tone and motility of the gallbladder in guinea pigs. These studies support the role of ICLCs in the generation and propagation of the spontaneous rhythmicity and excitability of the gallbladder. These observations were later confirmed by Huang *et al.*[27] using whole-mount preparations of the gallbladder and extrahepatic guinea pig biliary ducts. Xu *et al.*[28] demonstrated that cholecystokinin-A receptor on ICLCs, was essential for gallbladder muscle contractions in guinea pig muscle strips. Sun *et al.*[15] successfully identified ICLCs in the gallbladder of CD1 mice using confocal microscopy of whole-mount flat

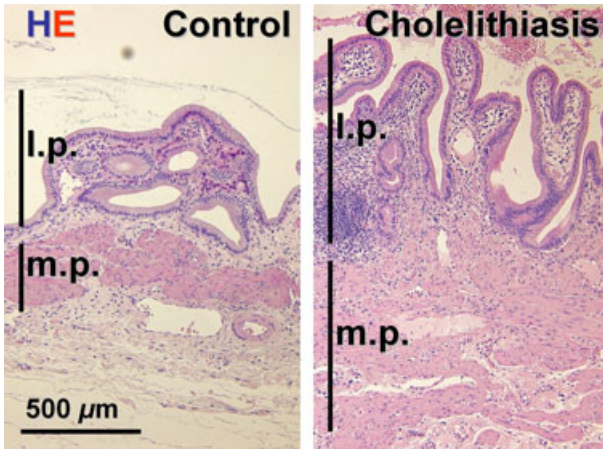

Figure 5 Comparison of the thicknesses of the wall of gallbladders from control group (left panel) and study group (right panel) in sections stained routinely with hematoxylin and eosin. l.p – lamina propria, m.p. – muscularis propria.

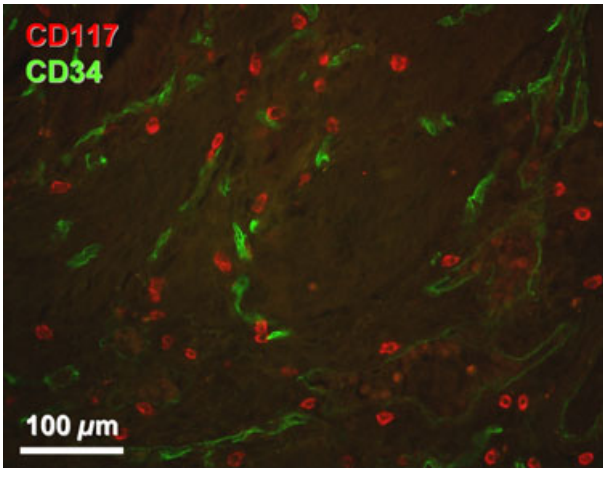

Figure 6 Cross-sections of the gallbladder wall from the control group stained for CD117 (red) and CD34 (green). No co-localization of CD117 and CD34 signals was observed.

preparations or enzyme-dispersed cells. These ICLCs were organized into a partially branched and partially spindle-shaped network.

The aim of the current study was to compare the location and number of ICLCs within the gallbladder wall of patients suffering from cholelithiasis and gallstone-free controls. The identification of ICLCs within the gallbladder wall cross-sections was based on a double immunofluorescence technique using anti-c-Kit and anti-tryptase antibodies to distinguish ICLCs from mast cells. Interstitial Cajal-like cells were predominantly located in the muscularis propria and the mast cells in the lamina propria. The ICLCs typically appeared individually or in small clusters of two to three cells, but they did not form a visible network. Hinescu *et al.* reported such a network, whereas Ahmadi *et al.* did not.[12,13] This discrepancy should be resolved in future studies given that the organization of ICLCs appears to be important for signal transduction and motility control in the gallbladder. However, networks are best seen in whole mounts (2D/3D imaging), therefore the failure to see networks in our study is due to the nature of preparations used. Thin paraffin sections did not allow proper determination of this architectural organization.

