ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

DETERMINATION OF THE RELATIONSHIP BETWEEN PYROPTOSIS AND OUTER MEMBRANE PROTEINS OF THE BACTERIA IN DIFFERENT PATIENT GROUPS ASSOCIATED WITH *HELICOBACTER PYLORI*

YAREN BÜYÜKÇOLAK
M.Sc. THESIS

DEPARTMENT OF MEDICAL BIOTECHNOLOGY

SUPERVISOR
Assist. Prof. Sinem Öktem Okullu

ISTANBUL-2023

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DECLARATION

I declare that this thesis work is my own work, I had no unethical behavior at any stage from the planning to the writing of the thesis, I obtained all the information in this thesis in accordance with academic and ethical and rules, I cited all the information and comments that were not obtained with this thesis work, and I provided resources in the list of references. I also declare that there was no violation of any patents and copyrights during the study and writing of this thesis.

20/06/2023
Yaren Büyükçolak

PREFACE AND ACKNOWLEDGEMENT

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LIST OF ABBREVIATIONS AND SYMBOLS

AGS	Human Gastric Adenocarcinoma
Alp	Adhesion-associated lipoprotein
ANOVA	Analysis of Variance
APS	Ammonium Persulfate
ASC	apoptosis-associated speck-like protein containing a caspase recruitment domain
ATADEK	Acıbadem Healthcare Institutions Medical Research Ethics Committee
ATPase	Adenosine triphosphate
Bab	Blood group antigen binding adhesins
BCA	Bicinchoninic acid assay
Bp	base pair
BSA	bovine serum albumin
C	Carbon
CagA	cytotoxin-associated gene A antigen
CagPAI	Cag Pathogenicity Island
CARD	caspase recruitment domain
CAR-T	Chimeric antigen receptor T
CEACAM	carcinoembryonic antigen-related cell adhesion molecules
CLO	*Campylobacter*-like organism
DAMP	Damage-associated molecular patterns
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ELISA	enzyme-linked immunoassay
EPIYA	motif glutamate-proline-isoleucine-tyrosine-alanine motif
EtBr	ethidium bromide
FBS	Fetal Bovine Serum
FlaA	flagellum A
g	gravitational force
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

GSDM	Gasdermin
Gzm	Granzyme
Hof	*Helicobacter* outer membrane protein family
Hom	*Helicobacter pylori* outer membrane family
Hop	*Helicobacte*r outer proteins
Hor	Hop-related proteins
HRP	horse radish peroxidase
H. pylori	*Helicobacter pylori*
IFN-γ	Interferon-gamma
IgG	immunoglobulin G
IL-6	Interleukin 6
IL-18	Interleukin 18
IL-1β	Interleukin 1 beta
kDA	Kilo Dalton
Lab	LacdiNac-binding adhesin
LPS	Lipopolysaccharide
MALT	mucosa-associated lymphoid tissue
MAPK	mitogen-activated protein kinase
MLKL	Mixed-lineage kinase domain-like pseudokinase
mRNA	messenger ribonucleic acid
MUC	Mucin
NAC	N-acetylcysteine
NCBI	National Center for Biotechnology Information
NF-KB	Nuclear Factor Kappa B
NOD	nucleotide-binding domain
NLR	nucleotide-binding oligomerization domain-like receptors
OipA	outer inflammatory protein A
OMP	outer membrane proteis
ORF	Open Reading Frame
PAMP	pathogen-associated molecular patterns
PBS	phosphate-buffered saline
pH	Power of Hydrogen

PI	propidium iodide
PIT	Pore-derived intracellular traps
PPI	proton pump inhibitors
PVDF	polyvinyl fluoride
PRR	Pattern recognition receptors
RIP	receptor-interacting protein
rpm	revolutions per minute
RT-PCR	real-time polymerase chain reaction
Sab	sialic acid-binding protein
SDS	Sodium Dodecyl Sulfate
SDS-page	SDS polyacrylamide gel
TBS	tris-buffered saline
TBS-T	Tris-buffered saline with 0.1% Tween 20
Th	T helper
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-alpha
UreA	Urease A
UreB	Urease B
V	Volt
VacA	vacuolating cytotoxin A
VacA I	intermediate allele of vacuolating cytotoxin A
VacA M	middle allele of vacuolating cytotoxin A
VacA S	signal-associated allele of vacuolating cytotoxin A

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ÖZET

H. pylori ile İlişkili Farklı Hasta Gruplarında Bakterinin Sahip Olduğu Dış Zar Proteinleri ile Piroptozis Arasındaki İlişkinin Belirlenmesi

Helicobacter pylori (H. pylori), mide epitelinin mukus tabakasını kolonize ederek; gastrit, ülser ve mide kanseri gibi hastalıkların gelişmesine neden olabilen bir patojendir. Bakterilerin dış zarındaki proteinler, patojenin mide epiteline tutunmasında görev alır ve spesifik yüzey proteinleri ile etkileşime girerek patojene bağlı hastalıkların gelişmesinde aktif rol oynar. Hücre içi patojen enfeksiyonu tarafından uyarılan konakçı savunma sistemi, pro-enflamatuar bir hücre ölümü olan piroptozu tetikleyebilir. Piroptoz bir patojen tarafından aktive edildiğinde, IL-1β ve IL-18 gibi enflamatuar sitokinler, kaspaz-1 tarafından pro-formlarından olgun formlarına dönüştürülür ve GSDMD proteini, ikiye ayrılır. Ortaya çıkan GSDMD-N-terminali hücre zarıyla bütünleşerek, zar yüzeyinde gözenekler oluşturur. Enflamatuar sitokinler hücre yüzeyindeki gözeneklerden dış ortama salınır ve hücre içine sıvı girmesi ile ozmotik lizis meydana gelir. Bu tez çalışması, patojenle ilişkili gastrit ve ülserli hastalarda *H. pylori*'nin dış zar proteinleri ve piroptoz arasındaki olası ilişkileri incelemeyi amaçlamıştır. Gönüllü hastaların midelerinin antrum bölgesinden alınan tek bir doku örneğinden; DNA, RNA ve protein izolasyonu yapılmış olup, bu dokularda bakteriyel dış zar virülans genlerinin gen düzeyinde ekspresyonu incelenmiştir ve *H. pylori* ile enfekte olan ve olmayan hasta gruplarında piroptoz belirteçlerinin ifadeleri karşılaştırılmıştır. Bu çalışma, patojen ilişkili hastalıklarda patojene ait virülans faktörleri ile piroptoz arasındaki ilişkiyi araştıran ilk çalışma olması nedeniyle literatüre katkı sağlamaktadır. *H. pylori'*ye ait dış zar proteinleri ile piroptoz arasındaki ilişkinin aydınlatılması, *H. pylori*'nin neden olduğu hücre içi değişiklikleri ve bakterinin patojenitesini anlamak ve tedavisi için yeni hedef mekanizmalar ve moleküller geliştirmek açısından önemlidir.

Anahtar Sözcükler: *Helicobacter pylori*, Dış membran proteinleri, Piroptoz, Programlı hücre ölümü,

ABSTRACT

Determination Of The Relationship Between Pyroptosis And Outer Membrane Proteins Of The Bacteria In Different Patient Groups Associated With *H. pylori*

Helicobacter pylori (H. pylori) is a pathogen that can colonize the gastric epithelium and cause the development of diseases such as gastritis, ulcers, and gastric cancer. The bacterial outer membrane proteins take part in the attachment to the gastric epithelium and play an active role in the development of pathogen-related diseases. The host defense system induced by intracellular pathogen infection can trigger pyroptosis, a proinflammatory cell death. When pyroptosis is stimulated by pathogen, IL-1β and IL-18 are activated by caspase-1, and the GSDMD protein splits into two, with the resulting GSDMD-N-terminal integrating into the cell membrane, resulting in pores in the cell membrane. Inflammatory cytokines are released from the pores, and osmotic lysis occurs. The aim of this thesis study is to examine relationships between pyroptosis and outer membrane proteins of the pathogen in patients with *H. pylori*-associated gastritis and ulcers. DNA, RNA, and protein were isolated from a single tissue sample taken from the volunteer's stomach, and the expression of bacterial outer membrane virulence genes was examined also, the expression levels of major pyroptosis markers were compared in *H. pylori*-infected and uninfected patient groups. This study contributes to the literature as it is the first to investigate the relationship between pathogen virulence factors and pyroptosis in pathogen-related diseases. Clarifying the relationship between *H. pylori*'s outer membrane proteins and pyroptosis will be important in terms of understanding the intracellular changes caused by *H. pylori* and the pathogenicity of the bacteria and developing new target mechanisms and molecules for its treatment.

Keywords: *Helicobacter pylori*, outer membrane proteins, pyroptosis, programmed cell death

1 INTRODUCTION AND AIM

Helicobacter pylori (H. pylori) infection is seen in more than half of the world's population and is the primary cause of many diseases such as acute and chronic gastritis, ulcers, and gastric cancer by colonizing the antrum and corpus of the human stomach. *H. pylori* is a spiral-structured, microaerophilic, gram-negative bacterium that moves via flagella, and the prevalence of *H. pylori* varies regionally and is lower in socioeconomically developed countries. Host, environmental conditions, and virulence genes of bacteria are effective in the pathogenesis of *H. pylori* and play complex roles in the development of pathogen-related diseases. The specific properties of bacteria that are effective in reaching the gastric epithelium, surviving in the acidic environment, and adhering to the epithelial surface by avoiding the peristaltic movements of the stomach are called virulence factors. *H. pylori* attaches firmly to the gastric epithelium using the adhesin proteins found in its outer membrane. The Hop family, which hosts 32 of these outer membrane proteins, is the best-known family of outer membrane proteins in *H. pylori*. However, the interactions between theseproteins and the host are not yet fully understood.

Pyroptosis is a lytic, inflammatory programmed cell death programmed against intracellular microbial pathogens. However, this beneficial mechanism can cause tissue damage by causing autoimmune and auto-inflammatory responses. In the mechanism of pyroptosis, pathogen-associated molecular patterns (PAMP) cause inflammasome formation. Caspase-1, activated by this inflammasome, causes the differentiation of IL-1β and IL-18 from the pro-form to the active form, thus splitting the GSDMD protein into two. The GSDMD-N terminal integrates into the cell membrane and forms pores. Inflammatory cytokines are released from the pores on the cell surface into the surrounding environment, and osmotic lysis occurs due to theentry of fluid into the cell.

This thesis study was carried out to examine possible relationships between pyroptosis and the outer membrane proteins of *H. pylori* in patients with pathogen-associated gastritis and ulcers. There is no study in the literature showing the

relationship between pyroptosis and the outer membrane virulence genes of *H. pylori* in pathogen-related diseases. Therefore, this thesis study is important as it is the first of its kind. Clarifying the relationship between *H. pylori*'s outer membrane proteins and pyroptosis will be important in terms of understanding the intracellular changes caused by *H. pylori* and the pathogenicity of the bacteria and developing new target mechanisms and molecules for its treatment. If the data obtained from this study is supported by further studies, it will be a precursor for the determination of target molecules and mechanisms in the development of new treatment methods.

2 BACKGROUND

2.1 Helicobacter pylori

2.1.1 Specific characteristics of *Helicobacter pylori*

Although the stomach's structure was assumed to be unfavorable for bacterial development from the discovery of bacteria until the end of the 19th century, with increasing microscope resolution, W. Jaworski first described the spiral-shaped microorganisms in animal stomachs in 1889. Working as a clinical pathologist at the Royal Perth Hospital in Australia, Dr. Warren observed a relationship between the number of bacteria and the severity of infection in gastric biopsy samples and tried to culture this bacterium with Professor Marshall, whom he worked with (1). For the first time, the bacterium obtained from the gastric biopsy of a duodenal ulcer patient was named Campylobacter-Like Organism (CLO) because it was thought to be a species of Campylobacter (2). However, when it was seen that the flagella structure was different from that of Campylobacter, it was regarded as a different genus of bacteria, and over time its name was changed to *Helicobacter pylori (H. pylori)* (1). Marshall infected himself by drinking the culture of the microorganism to show the relationship between the bacteria and the diseases, and the endoscopy performed on the 8th day revealed bacteria and active gastritis. Following this, Marshall and Warren showed that antibiotics can be used in the treatment of bacterial infections and received the Nobel Prize in medicine in 2005 for this study (3).

Helicobacter pylori is a spiral-shaped gram-negative bacteria with a diameter of 0.5 μm and a length of 3.5 micrometers (μm). It moves through its two to seven unipolar, sheathed flagella. Its spiral structure is advantageous because it travels swiftly, establishing the initial colony and ensuring the infection's persistence. It is known that bacteria get stressed in the face of problems such as nutritional deficiency and the presence of antibiotics. Bacteria under stress change shape and pass into the coccoid form. The microorganism that transforms into the coccoid form cannot be

cultured (4). Under less-than-ideal environmental circumstances, *H. pylori* can develop biofilm (5).

Biochemically, it exhibits catalase, oxidase, and urease activity. In particular, urease is vital for bacteria to survive in an acidic stomach environment (6). The only carbohydrate it can catalyze is glucose (7).

For *H. pylori* to thrive, a rich nutrient and serum are required, along with a microaerophilic environment with less oxygen than air. While it takes 2-4 days to grow on agar enriched with components such as blood, serum, and cholesterol, the culture time in liquid culture depends on the amount of inoculation. While the solid culture grows at 37°C in microaerophilic conditions, the liquid culture should be agitated at 150-180 revolutions per minute (rpm) at the same temperature (8,9).

Geographic location, socioeconomic conditions, age, and race all influence the frequency of *H. pylori* infection. The infection and maintenance processes cannot be accurately examined since the time of infection cannot be known (10). The infection, on the other hand, is assumed to be acquired throughout childhood. Infection with *H. pylori* is less common in developed countries than in developing and under developed countries. According to a 2013 study, the prevalence of *H. pylori* is 82.5 percent in Turkey (11).

There are various risk factors for *H. pylori* infection, such as the number of people living in the household, living in the same house as the infected individual, the number of people sharing the same bed, access to clean water, and the presence of a sewer system (12). It is thought that there is a relationship between infection and variables such as alcohol and cigarette consumption, the cooking status of foods, and dietary habits. There are different opinions about the relationship between blood groups and infection rates (12).

Helicobacter pylori is thought to have colonized the human stomach for thousands of years (13). Transmission of the pathogen can be from environment to person or from

person to person. Person-to-person transmission can take place in both vertical and horizontal directions. Vertical transmission refers to infections that occur within the same family, whereas horizontal transmission refers to infections that occur between persons who are not related (14). The disease, which may be passed from person to person via the gastro-oral, oral-oral, and fetal-oral pathways, can also be contracted by consuming infected water or food from the environment (15). The use of non-sterilized medical supplies can also cause the interpersonal transmission of pathogens (15).

2.1.2 Detection of *Helicobacter pylori* infection associated diseases and treatment

Helicobacter pylori infection is usually asymptomatic but may present with symptoms such as stomach pain, indigestion, bloating, vomiting, appetite loss, and weight loss. The methods used to detect the pathogen are classified as invasive and non-invasive testing. The main invasive methods are upper gastrointestinal endoscopy, and confocal laser endomicroscopy, which allow for examining the stomach histology and taking a piece if necessary. In addition, staining the bacteria with dyes such as silver dye and Giemsa through histology is also important in terms of understanding the type of infection. The culturing of the bacteria on the tissue taken is especially important for the detection of antibiotic resistance. It is a fast and inexpensive invasive method used to detect urease activity and ammonia formation with phenol red, which is one of the most important mechanisms for the colonization of the pathogen in the acidic environment of the stomach. In addition, polymerase chain reaction (PCR) and in-situ hybridization are methods used for this purpose (16).

Active and passive non-invasive diagnostics are available. Active tests provide detection of ongoing infections, while passive tests do not indicate whether the detected infection was experienced in the past or is part of an ongoing infection. Serological tests are based on the specific identification of anti- *H. pylori* -immunoglobulin G (IgG) antibodies in patient serum and a passive test technique and can be performed by techniques such as Elisa, and Western Blot. In addition, cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA) antibodies,

which are known to be very important in the virulence of the pathogen, can also be used as passive tests. Urine or saliva samples can be used as an alternative to serum in antibody tests, but these fluids have lower antibody content (16).

The 13C-urea (C: carbon) breath test is an active, non-invasive test based on the principle that urea labeled with ^{13}C or ^{14}C is given to the patient, and the patient will release the labeled carbon atom as carbon dioxide (CO_2) while converting urea-ammonia as a result of urease activity. In addition, stool antigen testing is also used to detect active *H. pylori* infection (16).

The *H. pylori* colonizes the duodenum and stomach. The infection can last a lifetime if the pathogen, which is commonly acquired in childhood, is not treated. An infection that is thought to be cured by unsuccessful treatment may recur. Although the pathogen colonizing the stomach causes digestive system-related diseases such as gastritis, ulcer, gastric cancer, and mucosa-associated lymphoid tissue (MALT) lymphoma, it has been associated with many different diseases such as mineral and vitamin deficiency, allergic asthma, diabetes, and Alzheimer's (17).

2.1.2.1 Gastritis

Gastritis, which is generally accepted as the beginning of *H. pylori* infection, is an inflammation of the stomach lining. In the absence of pathogen, gastritis may also develop due to factors such as dietary habits and stress. The pathogen can cause a sudden (acute) or persistent (chronic) infection of the stomach lining. Untreated long-term infection is effective in the chronicity of the disease. The pathogen that causes degeneration of the epithelial membrane, disrupts the regular cell structure (18,19). With the loss of cells in the gastric mucosal layer and glands, the infection becomes atrophic. Whether gastritis is atrophic or not, alteration of acid secretion is effective in the ongoing evolution of the infection into ulcer or cancer (20,21). The effect of the pathogen on acid secretion is variable. While increased acid secretion has been observed in patients with duodenal ulcers, decreased acid secretion may result in

gastric cancer (22). Gastritis is characterized by hyperchromatic nuclei, cytoplasmic basophilia, and increased mitosis (18).

