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# Glial Cell Line-Derived Neurotrophic Factor, S-100 Protein and Synaptophysin Expression in Biliary Atresia Gallbladder Tissue

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## ABSTRACT

**Purpose:** Biliary atresia (BA) is a disease that manifests as jaundice after birth and leads to progressive destruction of the ductal system in the liver. The aim of this study was to investigate histopathological changes and immunohistochemically examine the expression of glial cell line-derived neurotrophic factor (GDNF), synaptophysin, and S-100 protein in the gallbladder of BA patients.
**Methods:** The study included a BA group of 29 patients and a control group of 41 children with cholecystectomy. Gallbladder tissue removed during surgery was obtained and examined immunohistochemically and histopathologically. Tissue samples of both groups were immunohistochemically assessed in terms of GDNF, S-100 protein, and synaptophysin expression. Expression was classified as present or absent. Inflammatory activity assessment with hematoxylin and eosin staining and fibrosis assessment with Masson's trichrome staining were performed for tissue sample sections of both groups.
**Results:** Ganglion cells were not present in gallbladder tissue samples of the BA group. Immunohistochemically, GDNF, synaptophysin, and S-100 expression was not detected in the BA group. Histopathological examination revealed more frequent fibrosis and slightly higher inflammatory activity in the BA than in the control group.
**Conclusion:** We speculate that GDNF expression will no longer continue in this region, when the damage caused by inflammation of the extrahepatic bile ducts reaches a critical threshold. The study's findings may represent a missing link in the chain of events forming the etiology of BA and may be helpful in its diagnosis.

**Keywords:** Biliary atresia; Glial cell line-derived neurotrophic factor; Synaptophysin; S-100 protein; Gallbladder

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**Conflict of Interest**

The authors have no financial conflicts of interest.

# INTRODUCTION

Biliary atresia (BA) is a disease that manifests as jaundice caused by neonatal hyperbilirubinemia and leads to progressive destruction of the ductal system in the liver. BA is categorized into three types. In the most common type, atresia is present in the entire extrahepatic ductal system [1].

Although BA was initially thought to be an untreatable disease, many studies have investigated it, and some patients can now be successfully treated [1]. However, its etiopathogenesis is still not fully understood [1-5].

The enteric nervous system (ENS) plays an important role in many physiological events of gastrointestinal system, such as peristalsis, mucosal blood flow, and water and ion transport. Developmental impairment of the ENS leads to significant problems in the functioning of the gastrointestinal system. Neurotrophic factors play an important role in the development of the ENS [6]. Glial cell line-derived neurotrophic factor (GDNF) is a protein that belongs in the transforming growth factor β-family and consists of 134 amino acids. It is vital for the development of the ENS [6,7].

Synaptophysin is a neuromuscular junction marker in the nervous system and is the main component of acetyl cholinesterase storage compartments in the junction area. Synaptophysin expression is used to evaluate neuromuscular junction innervations [8].

S-100 is a calcium-binding protein located in the region of central and peripheral nervous system ganglion cells [9].

The aim of this study was to investigate histopathological changes and immunohistochemically examine the expression of GDNF, synaptophysin, and S-100 in the gallbladder of BA patients.

# MATERIALS AND METHODS

## Study groups

This single-center case-control study was approved by the Scientific Research Publication Ethics Committee of the Faculty of Medicine of Inonu University Malatya/Turkey (No. 2018/9-1). The study protocol conformed to the provisions of the Declaration of Helsinki. Informed consent was obtained from all patients' parents.

The BA group consisted of patients diagnosed with BA by intraoperative cholangiography who had no concomitant diseases (such as Hirschsprung's disease or intestinal atresia) or other major anomalies, whose cholecystectomy samples were adequate and suitable for histopathological evaluation, and whose parents consented to their participation in the study. Patients who were diagnosed with BA but had concomitant diseases (such as Hirschsprung's disease, intestinal atresia, or necrotizing enterocolitis) or other major additional anomalies, whose gallbladder tissue was not adequate and/or suitable for histopathological evaluation, and whose parents did not consent to their participation were excluded. In this study, 34 BA patients were eligible for inclusion. Five patients could not be included for some reasons

(e.g., not enough gallbladder tissue is available, some patients parents not allowed). The BA group thus consisted of 29 patients. All patients underwent Kasai portoenterostomy.