The published data on ICLCs in the human gallbladder are very limited. Although Hinescu *et al.*[12] and Ahmadi *et al.*[13] have published related studies, only the research by Hinescu *et al.* has provided reliable statistical data on ICLCs in the gallbladder. They reported an approximate ICLC density of 100–110 cells/mm^2 in specimens stained with methylene blue. These cells were located in the lamina propria, with some very close to the epithelium, and in the connective tissue spaces between bundles of smooth muscle cells. In toluidine blue–stained slides, the ICLCs accounted for 5.5% of the subepithelial cells in the gallbladder wall. Our results showed a slightly lower ICLC density (50–60 cells/mm^2), but our assessment was performed on more selectively stained slides (i.e., double immunohistochemistry). Other technical aspects could also explain differences in the data. In general, the data concerning ICLCs distribution and number are conflicting for multiple reasons, including difficulties in visualizing ICLCs in formalin-fixed/paraffin-embedded tissues (despite heat-induced antigen retrieval), proper human tissue handling (e.g., fixatives) the use of antibodies with varying sensitivities, and a general lack of standardization for quantifying ICC and ICLC populations. An establishment of control values and standardized protocols for tissue collection, fixation, and ICC visualization are required for the field to progress.[7]

We selected one specific region of the gallbladder for the quantitative analysis of ICLCs to obtain reliable results. An assessment of the thickness of the gallbladder wall was necessary for data interpretation because the ICLCs were counted in the muscularis propria. We suspect that the decrease in ICLC density in the study group resulted from hypertrophy of the muscularis propria. Indeed, there was a significant 15% increase in the thickness of the gallbladder wall in the study group compared with the control group, although the ICLC count was reduced by 50%.

We report a significant decrease in the density of ICLCs in the gallbladder wall in patients with gallstones. The amount of ICLCs was decreased in all subgroups of patients with cholelithiasis and was unrelated to the inflammatory grade.

It is difficult to propose a unique, rational, and clear hypothesis for the loss of ICLCs due to an increased lithogenicity of bile, although there are several possibilities. We evaluated the cholesterol saturation index (CSI), which is an accepted parameter relevant to bile lithogenicity,[29] in both groups of patients. We found an increased CSI in the patients with gallstones (data not shown), which could be related to the loss of ICLCs. However, the mechanism underlying the destructive influence of bile on ICLCs remains unclear. Our findings are supported by Hu *et al.*[16] who demonstrated that the expression of c-Kit mRNA and c-Kit protein in the gallbladder wall were significantly decreased in the gallbladders of guinea pigs fed a high cholesterol diet. Because the authors did not exclude mast cells, which are known to express c-Kit, from the c-Kit assessment, the decrease in c-Kit levels could have been caused by the degranulation of mast cells and not only by damage to the ICLCs. However, the Hu *et al.* study provided insight into the pathogenesis of gallstone formation, similar to the studies by Xu and Shaffer,[30] by showing that gallbladder hypomotility resulted from increased cholesterol levels. The excess cholesterol in the smooth muscle of the gallbladder attenuates the ability of the muscle to contract as a result of alterations in signal transduction, changes in ion channel activity, decoupled membrane receptor–ligand interactions, and disturbances in contractile protein activity.[31] In the recent study performed on guinea pigs fed lithogenic diet, Lavoie *et al.*[32] reported cholesterol accumulation in gallbladder smooth muscles in the plasma membrane, especially membrane caveolae, leading to decrease in membrane fluidity. Subsequently, the rhythmic electric activity was altered. The hypertrophy was also detected in the muscularis propria and contractile response to an agonist was decreased. Because the ICC and ICLCs

require large amounts Ca^{2+} to generate rhythmic electrical activity, the disturbances of membrane and cytosolic calcium homeostasis would lead to disruption of the membrane potential oscillation in ICC/ICLCs, influencing the pacemaker function. So far, however, no direct link between cytosolic calcium disturbances and ICLCs cell death has been reported.

The observed decrease in the number of ICLCs in the gallbladder wall of patients with cholelithiasis in our study must be taken into consideration as a possible mechanism underlying gallbladder dysmotility resulting from the increased lithogenic properties of bile. The role of ICLCs in the regulation of bile duct motility appears to be an important pathological factor in gallstone disease.

Another important mechanism underlying ICLC loss that should be considered concerns chronic inflammatory processes involving the gallbladder wall. In this study, chronic inflammation was predominantly present in the gallbladders of patients with gallstones and was associated with a significant increase in the mast cell count in this group, although this increase was especially present in the lamina propria. Portincasa *et al.*[25] described the impaired motility of the gallbladder caused by mild inflammation. One can speculate that these disturbances may be due to the loss of ICLCs as a consequence of chronic inflammatory processes.