2.1.2.2 Peptic ulcer

Peptic ulcers are the wounds that stomach acid creates in the tissues as a result of damage to the mucous layer that protects the stomach and duodenum (the first part of the small intestine). Stomach ulcers usually occur in the transition zone from the antrum to the corpus, while duodenal ulcers generally occur in the duodenal bulb (23). Uncontrolled use of non-steroidal anti-inflammatory drugs, as well as *H. pylori* infection, are among the causes of ulcers. Ulcers are more common in places of the body where inflammation is the most acute (23).

In long-term infections, acid secretion decreases due to a decrease in the cells that secrete gastrin hormone, which increases gastric acid secretion in the antrum region of the stomach, and this situation has been associated with atrophic gastritis. If the infection persists, the disease may take the form of a gastric ulcer or intestinal metaplasia.

In some cases, the body produces more stomach acid than it needs, in response to the increased pH level due to urease activity and the loss of acid-secreting cells. Hypergastrinaemia occurs when the negative feedback regulation that controls the gastrin level is disrupted due to the pathogen (24). Excessive gastric acid secretion and accumulation of acid in the duodenum cause metaplasia in the duodenal bulb (25, 26). On the other hand, the developing immune response causes ulceration.

2.1.2.3 Gastric cancer

Atrophic gastritis, which is characterized by cell loss and a reduction in the size of cells due to long-term corpus-predominant gastritis, is often considered a precursor of gastric cancer. The formation of new structures resembling intestinal glands following the disappearance of the gastric glands is called intestinal metaplasia. This is followed by dysplasia, in which cells with hyperchromatic nuclei become irregularly

shaped (20). Not all dysplasia is cancer, but there is a cancer potential that should not be ignored. Diffuse-type gastric cancer and intestinal-type adenocarcinoma are two types of gastric cancer. Stomach cancer is the 4th most common cancer in the World and is especially common in East Asian countries (27). The illustration showing the formation of gastritis, ulcer and gastric cancer due to *H. pylori* infection is given in Figure 1.

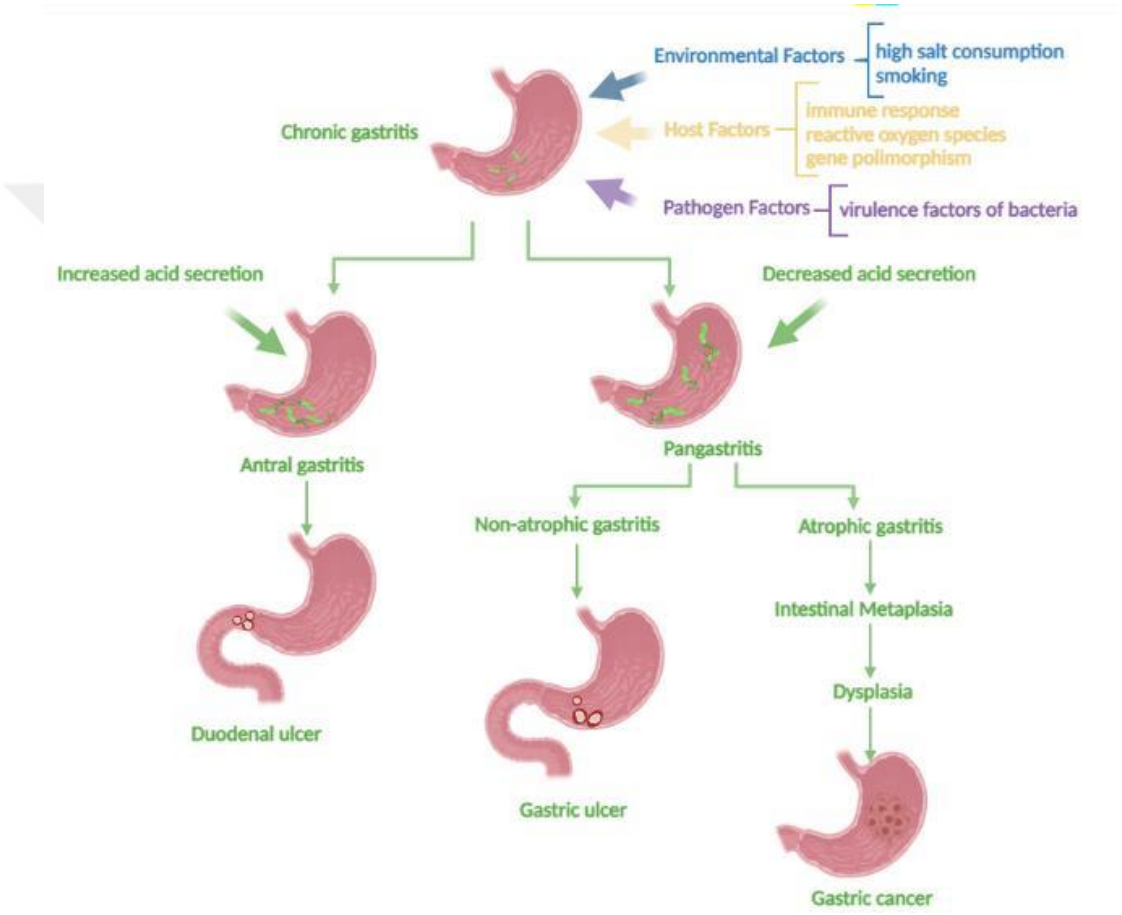


Figure 1. Pathway of *H. pylori*- associated gastrointestinal diseases

2.1.2.4 Others

Normal gastric tissue does not contain lymphoid tissue, but rarely this tissue produces a monoclonal population of B cells, and the cells eventually develop into Mucosa-associated lymphoid tissue (MALT) lymphoma (21). Almost all MALT patients have *H. pylori* infections (28).

It is known that *H. pylori* infection can also affect regions outside the digestive system and is associated with many other diseases. It is thought that the pathogen increases the risk of diseases such as Immune Thrombocytopenic Purpura (29) and B12 deficiency (30), and the decreasing prevalence of *H. pylori* increases the incidence of some diseases such as allergic asthma (31).

It is critical to develop effective treatment methods for *H. pylori* infection because it has the potential to recur if not treated completely and correctly. Increasing antibiotic resistance due to improper and irregular use of antibiotics is an important obstacle in the treatment of the pathogen. The antibiotic resistance of *H. pylori* may vary regionally due to different antibiotic consumption attitudes. In an *H. pylori* strain obtained from a patient with stomach cancer in Iran in 2010 (32), resistance to seven antibiotics was detected, including clarithromycin, metronidazole, amoxicillin, tetracycline, furazolidone, erythromycin, and ciprofloxacin

Combined antibiotics are generally used in the treatment of *H. pylori* infection, and the most commonly used antibiotics are those such as clarithromycin, metronidazole, and tetracycline. In a study conducted in Greece (33), between 2000 and 2010, more strains resistant to clarithromycin were found in the population over time. In order to find the most effective antibiotic in the treatment of the pathogen, it would be useful to examine the antibiotic susceptibility of strains worldwide.

Another method used in the treatment of *H. pylori* infection is proton pump inhibitors (PPIs). PPIs are basically antisecretory agents that prevent stomach acid secretion by inhibiting the adenosine triphosphatase (ATPase) enzyme. The increased gastric pH provides an unfavorable condition for acid-fast bacteria and a more neutral environment for the healing of scar tissue in the gastric epithelium. Because some antibiotics work more effectively at a neutral pH, PPIs are used with the antibiotic combination. In the past, dual therapies including an antibiotic and a PPI were actively used in the treatment of the pathogen, but with the increasing antibiotic resistance, this treatment has been replaced by triple or quadruple therapies containing more than one

antibiotic and antimicrobial agent. However, the potential side effects of long-term use of PPIs and antibiotics provide reasons for the development of new treatment methods.

In the treatment of *H. pylori* infection, some studies are carried out on alternative treatment methods such as using probiotics as adjuvants (34) or using the anti-mutagenic properties of some plant extracts to prevent the acquisition of antibiotic-resistance genes (35). Photodynamic therapy methods based on the conversion of molecular oxygen to reactive cytotoxic oxygen molecules using dye molecules known as photosensitizers and harmless visible light can be used in the treatment of *H. pylori* infection (36).

Vaccine studies to prevent *H. pylori* infection have gained importance nowadays, and these studies have been approached by various methods, such as the use of virulence factors of the bacteria as adjuvants (37) or the use of therapeutic antibodies (38). It is thought that nanoparticles or bacterial vesicles can be used as carrier molecules. However, for vaccines to be developed to protect against *H. pylori* infection, it is of great importance to better understand the functions of bacterial virulence factors and to identify potential vaccine targets.

It is known that virulence factors associated with disease-causing pathogens cause cellular damage by affecting mitochondrial and lysosomal functions, disrupting cellular ion balance, and producing endoplasmic stress (39). It is thought that *H. pylori*-induced cell damage formation, pro-inflammatory cytokine release, and cell death known as pyroptosis are effective. Treatment methods that can prevent pathogen-induced cell damage will have an important place in the eradication of *H. pylori*.

2.1.3 Virulence factors of *Helicobacter pylori*

The specific properties of the pathogen that support its survival and cause disease are called virulence factors. Virulence factors are effective for *H. pylori* to reach the gastric epithelium, adapt to the acidic gastric environment, and maintain the infection by adhering to the gastric epithelium without being affected by the peristaltic

movements of the stomach. Understanding the role and effects of these factors, which may vary according to bacterial strains, during infection is of great importance in order to analyze bacterial pathogenicity at the molecular level and to develop pathogen-specific treatments.

2.1.3.1 Flagella

The flagellum is the organ that provides movement in the colonization zone of the bacterium, and the number of flagella that *H. pylori* has varies between two and six. These sheathed flagella are about 3 μm and encoded by flagellum A (*flaA*) (40). Having a greater number of flagella is known to increase mobility (41). It was also observed that bacteria exposed to high acidity showed higher motility (42).

2.1.3.2 Urease

The stomach pH of a healthy person is around 1.5. One of the mechanisms that bacteria have for survival in the highly acidic stomach environment is urease activity. Although *H. pylori* is not the only bacterium showing urease activity, the presence of this immunogen is used as a marker in the diagnosis and follow-up of the infection. Seven urea genes are required for the synthesis of an active urease enzyme. Of these genes, urease A and B (*ureA* and *ureB*) are structural subunits, while the others are defined as accessory genes. The expression of *ureA* and *ureB* is sufficient to produce an apoenzyme (43,44).

Urea is hydrolyzed by urease enzyme activity to form ammonia and carbamate. The carbamate molecule spontaneously decomposes to form another ammonia molecule and carbonic acid. Carbonic acid and ammonia reach a condition of equilibrium, and an increase in pH occurs (43).

2.1.3.3 Vacuolating cytotoxin A (VacA)

Vacuolating cytotoxin A is synthesized as a large polypeptide and shows allelic variation according to the changes in its segregating regions, mainly s (signal), i

(intermediate), and m (middle) (45, 46). The main function of VacA is epithelial vacuolization and may affect mitochondrial cytochrome c release, membrane channel formation, and attachment to cell membrane receptors (47). It has been shown that strains expressing *vacA s1* and *vacA m1* alleles have a higher potential to develop a peptic ulcer and gastric cancer than *vacA s2* and *vacA m2* (48).

2.1.3.4 cag pathogenicity ısland (cagPAI)

Cag PAI is a 40 kb region of chromosomal deoxyribonucleic acid (DNA) encoding approximately 31 genes (45). The protein, called the cytotoxin-associated gene A antigen (CagA), is encoded by the cagA gene, located at one end of the PAI. After the bacteria attach to the gastric epithelium, the CagA protein is taken into the host cell via the type IV secretion system. CagA protein entering the host cell induces phosphorylation of tyrosine in the glutamate-proline-isoleucine-tyrosine-alanine (EPIYA) motifs, causing the infected cells to pass into the morphological form called the "hummingbird phenotype" (49,50). The EPIYA motifs may contain four different segments as A, B, C, D, and the appearance of these segments may vary regionally (46).

The presence of the *cagA* gene has been associated with peptic ulcer disease and an increased inflammatory response, which has been associated with the development of gastric cancer (51, 52). The co-existence of the *cagA* gene and the *vacA m1m2* allele are considered to be the most virulent strain of the bacterium (53).

2.1.3.5 Other membrane virulence factors

The outer membrane of gram-negative bacteria consists of two layers; the inner layer contains phospholipids, and the outer layer contains outer membrane proteins (OMPs). Adhesion, penetration, and immune response evasion are all functions of OMPs. These OMPs usually have several alleles, and the alleles perform different functions among themselves (54). The OMP expression levels are affected by phase

and allelic differences, as well as processes such as gene conversion and gene duplication (55).

There are 64 OMPs identified from *H. pylori* and these proteins were first categorized by Alm et al. In the study, which was aligned according to the N and C terminals, the proteins were: 21 Hop (*Helicobacter* outer protein), 12 Hor (Hop-related), 8 Hof (*Helicobacter* OMP family), 4 Hom (*H. pylori* outer membrane family), 6 iron-regulated, and 3 efflux pumps were classified as OMPs, although 10 OMPs could not be included in these categories (56). There are many studies investigating the effect of the Hop family, the largest OMP family of *H. pylori*, on the pathogenesis of bacteria-related diseases.

2.1.3.5.1 Blood group antigen binding adhesin (BabA, B, and C)

BabA (blood group antigen binding adhesin A) (HopS) protein, which mediates binding to fucosylated Lewis b (Leb) blood group antigens 0, A, and B in host gastric epithelial cells, is encoded by the *babA* gene and has two alleles, *babA1*, and *babA2*. However, of these alleles, only *babA2* is capable of producing an active protein (21). In addition, the *babA* gene has two paralogs, *babB (hopT)* and *babC (hopU),* and these genes take parts at three (A-B-C) different loci. Chromosomal loci in which bab genes are located may vary between strains, and bab genes may be chimeric with each other (57).

Although the presence of the *babA2* allele has been associated with peptic ulcers and gastric cancer, it is unclear whether the presence of this allele is a direct risk factor for diseases when concurrent with *vacA s1* and *cagA* (58, 59). BabA protein-mediated adhesion has been shown to increase the inflammatory response and induce DNA damage by causing regional breaks on double-stranded DNA (60, 61). Furthermore, N-acetylcysteine (NAC) has been demonstrated to disrupt the disulfide bonds at the BabA interaction site, impairing BabA binding (62).

The *babB* gene and the *babA* gene show homology specifically in regions 5’ and 3’, and mismatched portions of the two gene regions encode regions of the BabA protein that are effective in binding. Although the function of BabB and BabC has not been fully determined, BabB has been found to be effective in gastric lesion formation, and it is thought to be a predictor marker for people with severe dyspepsia (63, 64). However, further studies are needed to examine the role of this protein in the pathophysiology of diseases.

2.1.3.5.2 Adhesion -associated lipoprotein A and B (AlpA and AlpB)

AlpA/AlpB is encoded by the highly homologous *alpA* and *alpB* genes. Unlike similar homologous genes, AlpA and AlpB are always generated together. In a study by Jonge et al., strains without AlpA and AlpB proteins were shown to have limited colonization abilities (65). Similarly, it has been shown that the biofilm-forming ability of *ΔalpB* strains is reduced (66). It has been shown that AlpA and AlpB are effective in cellular adhesion and trigger proinflammatory responses that are effective in gastric damage by stimulating intracellular signals (67). Recombinantly produced AlpA and AlpB proteins were found to increase binding to laminin protein in the extracellular matrix (ECM), and laminin was thought to be the target of these proteins (68).

2.1.3.5.3 Sialic acid-binding adhesin A and B (SabA and B or HopP and O)

In gastric epithelium infected with *H. pylori*, the expression of the sialyl-dimeric-Lewis^{x} glycosphingolipid molecule is increased, and this molecule acts as a receptor for bacteria expressing the SabA protein on its surface. Increased expression of this receptor in the later stages of infection causes stronger bacterial adhesion via SabA (69). It was observed that *sabB*, the homolog of the *sabA* gene, was not effective in sialyl-Lewis x glycosphingolipid expression (69).

The CT base repeats in the open reading frame (ORF) at the 5' end of the *sabA* and *sabB* genes are vital for the production of functional proteins. Shifting ORFs due to altered CT repeats cause the stop codon to arrive prematurely, a condition called

"off status". Situations in which SabA or SabB proteins can be produced functionally are called "on status" (70). A similar situation occurs for the *oipA* and *hopZ* genes (71).

It was observed that the expression of SabA was increased by *H. pylori* when the pH of the stomach environment was decreased (72). The coexistence of BabA, SabA, and OipA was thought to be a predictive biomarker for gastric cancer (73). Gaudarzi et al., in a study in which they examined SabA expression in clinical diseases in Iran, showed that dyspepsia and atrophic findings were compatible with protein expression (74). On the other hand, it was shown that the off state of *sabB* was associated with duodenal ulcer (75).

2.1.3.5.4 LacdiNac-binding adhesin A (LabA or HopD)

The LabA protein is an adhesin that specifically binds the lacdiNAc motif on mucin 5AC (MUC5AC) (76). However, adequate studies have not been conducted to define its effects on the development of pathogen-related diseases.