The control group consisted of patients aged 0–18 years undergoing cholecystectomy due to cholecystitis who had not previously undergone gastrointestinal tract surgery and had no concomitant chronic diseases (such as ulcerative colitis, Crohn's disease, or diabetes mellitus), whose cholecystectomy samples were adequate and suitable for histopathological evaluation, and whose parents consented to their participation. Adult patients, patients who had previously undergone gastrointestinal tract surgery or had concomitant liver or chronic diseases or other major anomalies, whose parents did not consent to their participation, and whose gallbladder tissue samples were insufficient and/or unsuitable for histopathological evaluation were excluded. According to the inclusion and exclusion criteria, 41 patients were included in the control group, 28 of whom underwent laparoscopic and 13 underwent open cholecystectomies.

### Preparation of gallbladder tissue samples for histopathological examination

For both groups, full-thickness samples of gallbladder tissue removed during surgery were obtained and studied. The tissue samples were obtained from slightly above the cystic canal. The samples were rinsed with normal saline and immersed in 10% formalin for 24 hours. They were then buried in paraffin blocks. Sections 5 μm thick were cut and stained with hematoxylin and eosin and Masson's trichrome. The prepared samples were examined histopathologically by light microscopy for signs of fibrosis and inflammation.

### Immunohistochemical methods

Sections were placed on positively charged slides and rehydrated through 100%, 95%, and 70% alcohol series. For antigen retrieval, the slides were placed in a pressure cooker (Retriever 2100; Aptum, Southampton, UK) and heat-treated in citrate buffer (pH 7.6; Thermo Scientific, Fremont, CA, USA) at 121℃ for 15 minutes. After cooling to room temperature, the slides were immersed in phosphate-buffered saline (PBS). They were then treated with 3% hydrogen peroxide for 10 minutes and washed with PBS at room temperature. Then, protein V-blocking solution (Thermo Scientific) was dropped on the sections for 5 minutes. After removal of the protein-blocking agent, the sections were incubated with GDNF (sc-13147; Santa Cruz Biotechnology, Dallas, TX, USA; dilution ratio: 1:300), synaptophysin (sc-17750; Santa Cruz Biotechnology; dilution ratio: 1:300), and S-100 (sc-53438; Santa Cruz Biotechnology; dilution ratio: 1:300) monoclonal antibodies for 1 hours at room temperature. The slides were washed with PBS, incubated with biotinylated goat anti-polyvalent secondary antibody for 10 minutes at room temperature, and again washed with PBS. Streptavidin peroxidase was then applied for 10 minutes at room temperature, and the slides were washed with PBS. In all these steps, a polyvalent HRP kit (Thermo Scientific) for immunohistochemistry staining was used according to the manufacturer's protocol. Immunohistochemistry staining was completed with the application of AEC chromogen (Thermo Scientific)+AEC substrate buffer (Thermo Scientific) for 15 minutes at room temperature. The slides were again washed with PBS and immersed in distilled water. The sections were counterstained with Mayer's hematoxylin for 2 minutes. Excess hematoxylin was removed with tap water and then distilled water. Lastly, the slides were mounted in an aqueous mounting medium (Thermo Scientific).

**Table 1.** Gallbladder tissue inflammatory activity ratings

| Inflammation parameter | Mild | Moderate | Severe |
|---|---|---|---|
| Degree of inflammatory mononuclear infiltrate | Diffuse, not more than 10 inflammatory cells per HPF in any layer | Diffuse, between 11 to 30 cells per HPF | Diffuse, more than 31 cells per HPF or follicular |
| Degree of activity | Only vascular congestion and/or prominent eosinophils were observed (less than 10 neutrophils/HPF) | Activity showed the previous changes and neutrophilic infiltrate with permeation (10–30 neutrophils/HPF) | Activity also showed erosion (with more than 30 neutrophils/HPF) |

HPF: high power field.