The apoptotic mechanisms leading to the loss of gallbladder ICLCs should be considered. Apoptosis of ICCs might be caused by multiple processes, including chronic inflammatory reactions.[7,33] As reported by Gibbons *et al.*,[33] the level of apoptosis in ICCs in the healthy colon indicates that these cells must be continually replaced to maintain intact networks. It is possible that the reduced number of ICLCs in the inflamed gallbladder wall in patients with gallstones could be caused by the apoptosis of ICLCs that exceeded their regeneration. Finally, the reduced number of ICLCs might be of a primary (hereditary) origin caused by trans-differentiation of the ICC precursor cells in the smooth muscles in the gut.[34] Thus, gallstone formation might be related to primary disrupted gallbladder motility in patients with gallstone disease. However, there is no evidence for this in the clinical and experimental reports on gallbladders or biliary ducts. It is possible that all of the mechanisms described above are involved in the reduced ICLC density observed in the gallbladders of patients with gallstones.

In conclusion, damage to ICLCs in the gallbladder wall of patients with cholelithiasis could be related to an increased bile lithogenicity index or chronic inflammatory processes involving a gallbladder filled with gallstones. Both mechanisms may participate in gallstone formation by reducing the number of ICLCs in the gallbladder wall. These observations will contribute to further advancements in developing new treatment strategies for gallstones.

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DISCLOSURE

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

AP and KG were involved in study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, statistical analysis, obtained funding, study supervision, and final acceptance of the manuscript; AM was involved in study concept and design, acquisition of data, critical revision of the manuscript for important intellectual content, obtained funding, study supervision, and final acceptance of the manuscript; MG contributed to histology – technical support, acquisition of data, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, and final acceptance of the manuscript; KS was involved in study concept and design, acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, statistical analysis, study supervision, and final acceptance of the manuscript; JW was involved in drafting of the manuscript, critical revision of the manuscript for important intellectual content, obtained funding, study supervision, and final acceptance of the manuscript; JK contributed toward acquisition of data, critical revision of the manuscript for important intellectual content, obtained funding, study supervision, and final acceptance of the manuscript; PT was involved in study concept and design, critical revision of the manuscript for important intellectual content, study supervision, and final acceptance of the manuscript.

REFERENCES

1 Faussone-Pellegrini MS, Cortesini C, Romagnoli P. Ultrastructure of the tunica muscularis of the cardial portion of the human esophagus and stomach, with special reference to the so-called Cajal's interstitial cells. *Arch Ital Anat Embriol* 1977; **82**: 157–77.