2.1.3.5.5 HopZ

The *hopZ* gene has two known allelic variants (I and II), and despite the fact that *hopZ* and *hopQ* are genetically related, no recombination was found between the two genes (77). Expression of the *hopZ* gene is regulated during the infection process according to the CT repeats located at the 5' end (78). During infection of the AGS (human gastric adenocarcinoma) cell line with *ΔhopZ* strains, weaker binding was observed compared to normal strains, and this protein was found to be effective in binding (79). In previous studies, a relationship between HopZ and pathogen-related chronic diseases could not be determined. However, the *hopZ* "off" status has been associated with MALT lymphoma (51).

2.1.3.5.6 HopQ

HopQ protein, known to interact with carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) (80), has been shown to be effective in the

translocation of CagA antigen from gastric epithelial cells, stimulate mitogen-activated protein kinase (MAPK) signaling, and enhance interleukin-8 (IL-8) secretion (81). The *hopQ* gene has two known alleles (I and II), and both alleles have been associated with gastric cancer, but gastric ulcer has only been associated with the *hopQ1* allele (82). Additionally, in a study conducted, it was observed that the *hopQ* gene was frequently found together with *vacA s1m1*, and *cagA,* and this association was associated with the development of gastric cancer (83).

2.1.3.5.7 Outer Inflamatory Protein A (OipA or HopH)

The functional status of OipA (outer inflammatory protein A) is regulated by slipped strand mispairing with respect to the CT repeats at the 5' end of the *oipA* gene (84). There are conflicting ideas about whether the OipA protein increases IL-8 production and stimulates the pro-inflammatory response, so its effect on this mechanism is controversial (59, 85). It has been suggested that Mongolian gerbils cannot be infected using *ΔoipA* strains (86), but in another study, the same animals could be infected using *ΔoipA* strains (87). In the study in which the infection was carried out successfully, experimental animals infected with *ΔoipA* strains did not develop gastric cancer, nevertheless, 27% of experimental animals infected with the *H. pylori* 7.13 strain developed gastric cancer (87). This showed that *oipA* could be used as a vaccine target, and in a study (88), a Th1/Th2 (T helper 1/2)-mediated immune response was created in mice by using OipA-DNA-based vaccines, and it was thought to be effective against *H. pylori* infection.

In particular, the "on" status of *oipA* has been associated with pathogen-related diseases such as peptic ulcer and gastric cancer (89), but since it is epidemiologically associated with other virulence factors such as *cag PAI*, *vacA s1*, *vacA m1*, there is a need for further studies to explore the functions of a functional OipA protein in the pathogenesis.

There are still many questions about the role of bacterial virulence factors in interactions with the host, what functions they have in the pathogenesis of diseases,

and what potential they have in the treatment of the pathogen. Examining the relationship between these virulence factors, pathogen-related diseases, and each other is important in understanding the nature of diseases. The detection of changes caused by OMPs within the host may provide targets for the development of vaccines against the pathogen.

2.2 Types of Eukaryotic Cell Death

Cell death is a condition in which the integrity of the cell is disrupted and the cell becomes unable to perform its vital functions. In multicellular organisms, cell death occurs as a result of specific signaling events to maintain homeostasis and prevent cellular harmful events. Cell death may occur naturally in order to replace the aging cell with a new one, or it may occur due to disease, injury, infection, or the death of the organism to which the cell belongs. If cell death is genetically controlled and provides an advantage to the organism during its life cycle, it is called programmed cell death. Pathological cell death adversely affects natural functions and is called non-programmed cell death.

2.2.1 Nonprogrammed cell death

Necrosis, which is non-programmed cell death, results from factors outside the cell or tissue, such as infection or trauma, and results in the disordered digestion of cell components. Necrosis is a form of cell death in which the cell is damaged so severely that it cannot function with a sudden shock, and because cell-environment homeostasis is disrupted, the cell swells and membrane integrity is impaired.

Necrosis causes an inflammatory response that attracts phagocytes to remove dead cell components and may result in secondary tissue damage followed by a severe leukocyte response (90).

2.2.2 Programmed cell death

Programmed cell death is classified as apoptosis, autophagy, and necrotic cell death according to their formation mechanisms. Conditions such as inadequate nutrition, senility, diminished blood supply, and loss of innervation can be the driving force in the formation of programmed cell death.

Apoptosis occurs in order to protect cell populations according to the changing organism dynamics during development and aging processes, but it has also been shown that it can occur to protect from factors such as diseases or harmful agents (91). Apoptosis is characterized by chromatin condensation and nuclear fragmentation, plasma membrane swelling, and cell shrinkage. Unlike necrosis, in apoptosis, the remnants of the cell are enclosed in small sacs called apoptotic bodies, without being released into the external environment, so that the inflammatory response is not stimulated. It is estimated that approximately 1×10^9 cells per day undergo apoptosis in the human body (92).

Apoptosis occurs due to the activation of molecules known as cysteine aspartate-specific proteases (caspase) (91). Caspases can be divided into effector and initiator caspases according to their function; the N terminal region of initiator caspases is long to allow interaction with other protein structures, whereas the N thermal regions of effector caspases are short regions (93).

In the cell, apoptosis occurs as a result of caspase activation mediated by death receptors (DR) as an extrinsic pathway or mitochondria-dependent intrinsic pathway. In the extrinsic pathway, the enzymatic cascade is activated by proapoptotic ligands interacting with DRs (94). If it is in the intrinsic apoptotic pathway, cytosolic cytochrome c release increases according to mitochondria due to DNA damage or cytotoxic drugs, and this molecule supports the formation of apoptosomes (95).

During cellular development, in stress-induced autophagy, some proteins and organelles are recovered through the autophagosome. Autophagy is effective in cell differentiation and tissue remodeling (96). Autophagy, which is known to limit the

powers of oxidative damage produced in mitochondria, can be thought of as protective against aging that occurs as a result of this damage (97). Autophagy is also involved in the suppression of tumor growth, the elimination of misfolded proteins, the eradication of intracellular microorganisms, and antigen presentation (98-100). It is known that uncontrolled autophagy is effective in diseases such as Parkinson's and Alzheimer's (101,102). Vacuolization, cytoplasmic content degradation, and light chromatin condensation are all morphological characteristics of autophagy (103).

Sometimes the cell chooses other ways than apoptosis and autophagy to kill itself in a controlled manner. The necrotic cell deaths performed to achieve homeostasis are called necroptosis, oncosis, and pyroptosis.

Necroptosis is a controlled form of necrosis induced by the expression of DR ligands when favorable conditions for apoptosis are not provided. In the cell undergoing apoptosis, necroptosis occurs as a result of the inability of caspase-8 to be activated and the nuclear factor kappa B (NF-κB) pathway not supporting the cell (104). The formation of necroptosis is dependent on the activation of receptor-interacting protein 3 (RIP3) by RIP1 kinase and mixed lineage kinase domain-like pseudokinase (MLKL) (105,106). Necroptosis is a viral defense mechanism that allows the cell to go into suicide mode in order to prevent virus replication (107). Characteristic features of necroptosis are mitochondrial and cellular swelling and disruption of membrane integrity.

Oncosis is characterized by accidental cell and organelle swelling, blistering, and increased membrane permeability (108,109). The oncosis process results in the depletion of cellular energy stores and the malfunction of ionic pumps in the plasma membrane. Toxic chemicals that interfere with ATP synthesis or mechanisms that promote unregulated cellular energy consumption can cause oncosis (108). Extracellular leakage of cellular content associated with membrane rupture as a result of oncosis often promotes an inflammatory response in adjacent tissue, causing further cellular damage (109).

2.2.2.1 Pyroptosis

Pyroptosis is a proinflammatory cell death regulated by caspases and was first described by B. Cookson in 2001 (110). Prior to the definition of pyroptosis, this cell death was mistaken for apoptosis because it is caspase-dependent and caused by DNA damage and nuclear condensation. In later studies, the difference between pyroptosis and apoptosis was observed by detecting morphological changes such as the intact nuclear structure and the formation of pores in the cell membrane (111). Contrary to apoptosis, the pyroptotic cell can be stained with low molecular weight dyes such as propidium iodide (PI) and ethidium bromide (EtBr) according to pores in the cell membrane during pyroptosis (112). Unlike other caspases that are involved in apoptosis, caspase-1 is essential in pyroptosis, and it cannot occur in the absence of this protease (113).

Pyroptosis activated by intracellular or extracellular stimuli such as bacteria, viruses, toxins, and chemotherapy drugs can cause inflammation. During pyroptosis, pyroptotic bodies are formed (114). Studies are continuing to understand the formation mechanisms of pyroptosis and its effects on diseases.

2.2.2.1.1 Mechanism of pyroptosis

The formation of pyroptosis can occur in different ways, and studies are still ongoing to understand some of these pathways. For instance, in 2020, the granzyme-mediated pathway was defined as the state in which caspase 3 is activated due to the release of (Granzyme B) GzmB and the cell enters pyroptosis as a result of the division of gasdermin (GSDME) in chimeric antigen receptor T (CAR T) cells (115). Similarly, GSDMB cleavage and pyroptosis were observed in cytotoxic T cells due to GzmA (116).

Alternatively, with stimulants leading to caspase 3 activation, pyroptosis can occur in tumor cells with GSDME cleavage due to the release of apoptosis-inducing molecules by mitochondria due to DNA damage, a condition called the caspase 3

mediated pathway (117). Increasing the expression of GSDME, which is silenced by methylation in tumor cells, by chemotherapeutic drugs may change the substrate to which caspase 3 binds, causing the cell to enter pyroptosis instead of apoptosis (118).

Pyroptosis can occur via caspases 4-5 in humans and caspase 11, which has similar functions in mice, in what is called the non-canonical pathway. The non-canonical pyroptosis pathway that occurs directly by recognizing LPS may also be activated by recognizing pannexin-1 (119,120). It is also possible that there are other proteins that activate the pathway.

The canonical pathway is the best-understood pathway of pyroptosis, accompanied by GSDMD, IL-1β, IL-18, and inflammasome formation. In response to pathogenic microorganisms, oligomerization is induced at intracellular pattern recognition receptors (PRR) and combines with caspase precursors and an apoptosis-associated speck-like protein containing a CARD (ASC or PYCARD) to form inflammasome complexes (121). PRRs typically include receptors such as toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), and PRRs recognize specific pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (122). After PRRs recognize DAMPs and PAMPs that bind the NLR family pyrin domain containing 3 (NLRP3) to caspase-1, they associate with ASC to induce inflammasome formation. Caspase 1, which is activated due to inflammasome formation, allows IL-1β and IL-18 to pass from the precursor form to the active form. At the same time, activated caspase 1 cleaves GDSMD into N-terminal and C-terminal. While the C-terminal remains in the cytoplasm, the N-terminal integrates into the cell membrane, causing pore structures to form (123). The inner diameter of the pores is 15 nm, whereas the outside diameter is around 32 nm, and the cell may repair itself if the number of pores is low (124). While IL-1β, IL-18, and potassium are secreted outside the cell due to the pores formed, sodium and water enter the cell so osmotic lysis occurs (125). In addition, depending on the IL-1β, and IL-18 released from the cell, an immune response occurs against pyroptosis. Illustration showing canonical and noncanonical pathway mediated pyroptosis is given in Figure 2.

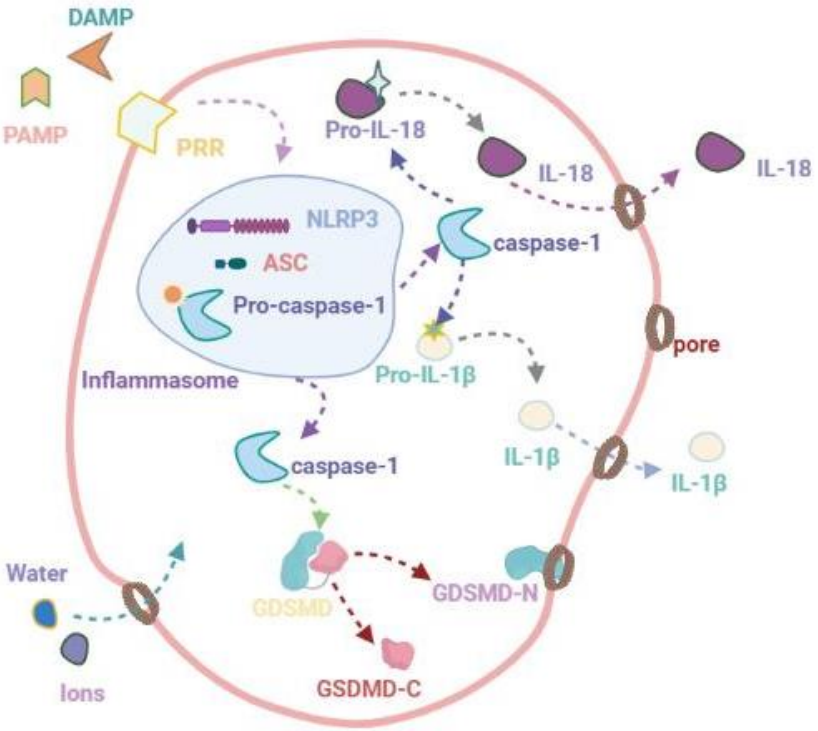
In some cases, pore-derived intracellular traps (PIT) can be created without releasing the cell contents to the external environment, and the ruptured cell membrane retains organelles and intracellular bacteria without dispersing to a large extent, a situation called efferocytosis. Efferocytosis is the process by which one cell is phagocytosis to another. While apoptotic bodies formed after apoptosis are efferocytosed by macrophages, PITs are efferocytosed by neutrophils during pyroptosis, this process is important in the recovery of intracellular pathogens (126,127).

Extracellular IL-1β affects the chemotactic migration of leukocytes, cytokine release, and vasodilation. Moreover, uncontrolled IL-1β release may be effective in the development of autoimmune diseases (124). IL-18 stimulates the release of the interferon-gamma (IFN-γ) cytokine, which induces the activation of T lymphocytes and macrophages (126). Uncontrolled pyroptosis can cause tissue damage, autoimmune disease development, and organ failure (127).

2.2.2.1.2 Detection of pyroptosis

Microscopic monitoring of changes in cell morphology can be used to detect pyroptosis (128). In addition, Annexin V/propidium iodide (Annexin V/PI) staining can be used for flow cytometry and microscope-mediated imaging of cellular pyroptosis (129). Moreover, Western Blot, real-time polymerase chain reaction (RT-PCR), immunohistochemistry, immunofluorescence, and enzyme-linked immunoassay (ELISA) methods can be used to detect biomarkers such as GSDM cleavage, caspase, and interleukin activation (128, 130).

A.



B.

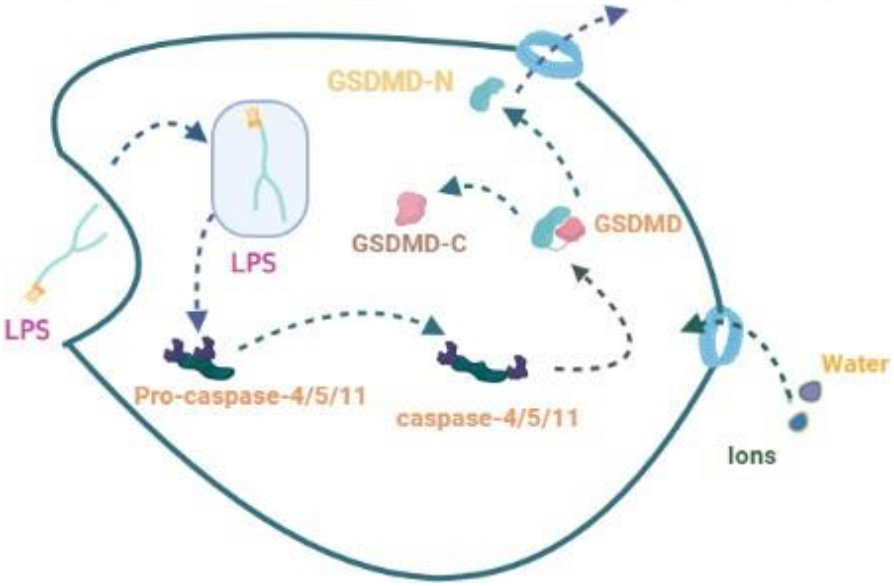


Figure 2. A. Canonical pyroptosis pathway promoter by activator signaling mediated by NLRP3 inflammasome formation. B. LPS-stimulated non-canonical pyroptosis pathway

2.2.2.1.3 Pyroptosis and gastric tissue damage

Although pyroptosis is a mechanism for the organism's advantage, it can cause irreversible damage to the organism due to some diseases and inflammatory disorders. The effects of pyroptosis at the cellular level can be seen in whole tissues. The effects of pyroptosis in the surrounding tissues are mainly based on the release of IL-1β and IL-18, but also include the cytokine storm created by other pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and IL-6 (131).

While pyroptosis is seen in all cell types, diseases that develop due to pyroptosis getting out of control also affect the digestive system. It has been observed that pyroptosis is effective in the pathogenesis of alcohol-induced gastritis, and the use of caspase-1 inhibitors can reduce cellular damage (132). Moreover, it is well known that IL-1β increases the risk of spontaneous gastric inflammation and the formation of gastric cancer (133). In gastric cancer, it has been observed that the expression of GSDMD is decreased, the phosphatidylinositol 3 kinase/protein kinase B pathway, which has been associated with proliferation and differentiation, is activated, and cells move from the S phase to G2 more rapidly (134). It has also been observed that chemotherapeutic drugs lead cells to GSDME-mediated pyroptosis rather than apoptosis in gastric cancer (135). The association of GSDMs with gastric cancer is very variable, with GSDMA, GSDMC, and GSDMD being down-regulated in the presence of gastric cancer (136-138), and overexpression of GSDMB in gastric cancer cells (137). In addition, it was observed that epithelial inflammation and hyperplasia were alleviated when IL-18 was given to NLRP3-deficient mice (139). In the presence of gastric cancer, famotidine was found to induce pyroptosis in a GSDME-dependent manner via NLRP3 activation (140).