**Table 2.** Gallbladder tissue fibrosis ratings

| Fibrosis parameter | Definition | Score |
|---|---|---|
| No fibrosis | No fibrosis detected | 0 |
| Mild | Fibrosis in lamina propria (uneven collagen depositionin 20% of the material) | 1 |
| Moderate | Fibrosis, including muscularis propria (uneven collagen deposition in 21–70% of the material) | 2 |
| Severe | Fibrosis covering all areas (uneven collagen or lamellar fibroplasia in 71% of the material) | 3 |

### Evaluation of sections for fibrosis and inflammatory activity

Inflammatory activity assessment with hematoxylin and eosin staining and fibrosis assessment with Masson's trichrome staining were performed for tissue sample sections of both groups as described by Barcia [10]. These evaluations also have ratings. The rating systems used are defined for inflammation and fibrosis in gallbladder tissue. The gallbladder inflammatory activity and fibrosis ratings are displayed in **Tables 1** and **2**, respectively.

### Immunohistochemical evaluation of gallbladder tissue samples

Tissue samples of both groups were immunohistochemically assessed in terms of GDNF, S-100, and synaptophysin expression. Expression was classified as present or absent. Positive control tissue samples were used for the immunohistochemical examination of GDNF, S-100, and synaptophysin expression.

### Statistical analysis

The normality of the data was evaluated by the Shapiro-Wilk test. Quantitative data were expressed as medians and ranges. The Mann-Whitney U-test was used for comparisons of quantitative data. Qualitative data were expressed as absolute numbers and percentages. Comparisons of qualitative data were performed by Pearson's chi-square test, Fisher's exact test, or a continuity correction chi-square test as appropriate. In all analyses, a two-sided $p$-value of less than 0.05 was considered statistically significant.

## RESULTS

The median age of the BA group patients at the time of surgery was 63 days, ranging between 45 and 101 days. The median age of the control group patients was 13 years, ranging between 5 and 17 years.

There was no statistically significant difference between the two groups in terms of postoperative complications (**Table 3**). In the BA group, minor upper gastrointestinal bleeding due to steroid treatment was observed in two patients, wound infection in one patient, and incisional hernia in one patient. Hepatic portoenterostomy revision was performed on one patient. In the control group, incisional hernia was observed in one patient and wound infection in two patients.

**Table 3.** Statistical analysis results of immunohistochemistry and histopathological evaluation data

| Parameters | Biliary atresia | Control | $p$-value |
|---|---|---|---|
| Complication | | | 0.168 |
|    Positive | 24 (82.7) | 38 (92.7) | |
|    Negative | 5 (17.2) | 3 (7.3) | |
| Synaptophysin expression | | | <0.001 |
|    Negative | 29 (100.0) | 0 (0.0) | |
|    Positive | 0 (0.0) | 41 (100.0) | |
| S-100 expression | | | <0.001 |
|    Negative | 29 (100.0) | 0 (0.0) | |
|    Positive | 0 (0.0) | 41 (100.0) | |
| GDNF expression | | | <0.001 |
|    Negative | 29 (100.0) | 0 (0.0) | |
|    Positive | 0 (0.0) | 41 (100.0) | |
| Ganglion cell | | | <0.001 |
|    Negative | 29 (100.0) | 0 (0.0) | |
|    Positive | 0 (0.0) | 41 (100.0) | |
| Fibrosis in stain of masson's trichrome | | | <0.001 |
|    Negative | 4 (13.7)[*] | 28 (68.3)[†] | |
|    Mild | 3 (10.3)[*] | 10 (24.4)[*] | |
|    Moderate | 3 (10.3)[*] | 3 (7.3)[*] | |
|    Severe | 19 (65.5)[*] | 0 (0.0)[†] | |
| Chronic Inflammation in H&E | | | 0.189 |
|    Negative | 2 (6.8) | 0 (0.0) | |
|    Mild | 27 (93.1) | 41 (100) | |
| Acute Inflammatory Activity in H&E | | | 0.008 |
|    Negative | 20 (68.9) | 39 (95.1) | |
|    Mild | 9 (31.0) | 2 (4.9) | |

Values are presented as number (%).
GDNF: glial cell line-derived neurotrophic factor.
The two groups were found to differ only in negative and severe categories. The difference between the mild and moderate categories was not found to be meaningful.
[*],[†]Two groups differs in categories where they have different superscript letters.