2 Faussone-Pellegrini MS, Thuneberg L. Guide to the identification of

interstitial cells of Cajal. *Microsc Res Tech* 1999; **47**: 248–66.
3 Thuneberg L. Interstitial cells of Cajal: intestinal pacemaker cells? *Adv Anat Embryol Cell Biol* 1982; **71**: 1–130.
4 Huizinga JD, Thuneberg L, Kluppel M *et al.* W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. *Nature* 1995; **373**: 347–9.
5 Koh SD, Sanders KM, Ward SM. Spontaneous electrical rhythmicity in cultured interstitial cells of cajal from the murine small intestine. *J Physiol* 1998; **513**: 203–13.
6 Farrugia G. Interstitial cells of Cajal in health and disease. *Neurogastroenterol Motil* 2008; **20**(Suppl. 1): 54–63.
7 Huizinga JD, Zarate N, Farrugia G. Physiology, injury, and recovery of interstitial cells of Cajal: basic and clinical science. *Gastroenterology* 2009; **137**: 1548–56.
8 Lammie A, Drobnjak M, Gerald W *et al.* Expression of c-kit and kit ligand proteins in normal human tissues. *J Histochem Cytochem* 1994; **42**: 1417–25.
9 Huizinga JD, Faussone-Pellegrini MS. About the presence of interstitial cells of Cajal outside the musculature of the gastrointestinal tract. *J Cell Mol Med* 2005; **9**: 468–73.
10 Popescu LM, Faussone Pellegrini MS. TELOCYTES - a case of serendipity: the winding way from Interstitial Cells of Cajal (ICC), via Interstitial Cajal-Like Cells (ICLC) to TELOCYTES. *J Cell Mol Med* 2010; **14**: 729–40.
11 Faussone Pellegrini MS, Popescu LM. Telocytes. *BioMol Concepts* 2011; **2**: 481–9.
12 Hinescu ME, Ardeleanu C, Gherghiceanu M *et al.* Interstitial Cajal-like cells in human gallbladder. *J Mol Histol* 2007; **38**: 275–84.
13 Ahmadi O, Nicholson Mde L, Gould ML *et al.* Interstitial cells of Cajal are present in human extrahepatic bile ducts. *J Gastroenterol Hepatol* 2010; **25**: 277–85.
14 Lavoie B, Balemba OB, Nelson MT *et al.* Morphological and physiological evidence for interstitial cell of Cajal-like cells in the guinea pig gallbladder. *J Physiol* 2007; **579**: 487–501.
15 Sun X, Yu B, Xu L *et al.* Interstitial cells of Cajal in the murine gallbladder. *Scand J Gastroenterol* 2006; **41**: 1218–26.
16 Hu WM, Luo HS, Ding XW *et al.* Expression of c-kit messenger ribonucleic acid and c-kit protein in the gallbladders in guinea pigs of high cholesterol diet. *Dig Dis Sci* 2009; **54**: 1651–5.
17 Ortiz-Hidalgo C, de Leon Bojorge B, Albores-Saavedra J. Stromal tumor of the gallbladder with phenotype of interstitial cells of Cajal: a previously unrecognized neoplasm. *Am J Surg Pathol* 2000; **24**: 1420–3.
18 Mendoza-Marin M, Hoang MP, Albores-Saavedra J. Malignant stromal tumor of the gallbladder with interstitial cells of Cajal phenotype. *Arch Pathol Lab Med* 2002; **126**: 481–3.
19 Park JK, Choi SH, Lee S *et al.* Malignant gastrointestinal stromal tumor of the gallbladder. *J Korean Med Sci* 2004; **19**: 763–7.
20 Furihata M, Fujimori T, Imura J *et al.* Malignant stromal tumor, so called ''gastrointestinal stromal tumor'', with rhabdomyomatous differentiation occurring in the gallbladder. *Pathol Res Pract* 2005; **201**: 609–13.
21 Apstein MD, Carey MC. Pathogenesis of cholesterol gallstones: a parsimonious hypothesis. *Eur J Clin Invest* 1996; **26**: 343–52.
22 Portincasa P, Moschetta A, Palasciano G. Cholesterol gallstone disease. *Lancet* 2006; **368**: 230–9.
23 Pomeranz IS, Shaffer EA. Abnormal gallbladder emptying in a subgroup of patients with gallstones. *Gastroenterology* 1985; **88**: 787–91.
24 Sharma BC, Agarwal DK, Dhiman RK *et al.* Bile lithogenicity and gallbladder emptying in patients with microlithiasis: effect of bile acid therapy. *Gastroenterology* 1998; **115**: 124–8.
25 Portincasa P, Di Ciaula A, Vendemiale G *et al.* Gallbladder motility and cholesterol crystallization in bile from patients with pigment and cholesterol gallstones. *Eur J Clin Invest* 2000; **30**: 317–24.
26 Balemba OB, Bartoo AC, Nelson MT *et al.* Role of mitochondria in spontaneous rhythmic activity and intracellular calcium waves in the guinea pig gallbladder smooth muscle. *Am J Physiol Gastrointest Liver Physiol* 2008; **294**: G467–76.
27 Huang Y, Mei F, Yu B *et al.* Distribution of the interstitial Cajal-like cells in the gallbladder and extrahepatic biliary duct of the guinea-pig. *Acta Histochem* 2009; **111**: 157–65.
28 Xu D, Yu BP, Luo HS *et al.* Control of gallbladder contractions by cholecystokinin through cholecystokinin-A receptors on gallbladder interstitial cells of Cajal. *World J Gastroenterol* 2008; **14**: 2882–7.
29 Swell L, Bell CC Jr, Gregory DH *et al.* The cholesterol saturation index of human bile. *Am J Dig Dis* 1974; **19**: 261–5.
30 Xu QW, Shaffer EA. The potential site of impaired gallbladder contractility in an animal model of cholesterol gallstone disease. *Gastroenterology* 1996; **110**: 251–7.
31 Jennings LJ, Xu QW, Firth TA *et al.* Cholesterol inhibits spontaneous action potentials and calcium currents in guinea pig gallbladder smooth muscle. *Am J Physiol* 1999; **277**: G1017–26.
32 Lavoie B, Nausch B, Zane EA *et al.* Disruption of gallbladder smooth muscle function is an early feature in the development of cholesterol gallstone disease. *Neurogastroenterol Motil* 2012; **24**: e313–24.doi: 10.1111/j.1365-2982.2012.01935
33 Gibbons SJ, De Giorgio R, Faussone-Pellegrini MS *et al.* Apoptotic cell death of human interstitial cells of Cajal. *Neurogastroenterol Motil* 2009; **21**: 85–93.
34 Torihashi S, Nishi K, Tokutomi Y *et al.* Blockade of kit signaling induces transdifferentiation of interstitial cells of cajal to a smooth muscle phenotype. *Gastroenterology* 1999; **117**: 140–8.