2.2.2.1.4 Pyroptosis and *Helicobacter pylori* infection

Microbial infection is the main cause of pyroptosis, and the relationship between *H. pylori* infection and pyroptosis is very important in understanding the pathogenesis of pathogen-induced diseases. PAMPs from *H. pylori* induce the production of pro-inflammatory cytokines and chemokines by PRRs in the host's innate immune system

(141). Inflammatory cytokines such as TNFα, IL-1, IL-6, IL-8, IL-18, and IL-23 are produced when these transcription factors are activated, resulting in an inflammatory state (142). In mice, transgenic expression of Il-1β was seen to promote gastric dysplasia and cancer formation (128). Inflammation is also effective in gastric carcinomas caused by *H. pylori*.

While the distinction between apoptosis and pyroptosis has not yet been determined, it has been observed that *Shigella* invades the colonic mucosa of the human host and triggers programmed cell death in macrophages due to infection of phagocytic cells in the lamina propria, a process thought to be apoptosis (143, 144). Subsequent studies have shown that caspase-1-dependent pyroptosis is associated with infection by pathogens such as *Listeria*, *Legionella pneumophila*, *Yersinia pseudotuberculosis*, *Salmonella*, and *Pseudomonas aeruginosa* (145,146).

Human host cells contain numerous receptors such as (Toll-like receptor 2) TLR2 and nucleotide-binding domain 2 (NOD2) that are also targeted by *H. pylori*, and these bacteria-host interactions lead to the expression of NLRP3, IL-1β and IL-18 precursors. The binding of NLRP3 and ASC with a secondary signal stimulates autoproteolytic cleavage of pro-caspase-1, resulting in the processing and release of mature IL-1β and IL-18 cytokines (147). Interestingly, NODs directly interact with caspase-1 by processing IL-18 in a manner independent of the canonical inflammasome proteins, NLRP3 and ASC. This indicates that IL-18 is important in maintaining tissue homeostasis and protecting the stomach against pre-neoplastic changes due to *H. pylori* infection (148). Mucin-1 (MUC1), which is highly expressed on the gastric epithelial cell surface, protects the epithelial lining against inflammation (149). In patients infected with *H. pylori*, MUC1-mediated NLRP3 has been shown to induce inflammation through the NF-κB pathway (150).

It was observed that the expression of precursor forms of inflammatory cytokines increased in immune cells due to *H. pylori* infection. It was observed that *H. pylori* infection increased the expression of IL-18 at the messenger ribonucleic acid (mRNA) level, but no relationship was found between infection, caspase-1 activation, and IL-

18 secretion (151). Administration of exogenous control activators such as nigericin or adenosine triphosphate (ATP) to infected cells has been shown to result in NLRP3 inflammasome formation and a large transition from the precursor IL-1β form to the mature IL-1β form. This suggests that chronic *H. pylori* infection in humans regulates inflammasome activation and pyroptosis for bacterial persistence (152).

During *H. pylori* infection, the polymorphism of Il-1β has been shown to affect the prognosis of pathogen-related diseases (153, 154). It has also been suggested that caspase-4/11 may be a drug target in the treatment of *H. pylori*-associated peptic ulcers (155). In another study, 618 volunteers with gastric cancer were divided into two groups according to the expression levels of pyroptosis markers, and these groups were placed on a pyroptosis score scale by examining the active behaviors in the immune system and the proteins they encode. The obtained score scale was adapted to 7 gastrointestinal system cancers and showed consistent results regarding the survival and recurrence rates of the patients. It was observed that gastric cancer patients infected with *H. pylori* scored higher on this scale than normal patients, that is, *H. pylori* infection was directly related to pyroptosis (152).

It is known that *H. pylori* infection reduces micro-ribonucleic acid-22 (miRNA-22) expression and induces NLRP3 inflammation due to CagPAI (156). In addition, it has been shown that IL-1β release from macrophages increases gastric cancer due to NLRP3 inflammasome formation (154). In infection experiments using CagA+ and CagA- *H. pylori* strains in 2021, the expression levels of NLRP3, ASC, IL-1β and IL-18 were higher as a result of CagA+ *H. pylori* infection compared to CagA- strains (157). In addition, miRNA-223-3p-dependent NLRP3 expression, which has been associated with granulocytic differentiation, was not found to be dependent on virulence factors such as CagA, CagPAI, VacA, cholesterol-a-glucosyltransferase (Cgt), and FlaA (158). Similarly, infection with CagA+ strains has been shown to increase the NLRP3 inflammatory response and increase the potential for cancer and cell migration in the stomach by stimulating ROS formation. On the other hand, it has been suggested that the use of N-acetylcysteine (NAC) inhibits this and reduces the potential for cancer by suppressing the NLRP3 response (157). Also, NLRP3

transcription was shown to be induced by urease B, whereas IL-1β transcription was found to be stimulated by bacterial LPS (159).

Although inflammasomes have an important place in the response to the pathogen, they can trigger the formation of cancer and autoimmune diseases. At the same time, understanding the intracellular changes caused by the bacteria and the pathogenicity of the bacteria will be important in terms of developing new target mechanisms and molecules for its treatment. In the literature, there are various publications on the role of the virulence genes of *H. pylori* in bacterial infection and their effects on pathogen-induced diseases. However, determining the relationship between outer membrane virulence factors, which are known to be very effective in the attachment of *H. pylori* to stomach epithelium and cell death mechanisms, is very important for the development of new diagnosis and treatment methods for *H. pylori* infection, and also the relationship between these factors has not yet been clarified.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 *Helicobacter pylori* strain

H. pylori G27 strain, which is thought to express all virulence factors of *H. pylori*, was used in the study. This strain was kindly provided by Prof. Dr. Anne Müller from the Institute for Molecular Cancer Research at the University of Zurich and stored at -80°C in our laboratory.

Supplemented Colombia Agar was primarily used for the growth of *H. pylori*. The components of supplemented Colombia agar are given in Table 1-3. The content of 100 mL of liquid culture for *H. pylori* is given in Table 4.

Table 1. Colombian agar plate components

Component	Amount
Colombia Blood agar	1000 ml
Horse blood	50 ml
200X antibiotic cocktail	5 ml
1000X antibiotic cocktail	1 ml
Beta-cyclodextrin	5 ml (solved with dimethyl sulfoxide)

Table 2. 200X Antibiotic coctail components

Component	Amount
Vancomycin	100 mg
Cefsulodin	50 mg
Polymixin B	3,3 mg
Double distilled water (ddH_2O)	50 ml

Table 3. 1000X Antibiotic coctail components

Component	Amount
Trimetophrim	100 mg
Amphotericin B	160 mg
Dimethyl sulfoxide (DMSO)	20 ml

Table 4. *H. pylori* liquid culture medium components

Component	Amount
Brucella Broth	90 ml
Fetal Bovine Serum (FBS) (10%)	10 ml
Vancomycin (1000X)	10 µl

3.1.2 Primers

Table 5 lists the primers that were used in the study. Some of the primers used in the study were designed using the Primer Blast tool at the National Center for Biotechnology Information (NCBI) and considering the basic primer design rules. Some primers selected from the literature are given with their references.

Table 5. Primers, their product sizes and their references

Primer	Sequence	Base pair	References
ureA-F	TGATGGGACCAACTCGTAACCGT	244 bp	(160)
ureA-R	CGCAATGTCTAAGCGTTTGCCGAA		
ureB-F	AGTAGCCCGGTGAACACAACATCCT	645 bp	(160)
ureB-R	ATGCCTTTGTCATAAGCCGCTTGG		
cagA-F	TTGACCAACAACCACAAACCGAAG	183 bp	(161)
cagA-R	CTTCCCTTAATTGCGAGATTCC		
vacAs1s2-F	ATGGAAATACAACAAACACAC	259/286 bp	(162)
vacAs1s2-R	CTGCTTGAATGCGCCAAAC		
vacAm1m2-F	CAATCTGTCCAATCAAGCGAG	570/645 bp	(163)
vacAm1m2-R	GCGTCAAAATAATTCCAAGG		
alpB-F	TGCGACTGGTTCAGATGGTC	708 bp	This study
alpB-R	CTGAGCGTGGATTGGAAGGT		

Table 5 (cont'd). Primers, their product sizes and their references

alpA-F	GGCTTACGCTACTACGGCTT	214 bp	This study
alpA-R	GGCTGTTTCTTAGCGTGCTG		
sabA-F	TCGTCATCAGTGGCGTTTCA	621 bp	This study
sabA-R	GGTAGTTGGATTGGCCTGCT		
sabB-F	GCTATCAAATCGGCGAAGCG	234 bp	This study
sabB-R	CTTGCGCGGTGTTGTAGATG		
babA2-F	AATCCAAAAAGGAGAAAAAGTATGAAA	832 bp	(164)
babA2-R	TGTTAGTGATTTCGGTGTAGGACA		
babB-F	GGTGGGCCTATATCCACTGC	803 bp	This study
babB-R	TGAGTGCCAAAGTGAGCGAT		
babC-F	AACGGCGGTGTGTATCAGTT	755 bp	This study
babC-R	TGAGTGCCAAAGTGAGCGAT		
labA-F	GCAGCGTTCGTGAAAGACTC	294 bp	This study
labA-R	CGCATCAGGCAAGCTAGAGA		
hopQ-F	ACTCGGCTTCTGATGTGTGG	317 bp	This study
hopQ-R	TTTCACGCCCAATTCCATGC		
hopZ-F	AACGGTGCGATGAATGGGAT	436 bp	This study
hopZ-R	TCTTCACGCCTAGTTCCACG		
oipA-F	GTTTTTGATGCATGGGATTT	401 bp	(165)
oipA-R	GTGCATCTCTTATGGCTTT		
GAPDH-F	CTCATGACCACAGTCCATGC	129 bp	(166)
GAPDH-R	TTCAGCTCTGGGATGACCTT		
RT-NLRP3-F	CAACCTCACGTCACACTGCT	170 bp	(167)
RT-NLRP3-R	TTTCAGACAACCCCAGGTTC		
RT-ASC-F	CTGACGGATGAGCAGTACCA	224 bp	(167)
RT-ASC-R	CAGGATGATTTGGTGGGATT		

3.1.3 Commercial Kits

Commercial kits and their supplier company were used in the thesis study are shown in Table 6.

Table 6. Commercial kits with the supplier company

Commercial Kit	Supplier Company
Bacteria genomic DNA purification kit	GeneMark
ZR-Duet DNA/RNA MiniPrep Plus Kit	Zymo Research
PCR master mix II (5x)	GeneMark
High-Capacity cDNA Reverse Transcription Kit with RNAse Inhibitor	Applied Biosystems
Taq Pro Universal SYBR qPCR Master Mix	Vazyme
Clarity Western ECL Substrate	BIO-RAD
Pierce™ BCA Protein Assay Kit	Thermo Scientific

3.1.4 Antibodies

Antibodies, their supplier company, and dilution rates used in this study are shown in Table 7.

Table 7. Antibodies, their target protein sizes with the supplier company and dilution rates

Antibody	Target Protein Size (kDA)		Supplier Company	Dilution Rates
	Pro-Form	Active-Form		
Caspase-1	~45kDa	~23kDa	St. John's Laboratory	1:1000
GSDMD	~53kDa	~30kDa	St. John's Laboratory	1:750
IL-1β	~37kDa	~17kDa	St. John's Laboratory	1:2000
IL-18	~24kDa	~18kDa	St. John's Laboratory	1:1000
Beta-actin	——	~42kDa	Cell Signaling Technology	1:1000
Anti-rabbit IgG, HRP-linked Antibody	——	——	Cell Signaling Technology	1:2500

3.1.5 Antibiotics

Antibiotics and their supplier companys were used to grow *H. pylori* were given in Table 8.

Table 8. Antibiotics with the supplier company

Antibiotic	Supplier Company
Trimethoprim	ChemCruz
Amphotericin B	Bristol-Myers Squibb
Vancomycin HCl	Koçak Pharma
Cefsulodin	Koçak Pharma
Polymixin B Sulfate	ChemCruz
β-Cyclodextrin	SIGMA-ALDRICH

3.1.6 General biological and chemical materials

General biological and chemical materials that were used in this study are given with the supplier company in Table 9.

Table 9. General biological and chemical materials and their supplier company

Materials	Supplier Company
100bp DNA ladder	GeneMark
6x Loading Dye	GenerMark
Prestained Protein marker	GeneMark (10-180kda)
Agarose	NORGEN
50X TAE Buffer	GeneMark
Stabilyser Reagent	SIGMA-ALDRICH
FBS	Gibco
Ethidium Bromide Solution	SIGMA-ALDRICH
Brucella Broth	Remel
Columbia Blood Agar Base	OXOID
Acrylamide/Bis-acrylamide 30% solution	SIGMA-ALDRICH
Ethylenediaminetetra acetic acid (EDTA)	SIGMA-ALDRICH
Tetramethylethylenediamine (TEMED)	BioFroxx
Ammonium Per Sulfate (APS)	BioFroxx
Triton® X-100	Merck
Tween-20	SIGMA-ALDRICH
Glycine	BIO-RAD
Acetone ≥99%	Tekkim

Table 9 (cont'd). General biological and chemical materials and their supplier company

Trizma® Base	**SIGMA-ALDRICH**
Sodium Dodecycle Sulfate (SDS)	SIGMA-ALDRICH
4X Laemmli Sample Buffer	BIO-RAD
Ponceau S Solution	SIGMA-ALDRICH
Dry Milk Powder	PINAR
Ethanol %96	ISOLAB
Ethanol 100%	Merck
Methanol 100%	Merck
2-Propanol	Merck
Acetic Acid ≥99%	SIGMA-ALDRICH
Bovine Serum Albumin (BSA)	SIGMA-ALDRICH

3.1.7 Equipments

Equipments and their supplier company were used in this study, are listed with their supplier company in Table 10.

Table 10. Equipment with the supplier company

Equipment	**Supplier company**
Biosafety Class II Cabinet	Thermo Scientific
Thermo Cycler, PCR	BIO-RAD, T100
Thermo Cycler, RT-PCR	BIO_RAD CFX96 Touch Real-Time PCR Detection System
Water Purification System	Merck Millipore, Milli-Q® Advantage A10
NanoDrop	Thermo Scientific One^{C}
Light Microscopy	Leica DM500
Imaging System	BIO-RAD, ChemiDoc^{TM} MP
Jar Gassing System	Donwhitley Scientific
pH meter	Bench Meter Xs
Electrophoresis System	BIO-RAD, Mini Protean
Power Supply	BIO-RAD, Power^{TM} Pac Basic
Mini-Puroteain® Glass Plates	BIO-RAD, 1mM spacers
Mini-Puroteain® Short Plates	BIO-RAD

Table 10 (cont'd). Equipment with the supplier company

TransBlot Turbo™ Transfer System	**BIO-RAD**
Microcentrifuges	Thermo Scientific, MICROCL 21R and Thermo Scientific, MICROCL 17
Centrifuges	Beckman Coulter™ Allegra 64R and Thermo Scientific, SL16R
Vortex	bıoSan Combi-Spin FVL-2400N
Incubator	Thermo Scientific, HERATHERM
Shaking Incubator	N-BIOTEK, NB-205VL and Thermo Scientific, MAXQ 4450
Shaker	Witeg
Magnetic Shaker with Heat	Thermo Scientific, CIMAREC
Multi-Rotator	bioSan, MultiBioRS-24
Autoclave	Nüve steamArt
Dry Heat Sterilizer	Nüve FN120
Petri Dishes	ISOLAB
Pipettes	Eppendorf ResearchPlus, Ergo One and Thermo Scientific
Pipette Tips	RatioLab® and Greiner bio-one
Serological Pipette Tips	Greiner bio-one and ISOLAB
Pipet Controller	Thermo Scientific, Pipet Filler S1
Water Bath	EMCO, ESM-3710
Dry Block Heating Thermostat	BIOSAN, Bio TDB-100
Microwave	SAMSUNG T.D.S
Weighing Machine	UNIBLOC, SHIMADZU UW620H
Fridges	ARCTIKO and Kirsch
Homogenizer	BioSpec, Mini Bead Beater 16
Laboratory Hood	Wesemann, System Delta 30
Membrane	BioRad, Immun-Blot PVDF Membranes for Protein Blotting
Plate reader	BioTek, Power Wave xs2

3.2 Methods

3.2.1 Cultivation of *Helicobacter pylori* G27 strain and bacterial genomic DNA isolation

The culturing of the G27 strain, which is known to express all the virulence genes of *H. pylori*, was performed. Columbia agar was dissolved with double distilled water within the specified amounts and autoclaved. The temperature was adjusted by keeping it at 50°C for 1 hour so that the antibiotics would not degrade, and horse blood, 200X antibiotics, and 1000X antibiotic cocktails were added in the specified amounts. The selective medium prepared for *H. pylori* G27 was poured onto the plates and left for cultivation. An automatic gas system was used to provide ideal microaerophilic conditions for the bacteria spreading on the agar plates, and the culture was allowed to grow for 3–4 days at 37°C.

The motility and amount of bacteria collected by swap on agar plates were examined by a light microscope. Bacteria were taken into brucella broth containing vancomycin and FBS and cultured for 24 hours in a shaker incubator at 37°C and 180 revolutions per minute (rpm), also providing ideal microaerophilic conditions for bacteria with a gas system. The culture, which was determined by the light microscope to reach the desired density, was turned into a pellet by centrifugation at 3000 gravitational force (g) for 5 minutes. The supernatant was removed, and the pellet was stored at -80°C. All studies on *H. pylori* were carried out in a biosafety class II cabinet in the microbiology laboratory.