Although histopathologically detected in the control group, ganglion cells were not detected in gallbladder tissue samples of BA patients (**Table 3**).

Immunohistochemically, GDNF, synaptophysin, and S-100 expression was not detected in the tissue samples of the BA group (**Table 3**, **Figs. 1**-**3**). In contrast, GDNF, synaptophysin, and S-100 expression was present in all patients in the control group (**Table 3**, **Figs. 4**-**7**).
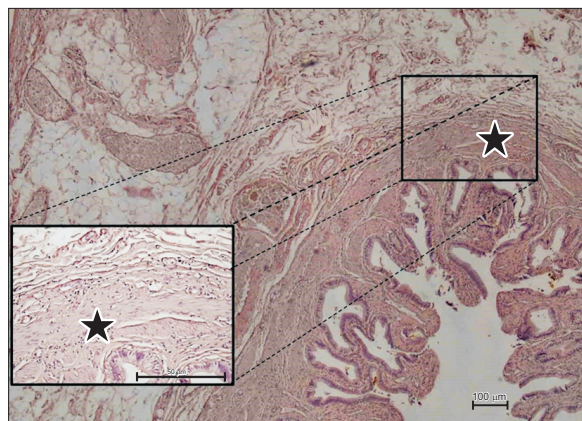


**Fig. 1.** Negative GDNF immunoreactivity in layers of gallbladder mucosa, submucosa, and tunica muscularis (asterisk). The ganglion structure is not monitored was showed in 40× and 200× magnification respectively.
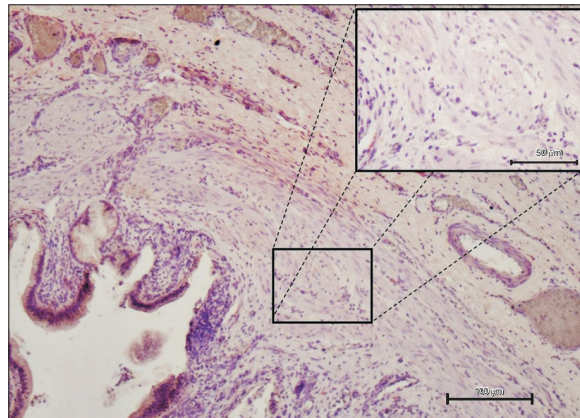GDNF: glial cell line-derived neurotrophic factor.

**Fig. 2.** Negative synaptophysin immunoreactivity in gallbladder tissue was showed in 100× and 400× magnification respectively.
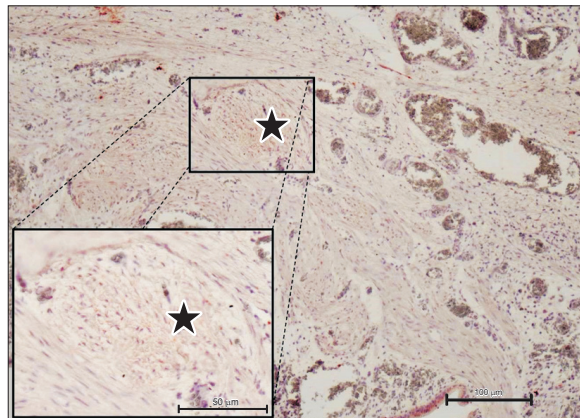


**Fig. 3.** Negative S-100 immunoreactivity (asterisk) in gallbladder tissue was showed in 100× and 400× magnification respectively.
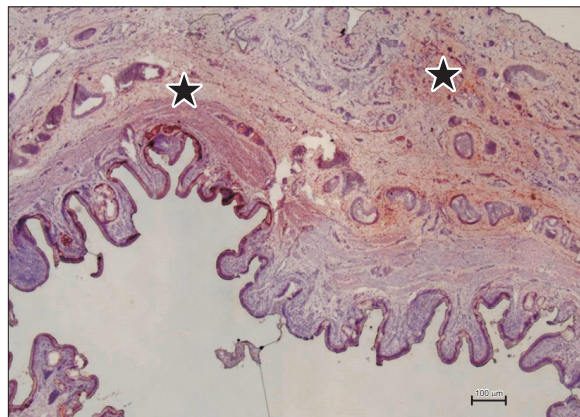


**Fig. 4.** Positive GDNF immunoreactivity (asterisk) in gallbladder tissue (40× magnification).
GDNF: glial cell line-derived neurotrophic factor.