Genomic DNA was isolated from the *H. pylori* G27 pellet stored at -80°C, following the GeneMark Bacteria genomic DNA purification kit instructions. The concentration of the obtained DNA was measured in the nanodrop device. DNA was stored at -20°C to be used as positive controls in PCR experiments.

3.2.2 Obtaining of patient gastric tissue samples

The number of samples to be included in the study was determined using statistical power analysis, suggesting that tissue collection from 22 gastritis and 22 ulcer patients and a control group not infected with *H. pylori* (5 gastritis patients, 5 ulcer patients, and 5 patients with normal histology) was necessary for the study to be meaningful. In the a priori power analysis for single factor ANOVA, the type 1 error was taken as 0.05 and the type 2 error as 0.20. Based on studies of similar effects in the literature, a medium-sized effect was determined as f = 0.4. In the analysis made for 3 independent groups, it was calculated how many individuals should be taken per group for 80% power.

The research permit required for the continuation of the study was obtained from Acıbadem Mehmet Ali Aydınlar University and Acıbadem Healthcare Institutions Medical Research Ethics Committee (ATADEK). Gastric tissue samples to be included in the study were obtained from volunteer patients who agreed to participate in the study by signing the informed consent form provided by Prof. Dr. Arzu Tiftikçi at Acıbadem Maslak Hospital, Gastroenterology Clinic. In this study, in which only adult patients were included, those who refused to sign the consent form, and patients with conditions such as kidney failure, liver failure, diabetes, and pregnancy were excluded from the study group.

Tissue samples taken from the antrum region of the stomach during gastroscopy were taken into 400 µl of stabilizer solution, which was provided to the doctor beforehand and stored at 4°C until the operation period. The *H. pylori*-infected patient samples were detected by pathology reports. After the samples were taken, the stabilizer solution was stored at -80°C, so the DNA, RNA, and proteins in the tissues were preserved.

3.2.3 Isolation of DNA, RNA and protein from patient tissue samples

H. pylori-infected 22 gastritis patients and 22 ulcer patients were selected from the collected tissues. As the control group, 5 gastritis and 5 ulcer patients, and 5 volunteers with normal histological features without bacterial infection were selected. Tissue samples stored at -80°C were fragmented using a BioSpec homogenizerthrough metal beads. DNA and RNA isolation were performed as specified in the protocol of the ZR-Duet DNA/RNA MiniPrep Plus kit, and acetone precipitate was continued for protein isolation. During the isolation period, four times the volume of cold acetone was added to the lysate expressing the protein content and incubated at -20 for one hour after vortexing. After centrifugation at 13000g g for 10 minutes, the supernatant was removed, and the protein-containing pellet was left to dry for 30 minutes under a hood to remove the remaining acetone. The resulting pellets were dissolved by vortexing in 200–300 µl, 160 millimolar (mM) Tris hydrochloride (Tris HCl; pH = 6.8) containing 2% sodium dodecyl sulfate (SDS).

The concentrations of the obtained DNAs and RNAs were measured in the nanodrop device and measured as ng/µl. During the measurement, absorbance 260/230 and absorbance 260/280 values were also collected, and the purity of the products was evaluated. DNA and RNA samples were stored at -80°C.

Pierce BCA protein assay kit was used to determine the concentration of protein samples. In this assay, which is based on the measurement of absorbance resulting from the reduction of Cu^{2+} molecules to Cu^{+} molecules by proteins, BSA protein was used in the preparation of assay standards. After the standards and proteins isolated from patient tissue samples were treated with kit reagents, absorbance values were read at 565 nm in a BioTek plate reader. A standard curve was created according to the absorbance values of the standards, and the concentrations of the samples were found through the obtained equation. The concentrations of the obtained proteins werein g/mL, and the proteins were stored at -20°C.

3.2.4 Investigation of the presence of bacterial virulence genes by convensiyonel PCR

Some of the primers used in PCR reactions were prepared in NCBI Primer Blast using the NCBI database, taking into account the basic primer design rules. In addition, some primers were taken from various studies in the literature. All primers used in the study are given in Table 5 together with their references. Primers were aliquoted to a concentration of 10 picomoles (pmol) and stored at -20°C. PCR experiments were carried out in a biosafety class II cabinet and Bio-Rad Thermocycler was used for the reactions. GeneMark PCR Master Mix II (5X) kit was used in the experiments, the components for each reaction are given in Table 11.

Table 11. PCR reaction components

Components	Volume (Final 25 µl)
5X Buffer	5 µl
Forward Primer (10 pmol)	2 µl
Reverse Primer (10 pmol)	2 µl
DNA	2,5 µl
DNAse RNAse free Water (up to 25 µl)	13,5 µl

First of all, the presence of *ureA* and *ureB* genes was confirmed in the DNA of the patient samples, and results consistent with the pathology reports were obtained in terms of the presence of *H. pylori* infection. Then, the presence of *alpA, alpB, sabA, sabB, hopQ, hopZ, oipA, labA, babA2, babB,* and *babC* genes in *H. pylori*-infected patient groups was investigated by PCR reaction. In addition, the presence of *cagA, vacA s1/s2,* and *vacA m1/m2,* which are important virulence factors of the bacteria, was investigated at the gene level in patient samples. The steps of the reaction are given in Table 12. The annealing temperature information for the different primers is provided in Table 13.

Table 12. PCR reaction conditions

Step		Temperature	Time (min:s)
Initial Denaturation	1. Step	94^0 C	3:00
Denaturation	2. Step	94^0 C	0:30
Annealing	3. Step	Variable according to primers	0:45
Extension	4. Step	72^0 C	1:00
Cycle Repeat	5. Step	Go to 2. Step	39 cycles
Final Extension	6. Step	72^0 C	5:00
Cooling	7. Step	4^0 C	5:00

Table 13. Annealing temperatures of different primers

Primer	Annealing Temperature
ureA	60^0 C
ureB	60^0 C
cagA	58^0 C
vacA s1/s2	58^0 C
vacA m1/m2	58^0 C
babA2	$55,5^0$ C
babB	58^0 C
babC	58^0 C
alpa	58^0 C
alpB	58^0 C
sabA	58^0 C
sabB	58^0 C
hopZ	58^0 C
hopQ	58^0 C
oipA	60^0 C
labA	58^0 C

As a result of the PCR reaction, the products were run in a 1.5% agarose gel with loading dye at 100 volts for 1 hour, and then the gel was stained with EtBr. The image of the gel was taken with the ChemiDoc device.

According to the results obtained, the distribution of virulence genes in gastritis and ulcer patients was examined with the Chi-square test, and the risk factors between genes and diseases were calculated. In cases of ulcers and gastritis, correlation matrices were created to examine the coexistence of genes. GraphPad Prism 9 was used for statistical studies.

3.2.5 cDNA synthesis for real time PCR

The cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Bioscience) from RNA samples obtained from patient tissues, using a Bio-Rad thermocycler. During the experiment, the protocols suggested by the kit were followed. 10 µl of 2X reverse transcriptase master mix, prepared as described in Table 14 was taken into the tube, and the RNA-nuclease-free water mixture prepared with each sample containing an equal amount of patient-derived RNA was added. The steps of cDNA synthesis are listed in Table 15. The obtained products were stored at -20°C.

Table 14. 2X reverse transcriptase master mix components

Components	Volume (Final 10 µl)
10X RT Buffer	2 µl
25X dNTP mix (100 mM)	0,8 µl
10X Random Primer	2 µl
MultiScribe Reverse Transcriptase	1 µl
RNase inhibitor	1 µl
Nuclease-Free H_2O	3,2 µl

Table 15. cDNA synthesis reaction conditions

Step	Temperature	Time (h:min)
1. Step	25^0 C	0:10
2. Step	37^0 C	2:00
3. Step	85^0 C	0:05

3.2.6 Investigation of the expression of NLRP3 and ASC levels by real time PCR

Using the obtained cDNA products, the expression levels of NLRP3 and ASC in patient samples were investigated at the RNA level. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene-specific primer was used as housekeeping in the experiments, and normalization was done according to the GAPDH expression of patient samples. Experiments were performed in Biosafety Class II cabinets, and the protocol suggested by the Taq Pro Universal SYBR qPCR Master Mix was followed. The content of the reaction and the reaction condition are given in Table 16 and Table 17. Primers used in RT-PCR experiments can be seen in Table 5. In the experiments performed using the CFX96 Touch Real-Time PCR Detection System, each sample was studied in duplicate. For the study, the experimental group was divided into 5, and samples belonging to uninfected gastritis patients, an ulcer patient, and a healthy volunteer were used in each group.

Optimization experiments were performed according to the expression levels of the GAPDH gene and the amount of cDNA to be used was determined. RT-PCR experiments were set up in duplicate to examine the expression of GAPDH, ASC, and NLRP3 in the samples of each patient group, preferring the cDNA concentration where the GAPDH samples had a CT (cycle threshold) value between 25 and 30 cycles. The samples obtained as a result of RT-PCR were run in a 2% agarose gel at 100 volts for 100 minutes, and images were taken with the ChemiDoc device after staining with EtBr dye.

Table 16. RT-PCR reaction components

Components	Volume (Final 20 µl)
Taq Pro Universal SYBR qPCR Master Mix	10 µl
Forward Primer (10 pmol)	0,4 µl
Reverse Primer (10 pmol)	0,4 µl
cDNA	Variable
DNAse RNAse free Water (up to 20 µl)	8,2 µl

Table 17. RT-PCR reaction conditions

Step		Temperature	Time (min:s)
Initial denaturation	1. Step	95^0 C	2:00
Denaturation	2. Step	95^0 C	0:10
Annealing	3. Step	58^0 C	0:10
Extension	4. Step	72^0 C	0:20
Cycle Repeat	5. Step	Go to 2. Step	39 cycles
Melting Curve	6. Step	72^0 C	5:00
	7. Step	62^0 C	0:05
Final Extension	8. Step	95^0 C	5:00
Cooling	9. Step	37^0 C	0:30

Analyses of RT-PCR experiments were performed using Microsoft Excel. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of each gene. Student t-test (unpaired) was used to compare ASC and NLRP3 expressions in different patient groups. In order to examine the relationship between ASC and NLRP3 values, a the Kruskal-Wallis test was applied for each patient. Graphpad prism (9.0.0) was used for statistical analysis. The alpha value was set at 0.05.

3.2.7 Evaluation of expressions of GSDMD Caspase-1 IL-18 and IL-1β by Western blot

In order to compare the expression levels of the markers known to be activated in the pyroptosis process, protein samples belonging to different patient groups were run on 12% (w/v) SDS-polyacrylamide gel (SDS-PAGE) and transferred onto the Polyvinylidene Fluoride (PVDF) membrane by the wet transfer method. The components of the different phases of the SDS-page gel are given in Tables 18 and 19.

Protein samples were diluted 4:1 with a Laemmli buffer (BioRad) mixture (including 10% beta-mercaptoethanol) and then denatured by heating at 95˚C for 5 minutes. Samples were loaded equally into each well in 12µl. 10-180 kDA A pre-stained marker (GeneMark) was used to follow the running of the proteins into the gel. The protein samples were run at 80 volts until they came out of the stacking gel, and

then the voltage was raised to 160 volts. The contents of the running buffer used for running are given in Table 20.

Table 18. Separating gel content for 10 ml

Components	Volume
Distilled Water	3,2 ml
30% Acrylamide Bis-acrylamide	4 ml
1.5 M Tris HCl pH 8.8	2,6 ml
10% SDS	100 µl
10% APS	100 µl
TEMED	10 µl

Table 19. Stacking gel content for 5 ml

Components	Volume
Distilled Water	2,975 ml
30% Acrylamide Bis-acrylamide	670 µl
0.5 M Tris HCl pH 6.8	1,25 ml
10% SDS	50 µl
10% APS	50 µl
TEMED	5 µl

Table 20. 10X running buffer components

Components	Amount
Tris Base	30 gr
Glycine	144 gr
SDS	10 gr
ddH_2O	up to 1L

Before transfer, the PVDF membrane (Bio-Rad) was activated by sequential washing with methyl alcohol, ddH_2O, and 1X transfer buffer. The components of the transfer buffer are given in Table 21.

Table 21. 10X transfer buffer components

Components	Amount
Tris Base	30 gr
Glycine	144 gr
ddH_2O	up to 1L

The transfer process, which took 16 hours with 20 volts, was carried out at 4°C. After the transfer, the membrane was blocked by incubating in an orbital shaker for 1 hour with TBS-T solution containing 5% (w/v) milk powder. The preparation of 10X TBS buffer (pH = 8) and 1X TBS-T is given in Tables 22 and 23. After that, the membrane was washed three times for five minutes using 1X TBS-T buffer. The membrane was cut to express portions of it comprising activated markers, and beta-actin was used as a positive control. Membranes were blotted by incubating at room temperature for 3 hours in the solution in TBS-T buffer prepared to express a 1:2000 dilution (v/v) of the targeted primary antibodies. The membrane was then rinsed three times for five minutes with 1X TBS-T buffer. The washed membrane was left for 1 hour at room temperature with the anti-rabbit IgG antibody selected as a secondary antibody and diluted with 1X TBS-T 5% milk solution to 1: 2500. Membranes were rinsed as previously described. Illuminances produced using a Clarity Western ECL substrate based on horseradish peroxidase (HRP) substrate were read through ChemiDoc devices. In the western blotting results, the area of the obtained bands was determined using Image Lab Software.

Table 22. 10X TBS buffer components

Components	Amount
Tris Base	28 gr
Sodyum Chloride (NACI)	88 gr
HCI	Up to the pH is equal to 8
ddH_2O	up to 1L

Table 23. 1X TBS-T buffer components

Components	Volume
10X TBS buffer	100 ml
Tween 20	1 ml
ddH_2O	up to 1L

After the first blotting, antibodies on the membrane were removed by stripping and reblotted with beta-actin for normalization. The contents of the strip buffer used for the stripping process are given in Table 24.

Table 24. Strip buffer components

Components	Volume
Glycine	15 gr
SDS	1 gr
Tween 20	10 ml
HCI	Up to the pH is equal to 2,2
ddH_2O	up to 1L

Following the first blotting, the membrane was washed three times with strip buffer for 10 minutes. Afterward, the membrane was washed twice with PBS and once with TBS-T buffer for 10 minutes, then it was taken into the milk powder-TBS-T solution and the second blotting process was started. The blotting process was carried out as previously stated.

Using Image Lab software, the volume occupied by the bands on the membrane was determined, and the band volumes in the active and pro states of GSDMD, caspase-1, IL-1β, and IL-18 proteins were normalized to beta-actin and examined to see how the protein expressions changed in a semi-quantitative manner. Microsoft Excel was used for the semi-quantitative analysis. In order to examine the relationship between caspase-1, GSDMD, IL18 and IL1β values, a Kruskal Wallis test was applied for each patient. Graphpad Prism (9.0.0) was used for statistical analysis. To investigate the relationship between active and pro forms of target markers, correlation

matrices were created, and the linearity and strength of the relationship between pro and active forms were evaluated.

3.2.8 Statistical analysis

In order to examine the effects off the expression of bacterial virulence genes and pyroptosis markers; on the pathogen-related diseases, the experimental results obtained were investigated using Graphpad Prism (9.0.0).

Using the results determined by qRT-PCR and Western Blot methods, a correlation matrix was created for each patient group and the synchronicity in the expression levels of the markers was examined. Investigating the frequency of the co-expressions of markers together, the Pearson r coefficient was calculated. At the same time, *p* values were calculated to examine the statistical significance of co-expressions of markers.

Furhermored, scatter plots were created to examine the relationship between pro and active forms of target markers for each patient group. GraphPad Prism (9.1.1) is used for this process.

In order to examine the relationship between the increased expression of pyroptosis markers and the virulence factors of *H. pylori*, the presence of virulence factors in patient tissues where each marker was upregulated was investigated.

Within the scope of this study, an illustration showing the process of examining the expression levels of *H. pylori* virulence genes and some pyroptosis markers in tissues collected from volunteers is given in Figure 3. This illustration was created by BioRender.

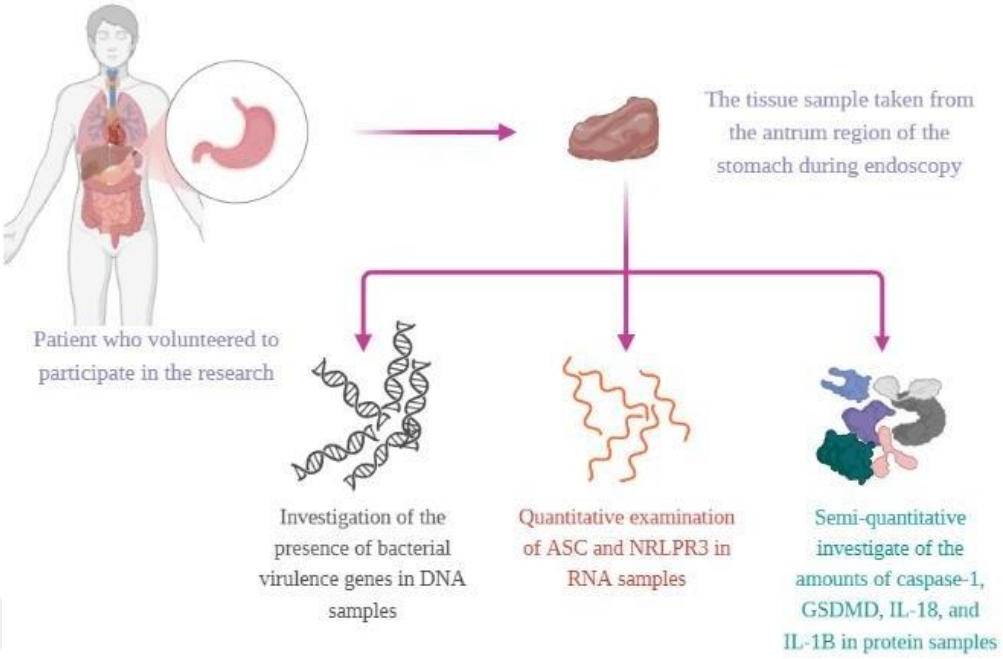


Figure 3. The methodology of examining the expression levels of *H. pylori* virulence genes and some pyroptosis markers in tissues collected from volunteers.