Histopathologically, the presence of fibrosis was significantly more frequent in tissue samples of the BA group than in those of the control group (**Table 3**, **Fig. 8**).
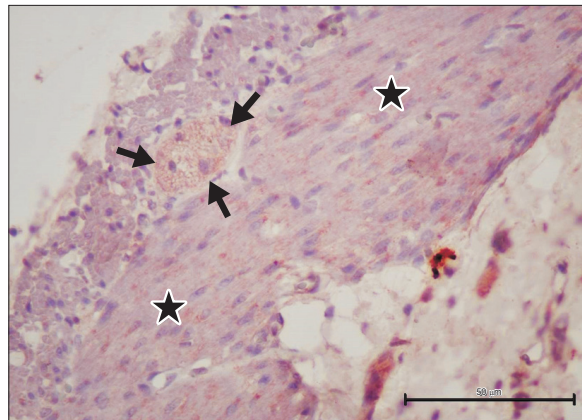
**Fig. 5.** Positive GDNF immunoreactivity in tunica muscularis (asterisk) and ganglion tissue (arrow) (400× magnification).
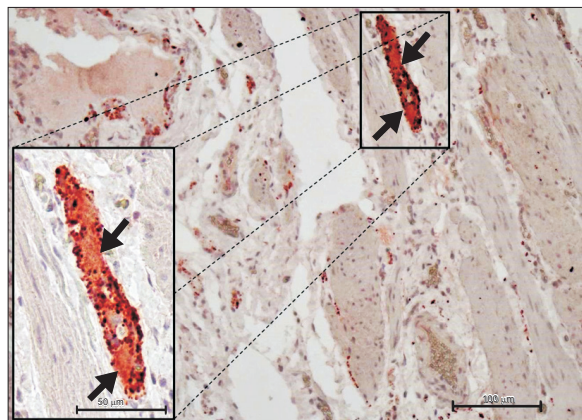GDNF: glial cell line-derived neurotrophic factor.



**Fig. 6.** Positive synaptophysin immunoreactivity in ganglion tissue (arrows) in the muscular gallbladder layer was showed in 100× and 400× magnification respectively.
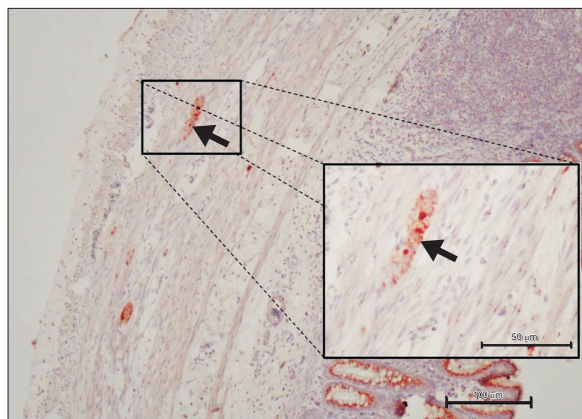


**Fig. 7.** Positive S-100 immunoreactivity in ganglion tissue (arrow) in the muscular gallbladder layer was showed in 100× and 400× magnification respectively.

Inflammation was evaluated with H&E staining and mild chronic inflammation was detected in BA group. Chronic inflammation in the BA group was present scattered in all areas which
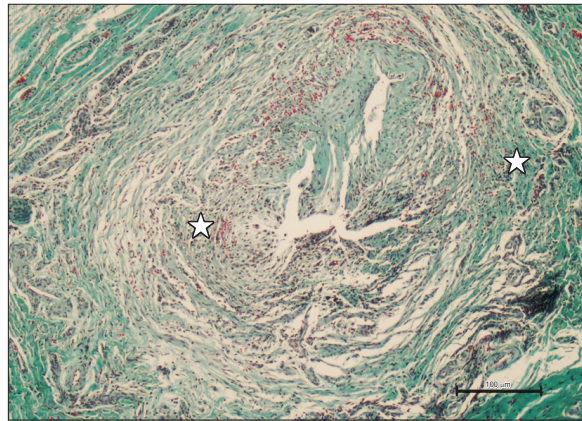
**Fig. 8.** Biliary atresia fibrosis (asterisk) on the gallbladder wall (Masson's trichrome staining, 100× magnification).

we can histologically identify because of atresia together with fibrosis. Chronic inflammation was present in the control group, but this inflammation was observed only in the subepithelial region. Although statistical analysis revealed no significant difference between the both groups in terms of chronic inflammation, there was a mild grade inflammation in the BA group, with fibrosis together in all layers (**Table 3**).

Acute inflammatory activity was evaluated with H&E painting and was slightly detected in the BA group. Statistically significant acute inflammatory activity was not detected in the control group. Although mild inflammatory activity was detected in the BA group, it was statistically significant (**Table 3**, **Fig. 9**).

Clinical, laboratory operative data and analysis of the patients in both groups are shown in **Table 4**. In 15 BA patients, the appearance and consistency of their intraoperative livers were poor (**Table 4**). The intraoperative liver appearance of 14 BA patients was good. All patients in the control group had good intraoperative liver appearance (**Table 4**).
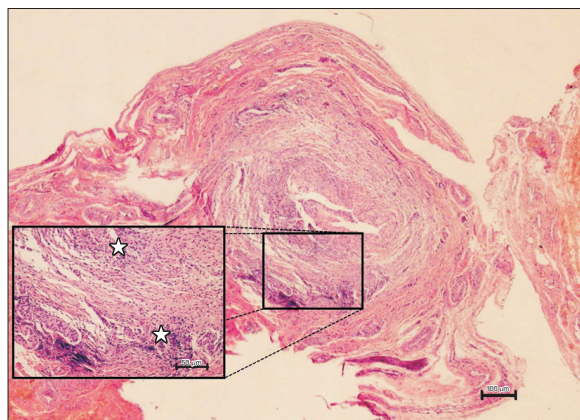


**Fig. 9.** Biliary atresia inflammatory activity (asterisk) on the gallbladder wall (Hematoxylin and eosin staining, 40× and 400× magnification).

**Table 4.** Statistical analysis of patients clinical, laboratory operative data

| Clinical, laboratory and operative data | Biliary atresia (n=29) | Control (n=41) | *p*-value |
|---|---|---|---|
| Operation time age | 63 (45–101) d | 13 (5–17) yr | |
| Operation time (min) | 140 (120–240) | 75 (60–120) | <0.001 |
| Appearance of the liver during the operation Good | 14 | 41 | <0.001 |
| Poorly | 15 | - | |
| Preoperative total bilirubin (mg/dL) | 10.1 (7–26.2) | 0.5 (0.4–1.5) | <0.001 |
| Preoperative direct bilirubin (mg/dL) | 7.7 (5.1–21.4) | 0.3 (0.1–1.1) | <0.001 |
| Postoperative 7th day total bilirubin (mg/dL) | 7.8 (3.8–20.4) | 0.4 (0.3–1.1) | <0.001 |
| Postoperative 7th day direct bilirubin (mg/dL) | 5.8 (2.2–13.2) | 0.2 (0.2–0.7) | <0.001 |
| Postoperative time to start oral feeding time (d) | 3 (3–5) | 1 (1–2) | <0.001 |
| Intensive care unit stay (d) | 5 (3–10) | 0 (0–1) | <0.001 |
| Hospitalization stay (d) | 7 (6–15) | 2 (2–4) | <0.001 |

Values are presented as Median (range) or number only.

## DISCUSSION

In this study, ganglion cells were not detected in gallbladder tissue samples of BA patients. Moreover, GDNF, synaptophysin, and S-100 expression was not found in the BA group. Histopathological examination revealed frequent fibrosis and mild inflammatory activity in BA patients.