4 RESULTS

4.1 Isolation of Bacterial Genomik DNA

DNA was isolated from the *H. pylori* G27 strain using the GeneMark Bacteria genomic DNA purification kit, and the amount of DNA obtained was determined by the Nanodrop device. The amount of bacterial genomic DNA obtained is 29.4 ng/ul. (Absorbance 260/280: 1,88; Absorbance 260/230: 2,22)

4.2 Extraction of DNA RNA and Protein

During the study period, 121 patient tissues were collected, and *H. pylori* infection was detected in 50 patients (41.2%) with pathology reports. Although two of these patients were listed as infected in the pathology reports, no bacteria were found in the tissue samples taken for the study. In addition, one patient was found to be chimerically infected and was excluded from the study.

Tissues from 22 gastritis and 22 ulcer patients were selected from the collected tissues, and DNA, RNA, and protein isolation were performed. In addition, tissue samples from 5 uninfected gastritis patients, 5 uninfected ulcer patients, and 5 volunteers with normal histology were included in the study as controls. Isolated DNA and RNA concentrations were measured with the NanoDrop device, and BCA assay was performed to determine protein concentrations.

The mean age of the patients included in the study is 46.5. The disease-age distribution of patients infected with *H. pylori* included in this study is given in Figure 4. The age distribution graph was created using GraphPad Prism (9.0.0.). A two-way ANOVA was applied to examine the age distribution in the diseases, and the distribution was not statistically significant.

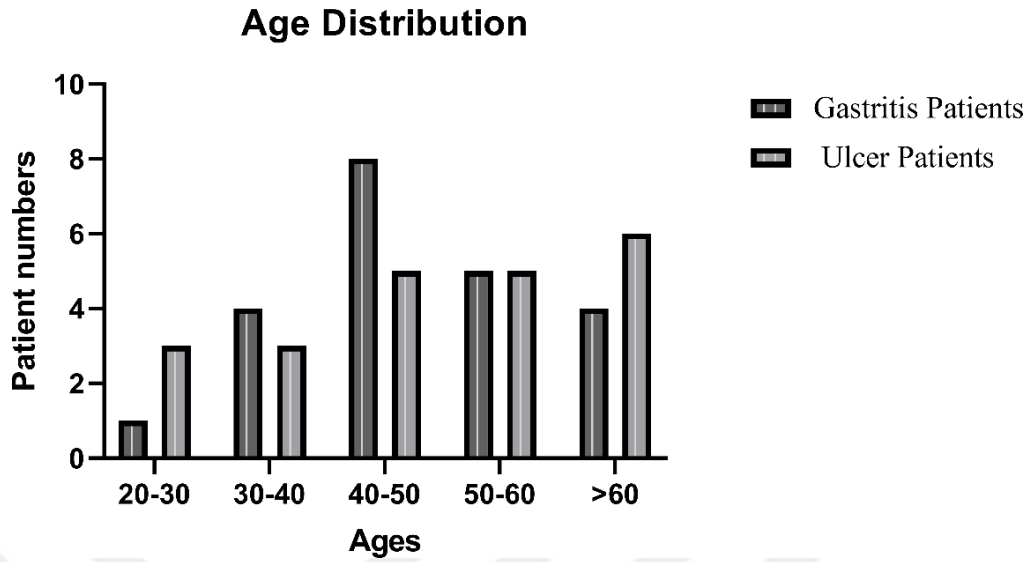


Figure 4. Age distribution graph.

The gender distribution of infected patients included in the study is 19/25 (the f/m ratio is 43%), and a two-way ANOVA was applied to examine the age distribution of the diseases. The gender distribution of diseases is given in Figure 5.

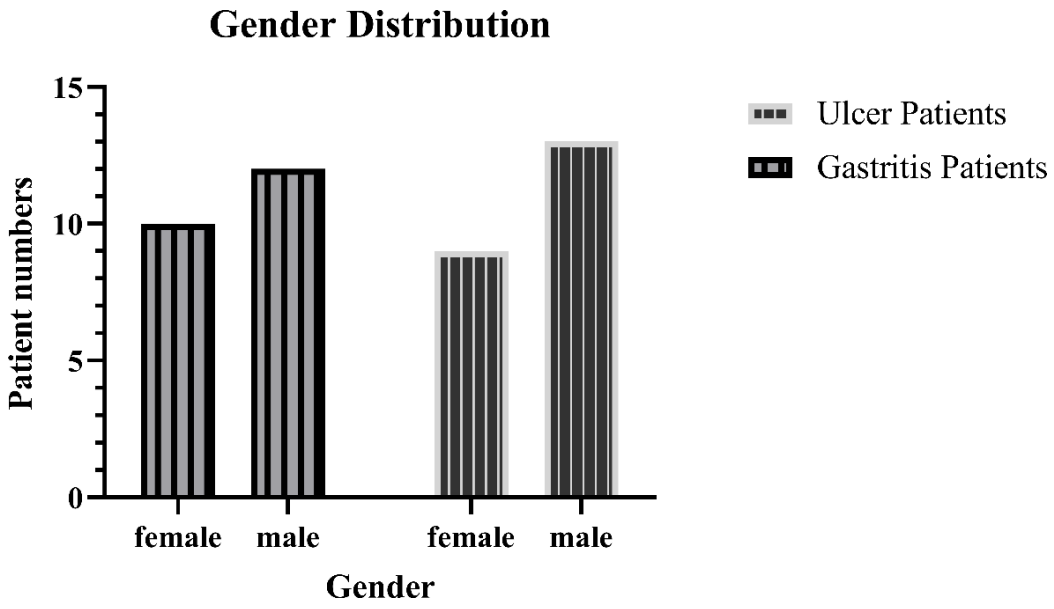


Figure 5. Gender distribution graph.

4.3 Investigation of the Presence of Bacterial Virulence Genes by Conventional PCR

The presence of virulence genes of *H. pylori* in DNA samples isolated from patient tissues was investigated by conventional PCR. At the end of PCR, samples were run on 1.5% agarose gel and visualized by staining with EtBr dye. Figure 6. shows the expressions of *H. pylori* virulence genes included in the study on the *H. pylori* G27 strain. The expression status of main virulence genes in patient tissues is shown in Table 26, and the expression status of outer membrane virulence genes is shown in Table 27. Heatmaps were created to simultaneously examine the expression of virulence genes in patients infected with *H. pylori*, these are shown in Figure 7. Heatmaps were created using GraphPad Prism (9.0.0).

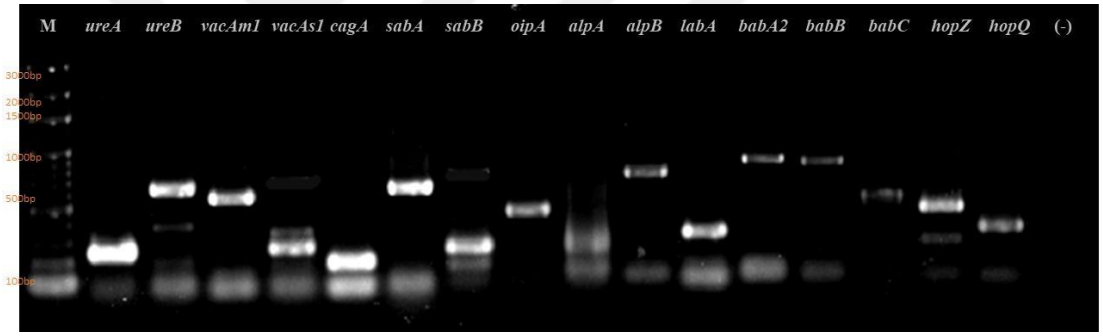


Figure 6. Agarose gel image showing the virulence genes included in the study.

Table 25. Expressions of bacterial main virulence genes in patient tissues

Sample No	*ureA*	*ureB*	*vacA*		*cagA*
			m1/m2	*s1/ s2*	
HpG1	(+)	(+)	m1	s1	(+)
HpG2	(+)	(+)	m1	s1	(+)
HpG3	(+)	(+)	m2	s1	(+)
HpG4	(+)	(+)	m1	s1	(+)
HpG5	(+)	(+)	m2	s1	(+)
HpG6	(+)	(-)	(-)	(-)	(-)
HpG7	(+)	(+)	m2	s1	(+)

Table 25 (cont'd). Expressions of bacterial main virulence genes in patient tissues

HpG8	**(+)**	**(+)**	**m1**	**s1**	**(+)**
HpG9	(+)	(+)	m1	s1	(+)
HpG10	(+)	(-)	m1	(-)	(-)
HpG11	(+)	(+)	m2	s1	(-)
HpG12	(+)	(+)	m1	s1	(-)
HpG13	(+)	(+)	m2	s1	(+)
HpG14	(+)	(+)	m1	s1	(-)
HpG15	(+)	(+)	m2	s2	(-)
HpG16	(+)	(+)	m2	s2	(-)
HpG17	(+)	(+)	m1	s1	(-)
HpG18	(+)	(+)	m2	s2	(-)
HpG19	(+)	(+)	m1	s1	(-)
HpG20	(+)	(+)	m2	s1	(-)
HpG21	(+)	(+)	m2	s1	(+)
HpG22	(+)	(+)	m2	s1	(-)
HpU1	(+)	(+)	m2	s1	(+)
HpU2	(+)	(+)	m2	s1	(-)
HpU3	(+)	(+)	m1	s1	(+)
HpU4	(+)	(+)	m1	s1	(+)
HpU5	(+)	(+)	m2	s1	(-)
HpU6	(+)	(+)	m2	s1	(+)
HpU7	(+)	(+)	m2	s2	(-)
HpU8	(+)	(+)	m2	s1	(-)
HpU9	(+)	(+)	m2	s1	(-)
HpU10	(+)	(+)	m2	s1	(+)
HpU11	(+)	(+)	m2	s1	(+)
HpU12	(+)	(+)	m1	s1	(+)
HpU13	(+)	(+)	m2	s1	(+)
HpU14	(+)	(+)	m1	s1	(-)
HpU15	(+)	(+)	m2	s2	(-)
HpU16	(+)	(+)	m1	s1	(+)
HpU17	(+)	(+)	m1	s1	(+)
HpU18	(+)	(+)	m2	s2	(-)
HpU19	(+)	(+)	m1	s1	(+)
HpU20	(+)	(+)	m1	s1	(+)

Table 25 (cont'd). Expressions of bacterial main virulence genes in patient tissues

HpU21	**(+)**	**(+)**	**m1**	**s1**	**(+)**
HpU22	(+)	(+)	m1	s1	(-)

Table 26. Expressions of bacterial outer membrane virulence genes in patient tissue sample

Sample No	***sabA***	***sabB***	***hopQ***	***hopZ***	***babA***	***babB***	***babC***	***alpA***	***alpB***	***labA***	***oipA***
HpG1	(+)	(+)	(+)	(+)	(-)	(+)	(-)	(+)	(+)	(+)	(+)
HpG2	(+)	(+)	(+)	(+)	(-)	(+)	(-)	(+)	(+)	(+)	(+)
HpG3	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(+)
HpG4	(+)	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(+)	(+)	(+)
HpG5	(+)	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(+)	(-)	(+)
HpG6	(-)	(-)	(-)	(+)	(-)	(-)	(+)	(-)	(-)	(-)	(-)
HpG7	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(+)
HpG8	(-)	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(+)
HpG9	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)
HpG10	(+)	(-)	(+)	(-)	(-)	(-)	(-)	(+)	(+)	(-)	(-)
HpG11	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(+)
HpG12	(+)	(+)	(+)	(+)	(-)	(+)	(-)	(+)	(+)	(+)	(-)
HpG13	(+)	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(+	(-)	(+)
HpG14	(+)	(+)	(+)	(+)	(+)	(-)	(-)	(+)	(-)	(+)	(-)
HpG15	(+)	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(-)	(-)	(+)
HpG16	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(-)	(+)
HpG17	(+)	(+)	(+)	(-)	(-)	(-)	(-)	(+)	(+)	(-)	(-)
HpG18	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(-)	(+)
HpG19	(+)	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(+)	(+)	(+)
HpG20	(+)	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(+)	(-)	(+)
HpG21	(+)	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(+)	(-)	(+)
HpG22	(+)	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(+)	(-)	(-)
HpU1	(+)	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(+)	(-)	(+)
HpU2	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(-)	(+)
HpU3	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(+)
HpU4	(-)	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(+)	(+)	(-)

Table 26 (cont'd). Expressions of bacterial outer membrane virulence genes in patient tissue sample

HpU5	**(+)**	**(+)**	**(+)**	**(+)**	**(-)**	**(-)**	**(+)**	**(+)**	**(+)**	**(+)**	**(+)**
HpU6	(+)	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(+)	(+)	(+)
HpU7	(+)	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(+)	(-)	(-)
HpU8	(+)	(+)	(+)	(+)	(-)	(+)	(-)	(+)	(+)	(-)	(-)
HpU9	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(-)	(+)
HpU10	(+)	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(+)	(-)	(+)
HpU11	(+)	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(+)	(-)	(+)
HpU12	(+)	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(+)	(+)	(+)
HpU13	(+)	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(+)	(+)	(+)
HpU14	(+)	(-)	(+)	(+)	(-)	(-)	(-)	(+)	(+)	(-)	(+)
HpU15	(+)	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(+)	(-)	(+)
HpU16	(+)	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(+)	(-)	(+)
HpU17	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(-)	(+)
HpU18	(+)	(-)	(+)	(+)	(+)	(-)	(-)	(+)	(-)	(-)	(+)
HpU19	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)
HpU20	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(-)	(+)
HpU21	(+)	(+)	(+)	(+)	(-)	(+)	(-)	(+)	(+)	(-)	(+)
HpU22	(+)	(+)	(+)	(+)	(-)	(+)	(-)	(+)	(+)	(-)	(+)

ureA gene was found in all gastritis and ulcer patients infected with *H. pylori; ureB, hopZ, hopQ*, and *alpA* genes were also seen in all ulcer patients. No variability was found in the incidence of the *sabB* gene in patients with gastritis and ulcers. The *babA2, babB, babC*, and *labA* genes were more common in gastritis patients than ulcer patients. *ureB, cagA, oipA, sabA, alpA, alpB, hopZ,* and *hopQ* genes were seen more frequently in ulcer patients than in gastritis patients. Alleles of the *vacA* gene are frequently seen in *s1* and *m2* alleles in ulcer patients, while the opposite is true for gastritis patients. None of the distributions of the bacterial virulence genes were found statistically significant in gastritis and ulcer patients

A.

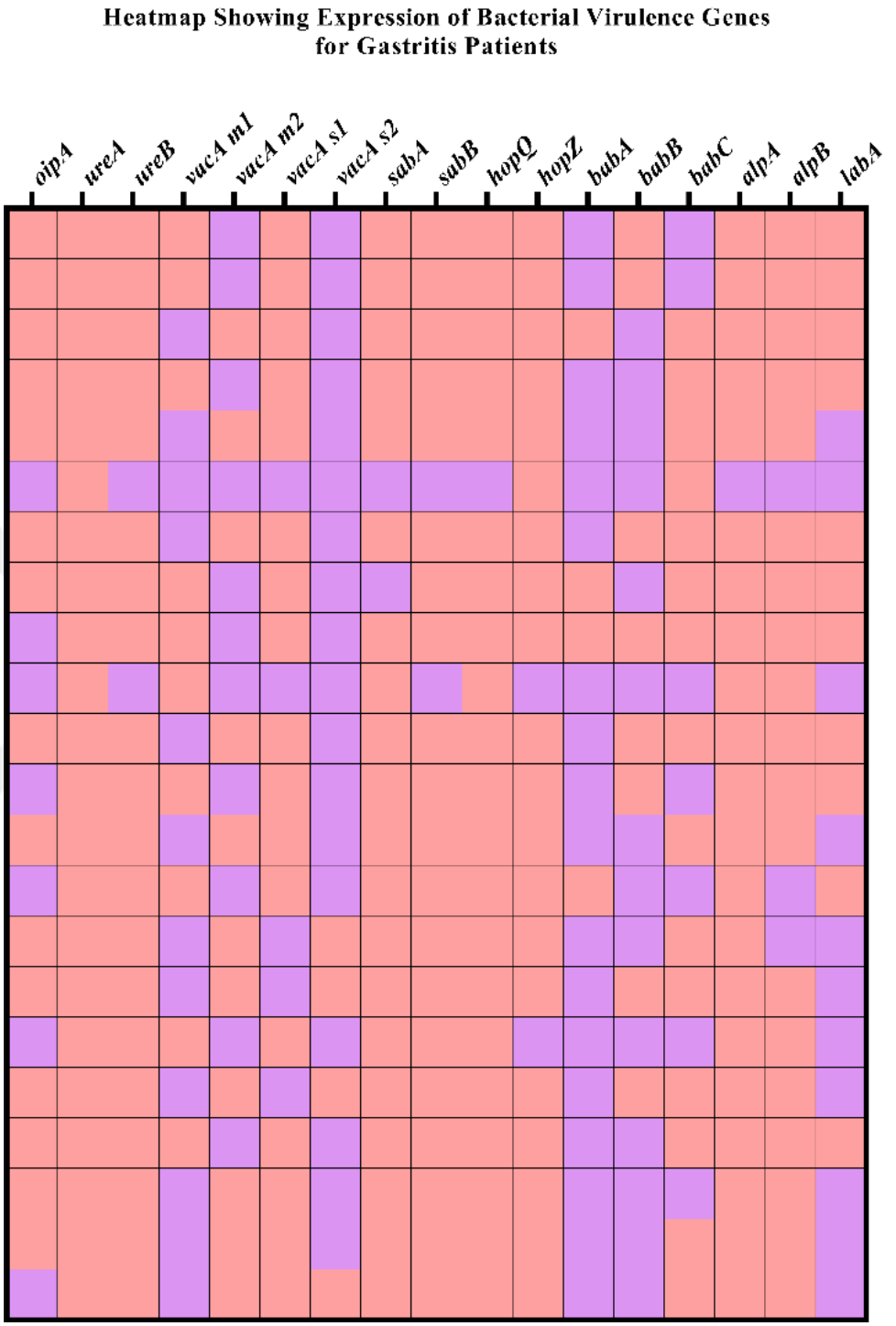


Figure 7. Heatmaps showing simultaneous expression of virulence genes in patients infected with *H. pylori.* **A.** Gastritis patietns, **B.** Ulcer patients, **C.** All patients

B.

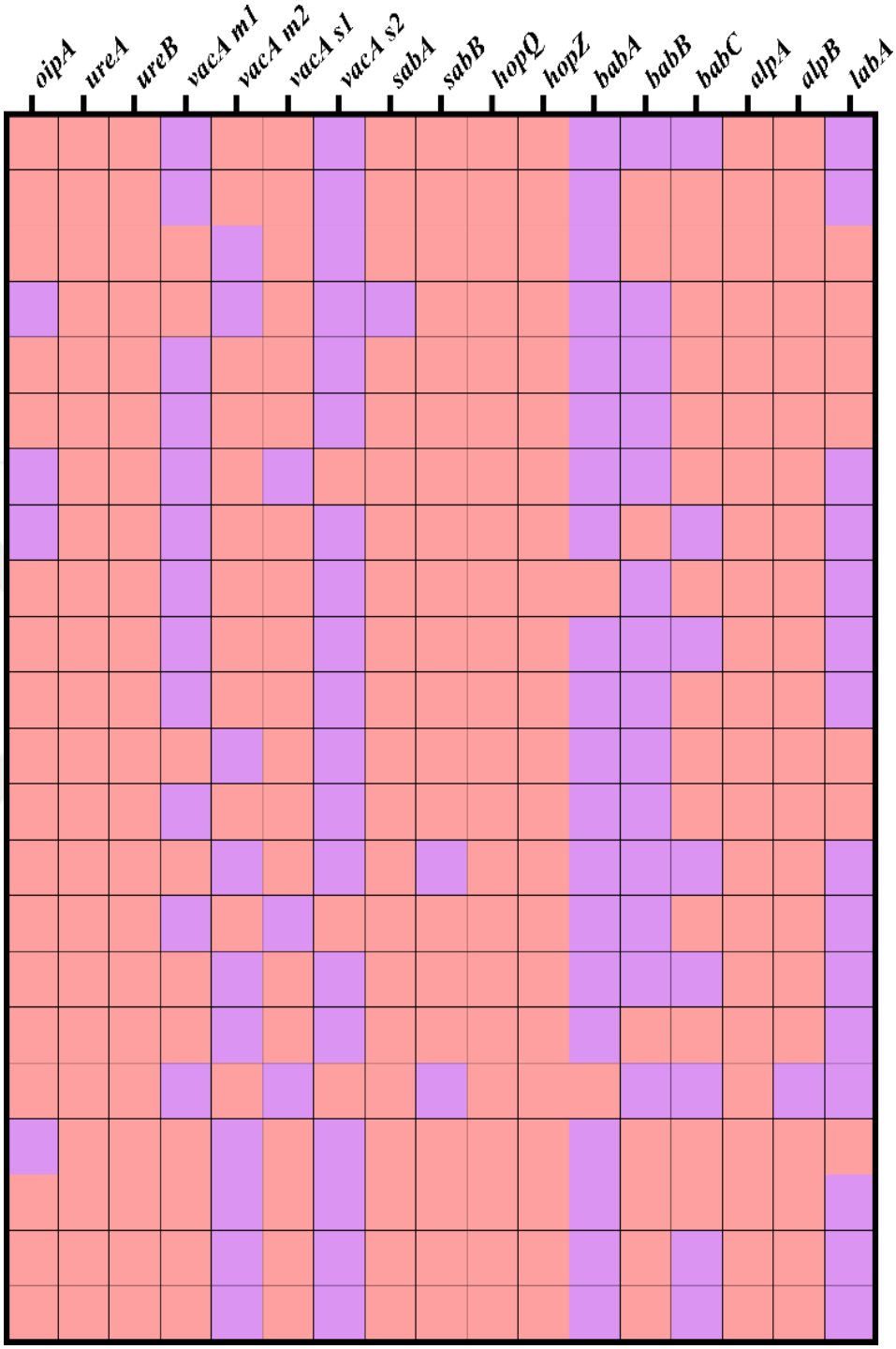


Figure 7 (cont'd). Heatmaps showing simultaneous expression of virulence genes in patientsinfected with *H. pylori*. **A.** Gastritis patietns, **B.** Ulcer patients, **C.** All patients

C.

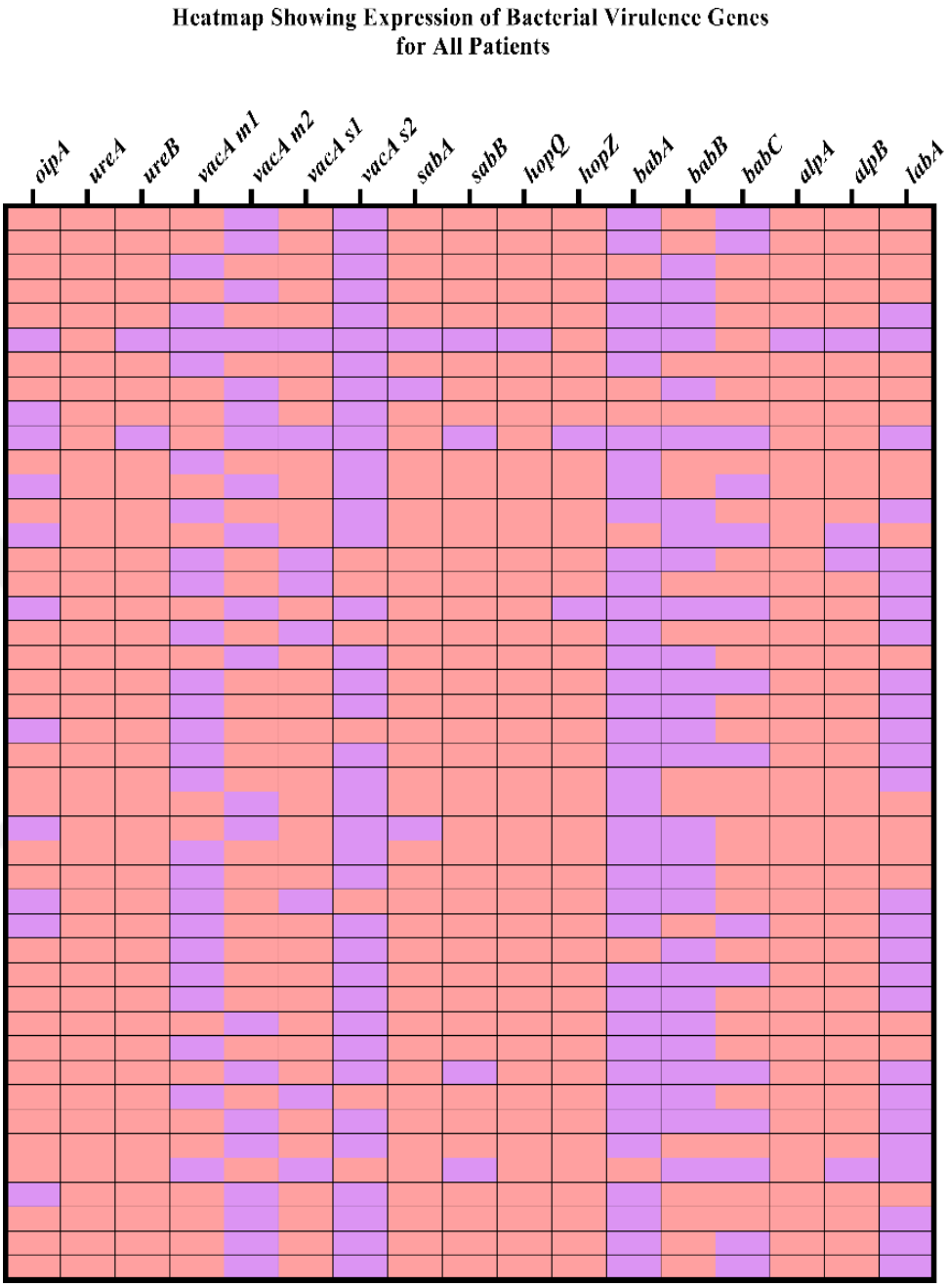


Figure 7 (cont'd). Heatmaps showing simultaneous expression of virulence genes in patientsinfected with *H. pylori.* **A.** Gastritis patietns, **B.** Ulcer patients, **C.** All patients

Chi-square Fisher's Excact test was performed using GraphPad Prism (9.0.0) to examine the distribution of virulence genes of bacteria in different patient groups. The frequency of virulence genes in patient groups and the *p* values and relative risks calculated using the Pearson's Chi-square Fisher's Excact test are given in Table 27.

Table 27. Incidance and *p* values and relative risks calculated according to the chi-square test of the distribution of bacterial virulence genes

Genes	**Situation**	**Incıdance of Gastritis**		**Incıdance of Ulcer**		**Relative Risk**	**p value**
		n	**(%)**	**n**	**(%)**		
ureA	**Present**	22	100	22	100	-	>>0,99
	Absent	0	0,0	0	0,0		
ureB	**Present**	20	90,9	22	100	2,1	0,49
	Absent	2	9,1	0	0,0		
cagA	**Present**	10	45,5	13	59,1	1,3	0,55
	Absent	12	54,5	9	40,9		
vacA	**s1**	17	77,3	19	86,4	-	-
	s2	3	13,6	3	13,6		
	Absent	2	9,1	0	0,0		
vacA	**m1**	10	45,5	10	45,5	-	-
	m2	11	50,0	12	54,5		
	Absent	1	4,5	0	0,0		
oipA	**Present**	15	68,2	18	81,8	1,4	0,3
	Absent	7	31,8	4	18,2		
babA2	**Present**	4	18,2	2	9,1	0,7	0,66
	Absent	18	81,8	20	90,9		
babB	**Present**	8	36,4	8	36,4	1	>>0,99
	Absent	14	63,6	14	63,6		
babC	**Present**	15	68,2	14	63,6	0,9	>>0,99
	Absent	7	31,8	8	36,4		
sabA	**Present**	20	90,9	21	95,5	1,4	>>0,99
	Absent	2	9,1	1	4,5		
sabB	**Present**	20	90,9	20	90,9	1	>>0,99
	Absent	2	9,1	2	9,1		
alpA	**Present**	21	95,5	22	100	2,1	>>0,99
	Absent	1	4,5	0	0,0		

Table 27 (cont'd). Incidance and *p* values and relative risks calculated according to the chi-square test of the distribution of bacterial virulence genes

alpB	**Present**	19	86,4	21	95,5	1,6	0,61
	Absent	3	13,6	1	4,5		
hopZ	**Present**	20	90,9	22	100	2,1	>>0,99
	Absent	2	9,1	0	0,0		
hopQ	**Present**	21	95,5	22	100	2,1	>>0,99
	Absent	1	4,5	0	0,0		
labA	**Present**	11	50,0	7	31,8	0,69	0,36
	Absent	11	50,0	15	68,2		

4.4 Investigation of the Expression of NLRP3 and ASC Levels by Real Time PCR

CT values for GAPDH, ASC and NLRP3 genes were obtained as a result of RT-PCR experiments performed to examine ASC, and NLRP3 expressions in patient samples. The gel images of the samples in which the GAPDH, ASC, and NLRP3 genes were amplified, obtained as a result of RT-PCR experiments, are given in Figure 8. The CT values were normalized using the $2^{-\Delta\Delta Ct}$ method. Normalization was done according to GAPDH expression.

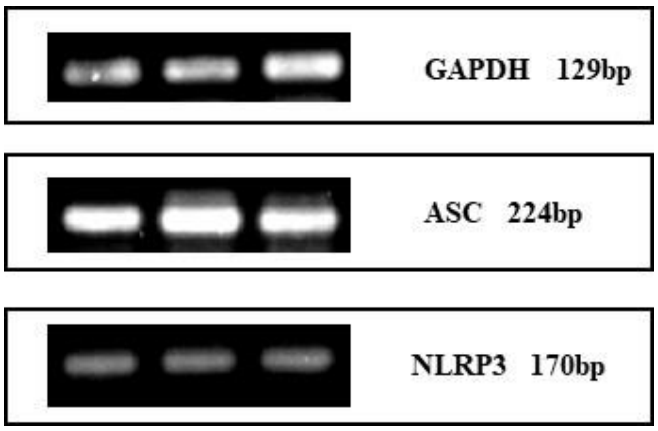


Figure 8. Gel images of GAPDH, ASC and NLRP3

The distribution of relative fold change ratios of the ASC and NRLP3 genes in different patient groups is given in Figure 9. The Kruskal-Wallis test was used to test the significance of the mean ratio of target markers to GAPDH expression in four different patient groups by using GraphPad Prism (9.0.0). The alpha value of 0.05 was chosen. The graphs was prepared according to the median with interquartile range value.

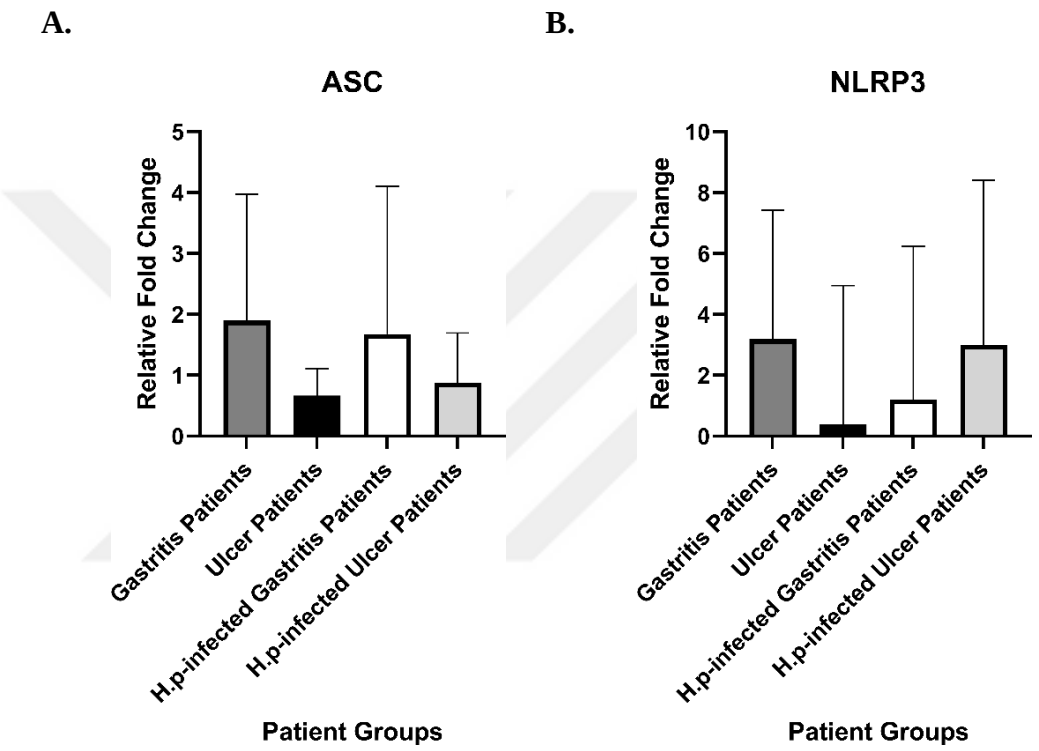


Figure 9. A. The change in fold change values in different groups of the ASC gene expressions, B. The graph showing the change in the fold change values in different groups of the NLRP3 gene expressions.

Changes in ASC and NLRP3 expressions were not significant for any patient group. It has been observed that the ASC gene is expressed more in gastritis patients than in ulcer patients. Expression of both ASC and NLRP3 was highest in uninfected gastritis patients. In addition, *H. pylori* infection increased the expression levels of both ASC and NLRP3 in ulcer patients.

4.5 Semi-quantitative Analysis of GSDMD Caspase-1 IL-18 and IL-1β by Western Blot

Protein samples isolated from patient tissues were transferred onto PVDF membranes following 12% SDS-page electrophoresis and bloted with GSDMD, caspase-1, IL-1β, and IL-18. For the normalization of samples, target proteins on each membrane were stripped and rebloted with beta-actin. The images obtained as a result of Western Blot were analyzed with the Image Lab program. The area occupied by the bands corresponding to the target proteins was calculated. Western blot results for active and pro-forms of target proteins are given in Figure 10.