Many studies have been conducted to illuminate the etiology of BA [2-5,11-22]. Some studies have suggested that inflammation caused by an infection that affects the bile duct during gestation or immediately after birth plays an important role [3,4]. Other studies have pointed to the role of defects caused by genetic mutations [17,20-22].

The ENS is vital for the proper functioning of the gastrointestinal tract. Between 5 and 12 weeks of gestation, enteric nerve cells spread from the neural crest and settle along the gastrointestinal tract. Migration occurs in the myenteric plexus within the lumen structure along the alimentary tract. Normal migration is quite complex and depends on several factors. The expression of neurotrophic factors such as GDNF plays a crucial role. If these neurotrophic factors are absent due to genetic defects, migration does not occur. In such cases, neural crest cells called ganglion cells are missing from the gastrointestinal tract wall [23]. The expression of GDNF, S-100, and synaptophysin in lumen wall tissue is inhibited in the absence of ganglion cells [6-9]. In our study, there was no ganglion cell in the gallbladder wall of BA group patients, but also no GDNF, SYP and S-100 immunreactivity.

Hirschsprung's disease involves missing ganglion cells in the bowel wall structure, which causes inhibition of GDNF, S-100, and synaptophysin expression [23].

GDNF is vital for the development of the ENS not only in the prenatal but also in the postnatal period [6]. An in vitro study found that GDNF reduced apoptosis in embryonic dopaminergic neurons, increasing their survival rate [24]. Moreover, Han et al. [25] reported a correlation between GDNF expression and intestinal smooth muscle cell growth. They found that reducing GDNF expression in normal rat intestinal smooth muscle tissue with inflammation played an active role in muscle tissue stricture formation. Thus, they speculated that GDNF could play an important role in the development of the intestinal stricture formation in inflammatory bowel diseases.

Some studies have suggested that GDNF is expressed only by the main enteric glial cells on the intestinal wall [26]. However, recent studies have reported that enterocytes and intestinal smooth muscle cells also contribute to GDNF expression significantly [27,28]. Although an in vitro study reported that pro-inflammatory cytokines increase GDNF expression [29], GDNF expression has been found to decrease with an unclear mechanism in inflammatory bowel diseases [26]. This decrease was claimed to destruct the desmosomes in intestinal epithelium which work as the building blocks of intestinal barrier [26]. This may be the beginning of a vicious cycle that aggravates inflammation.

Another important finding is that there is a relationship between a decrease in GDNF expression and pelviureteric junction [PUJ] obstruction. Demirbilek et al. [30] found that cases with PUJ stenosis exhibited a significant decrease in GDNF expression and developed fibrosis. This is in line with our study, where frequent fibrosis was observed in BA patients.

No previous studies have examined GDNF expression in the gallbladder tissue of BA patients. Our study is the first study to examine GDNF expression in gallbladder tissue for BA patients. However, Xia et al. [31] investigated intestinal GDNF expression in intestinal atresia. They found that miRNA211 expression was clearly higher in intestinal atresia patients than in controls, while GDNF was down-regulated at both the transcriptional and translational levels. The authors suggested that this could increase ganglion cell apoptosis and that ganglion cell dysplasia was associated with these findings. This is in line with our study, where GDNF expression was not observed in BA patients.

In our study, BA patients had both mild chronic inflammation and severe fibrosis in scattered all areas of gallbladder tissue. With these findings, we speculate that the absence of GDNF expression in the gallbladder tissues of BA patients may be related to each other.

Synaptophysin is an integral membrane glycoprotein expressed in nerve terminals and various neuroendocrine cells [32]. No previous studies have investigated synaptophysin in BA gallbladder tissue. Our study is the first study to examine Synaptophysin expression in gallbladder tissue for BA patients. However, Khen et al. [33] evaluated the expression of synaptophysin in tissue samples of 22 intestinal atresia patients and found that not only was the expression of synaptophysin in the proximal and distal segments of atresia decreased but also that the ENS did not develop well. Moreover, Ozguner et al. [34] found decreased expression of synaptophysin in the intestinal tissue in jejunoileal atresia. These studies' findings are consistent with our results. In this study, we used monoclonal antibodies to evaluate the expression of synaptophysin and found that there was not expression in BA patients.

Although, there are no other studies in the literature that support that our findings are characteristic findings to BA patients, our study is the first study to examine ganglion cell presence in gallbladder tissue of BA patients. The ganglion cells were absent from the gallbladder tissue of BA patients, whereas they were present in the control group. In Alagille's syndrome and nonsyndromic biliary hypoplasia is characterized by a paucity of intrahepatic ducts, and cholangiography demonstrates a diminutive biliary tree, which precludes the need for biliary reconstruction [1]. We did not include in this study to conditions such as Alagille's syndrome, where there is a stagnation in the bile flow but extrahepatic bile ducts are present. Because intraoperatively, we ended the operation without removing the gallbladder in patients where we detected the bile ducs tree in cholangiography. There is no study in the literature examining the expression of GDNF, S-100 protein and Synaptophysin in gallbladder

tissue in cases of stagnation in bile flow such as Alagille's syndrome. However, we speculate that it is maybe important to investigate the absence of glial cells in the stop of bile flow. In our further studies, we aim to examine the findings we found in this study in cases of stagnation in bile flow, such as Alagille's syndrome.

S-100 is a protein found in glial cells and is vital for neuronal development and differentiation [35]. A decrease in S-100 protein expression of intestinal tissue is used as a marker for the diagnosis of Hirschsprung's disease [36]. No previous studies have investigated the relationship between gallbladder S-100 protein expression and BA. Our study is the first study to examine S-100 expression in gallbladder tissue for BA patients. However, Wang et al. [37] found decreased S-100 expression in intestinal tissue in congenital intestinal atresia. In line with this finding, we found that S-100 was not expressed in BA gallbladder tissue.

We also think that there may be many components that affect inflammation in the BA development process. Lack of submucosa layer in the bile ducts may be one of these components. Normal extrahepatic bile ducts do not have a submucosa layer [38]. It has been claimed that the intestinal submucosa layer has an important function in inflammatory processes [39]. Although there has been no study on this subject, we believe that if there is an intestinal inflammation process affects the extrahepatic bile ducts, the bile ducts may be more vulnerable to inflammation due to lack of submucosa layer.

It is not clear whether these findings indicate causes or results of BA. The claims that GDNF is downregulated due to inflammation with any cause and that it leads to fibrosis in intestinal tissue is of great importance. Perhaps an inflammation in BA with autoimmune or infectious etiology may lead to GDNF downregulation in the extrahepatic bile duct and gallbladder tissue. We speculate that a genetic defect that meets this condition may be a missing link in the chain of events that lead to GDNF downregulation and a lack of ganglion cells. However, as most studies on the development of intestinal fibrosis with GDNF deficiency have been conducted in vitro, we believe that many more studies are necessary to test this hypothesis.

Our study had some limitations. This study, did not have the gallbladder tissue of healthy children consisting of normal individuals. We didn't have a control group that was in the same age group as the BA group. The numbers of the control group and the BA group, their gender, Bilirubin levels, operation times, were not equal.

In conclusion, this study examined gallbladder tissue of BA patients immunohistochemically and histopathologically; and found signs of increased inflammatory activity together mild chronic inflammation and fibrosis. We also found no GDNF, synaptophysin, and S-100 expression and no ganglion cells. We believe that these findings may help in the diagnosis of BA. We also speculate that they could represent a missing link in the chain of events that form the etiology of the disease. We speculate that GDNF expression will no longer continue in this region, when the damage caused by inflammation of the extrahepatic bile ducts reaches a critical threshold. In this situation absence of GDNF in the environment causes maybe destruction in the glial cells within the extrahepatic bile ducts. The glial cell destruction can cause damage to extrahepatic bile ducts and maybe resulting in acquired atresia. Extrahepatic bile ducts maybe more susceptible to this atresia process, than the intestines, because of the lack of a layer of submucosa. However, further research is needed to confirm these hypotheses.

## ACKNOWLEDGEMENTS

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