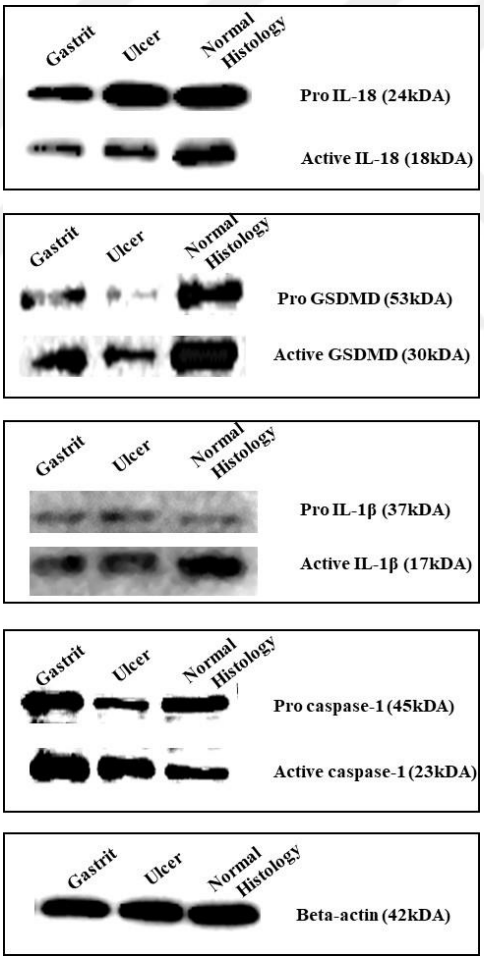


Figure 10. Western blot results of target proteins

Using Microsoft Excel, each sample was normalized to beta actin expression. The fold change values were calculated by calculating the ratio of the samples to the control group of patients with normal histology. The distribution of relative fold change values of the pro and active form of caspase-1, GSDMD, IL-18 and IL-1β in different patient groups is given in Figure 11. The graphs was prepared according to the median with interquartile range value. Kruskal Wallis test was applied to all patient groups for caspase-1, GSDMD, IL-18, and IL1β expression levels by using GraphPad Prism (9.0.0). The alpha value of 0.05 was chosen. It was found to be statistically significant when p <0.05. To investigate the relationship between mature and pro forms of target markers, correlation matrices were created, and the linearity and strength of the relationship between pro and active forms were evaluated.

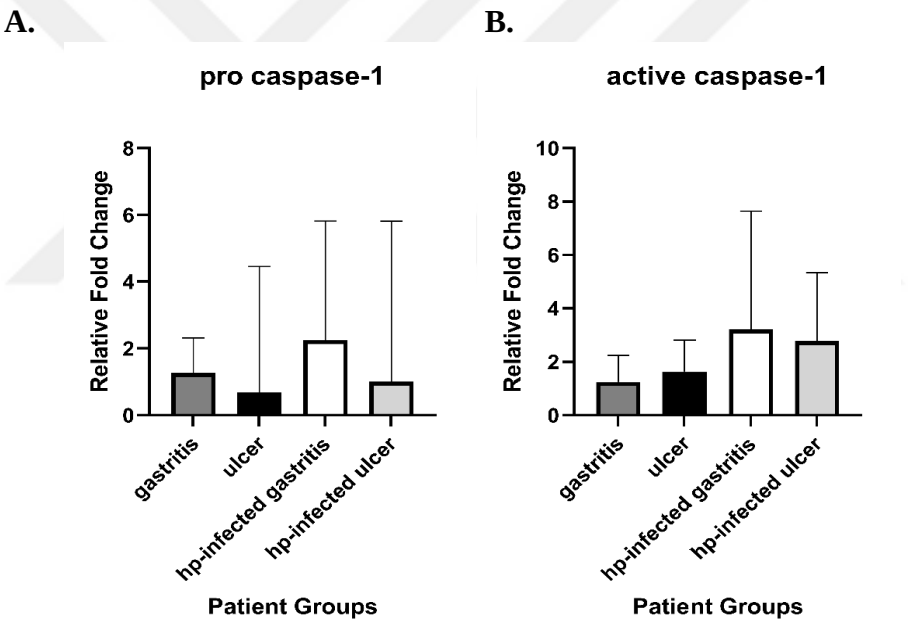


Figure 11. The ratio of the expression levels of pro and active forms of caspase-1, GSDMD, IL-1β and IL-18 to the expression level of beta actin in different patient groups. Normalization of the groups was made according to the patient group showing normal histology. A. Pro caspase-1, B. Active caspase-1, C. Pro GSDMD, D. Active GSDMD-N, E. Pro IL-18, F. Active IL-18, G. Pro IL-1β, H. Active IL-1β relative fold change ratios. (The symbol * represent p<0.05)

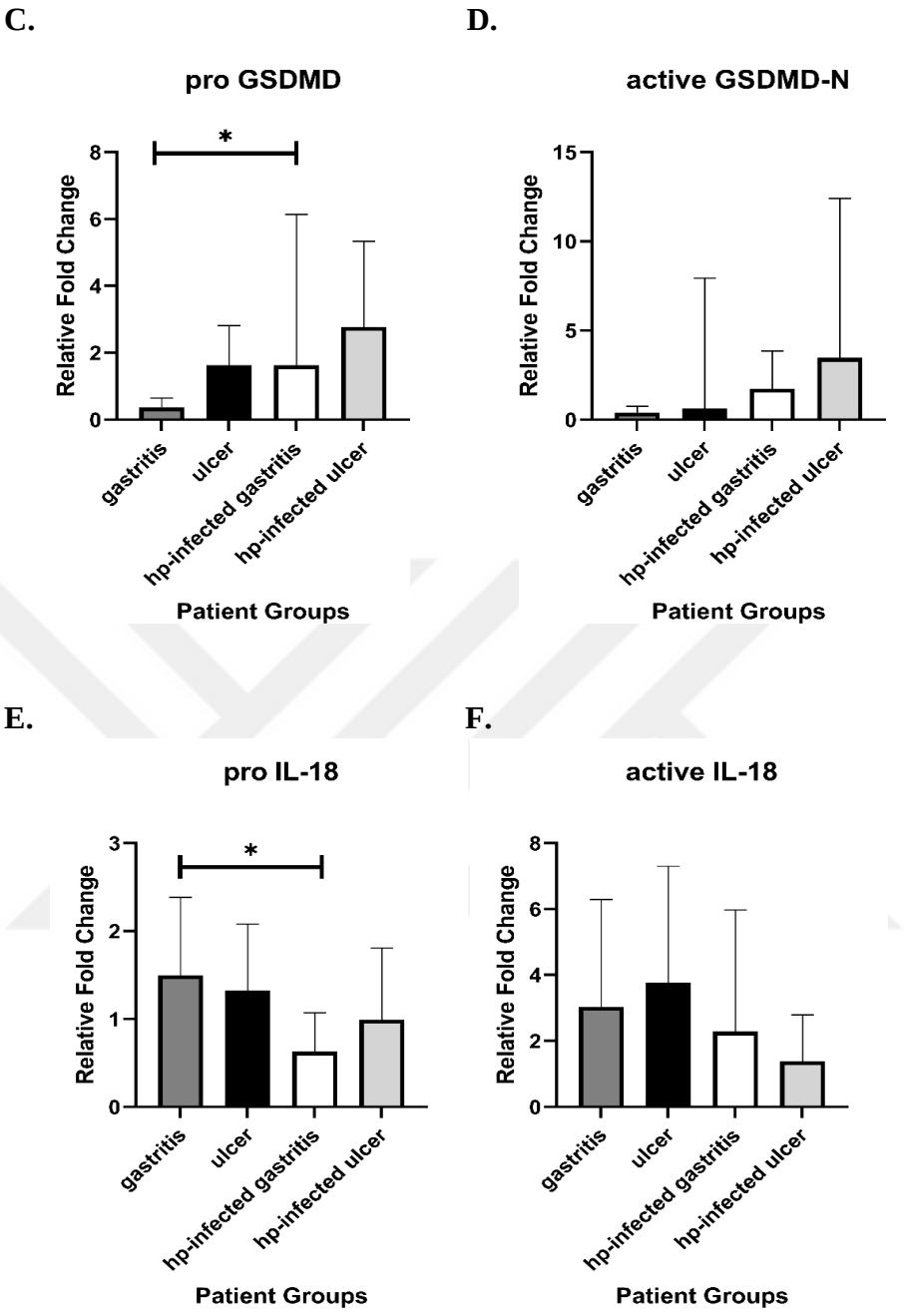


Figure 11 (cont'd). The ratio of the expression levels of pro and active forms of caspase-1, GSDMD, IL-1β and IL-18 to the expression level of beta actin in different patient groups. Normalization of the groups was made according to the patient group showingnormal histology. A. Pro caspase-1, B. Active caspase-1, C. Pro GSDMD, D. Active GSDMD-N, E. Pro IL-18, F. Active IL-18, G. Pro IL-1β, H. Active IL-1β relative foldchange ratios. (The symbol * represent $p<0.05$)

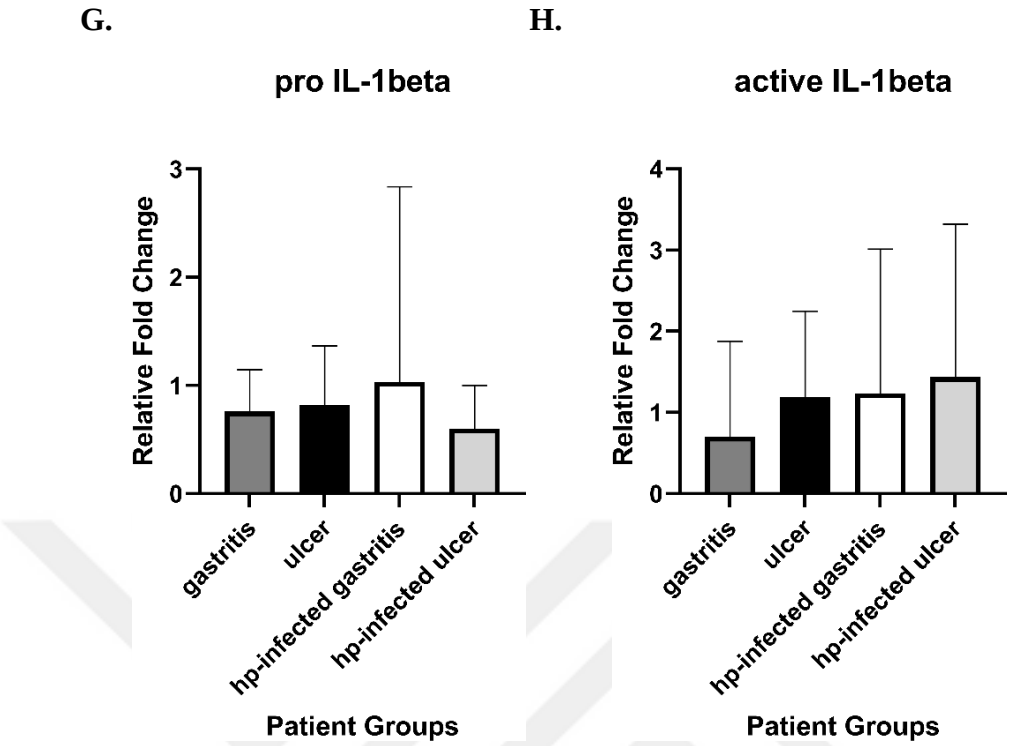


Figure 11 (cont'd). The ratio of the expression levels of pro and active forms of caspase-1, GSDMD, IL-1β and IL-18 to the expression level of beta actin in different patient groups. Normalization of the groups was made according to the patient group showingnormal histology. A. Pro caspase-1, B. Active caspase-1, C. Pro GSDMD, D. Active GSDMD-N, E. Pro IL-18, F. Active IL-18, G. Pro IL-1β, H. Active IL-1β relative foldchange ratios. (The symbol * represent $p<0.05$)

When the expression of caspase-1 was examined in both its active and mature forms, it was observed that the highest rate in both forms was found in patients with gastritis infected with *H. pylori*. Moreover, the presence of *H. pylori* infection increased the amount of caspase-1 in both ulcer and gastritis patients. The data obtained were not statistically significant. In patients with gastritis and ulcers accompanied by *H. pylori* infection, the pro- and active forms of GSDMD were expressed at a higher rate than in patients with control ulcers and gastritis; the pro-GSDMD variation between uninfected gastritis and infected gastritis expression levels was statistically significant. In addition, the expression level of both forms of GSDMD is higher in ulcer patients than in gastritis patients.

Expression of the pro and mature forms of IL-18 is decreased with *H. pylori* infection, especially the amount of pro-IL18, which is significantly higher in uninfected gastritis patients than in infected patients. Mature Il-1β was upregulated in the presence of ulcers in both control and infected patients.

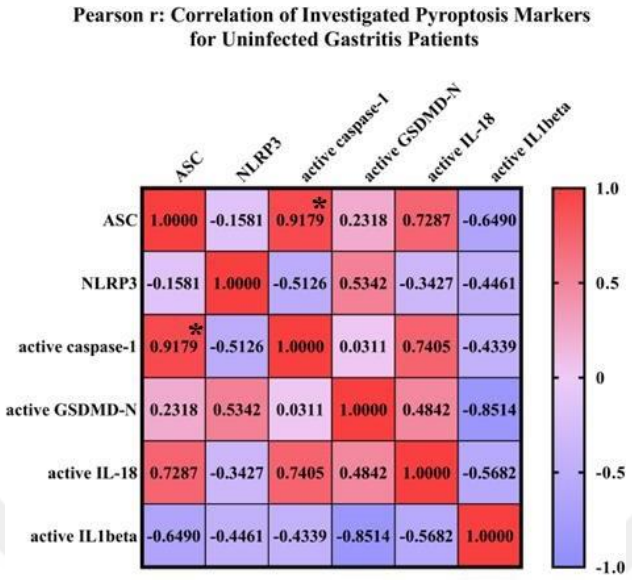
4.6 Statistical Analysis

Correlation matrices were created for each patient group using the results of the qRT-PCR and Western Blot techniques, and the synchronization in the expression levels of the markers was examined. While the Pearson r coefficient approaches 1 (shown in red) in cases where genes are encoded together, the Pearson r coefficient approaches -1 (shown in blue) when there is an opposite relationship between expression levels. In Figure 12 the patient groups with statistically significant relationship between them are shown with *. The symbol * represent $p<0.05$. Heatmaps were created using GraphPad Prism (9.0.0).

In uninfected ulcer patients, a significant correlation was seen between NLRP3 expression and IL-1β expression. Similarly, there is a relationship between the active forms of caspase-1 and IL-18. Besides, a negative correlation was observed between the expression levels of NLRP3 and ASC genes.

The correlation between expression levels of mature caspase-1 and mature-IL18 was significant in patients with gastritis infected with *H. pylori*. No relationship was found between other markers in this patient group. Similarly, active caspase-1 and GSDMD-N levels were found to be significantly correlated in *H. pylori*-infected ulcer patients.

A.



B.

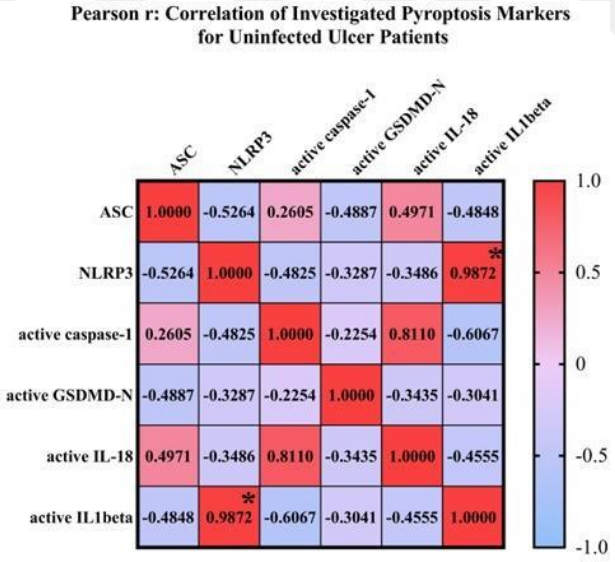
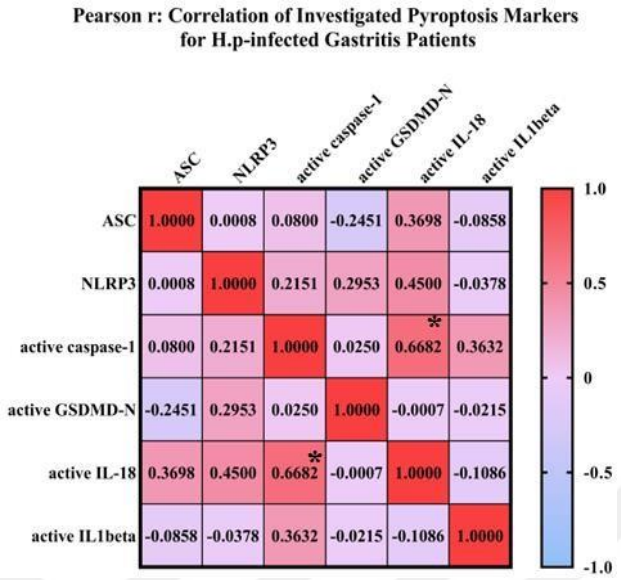


Figure 12. Heatmap graphics created to examine simultaneous changes in the expression of pyroptosis markers in different patient groups. The Pearson r coefficient and p values are shown in the figure as A. in uninfected gastritis patients, B. in uninfected ulcer patients, C. in H.p-infected gastritis patients and D. in H.p-infected ulcer patients. (The symbol * represent $p<0.05$.)

C.



D.

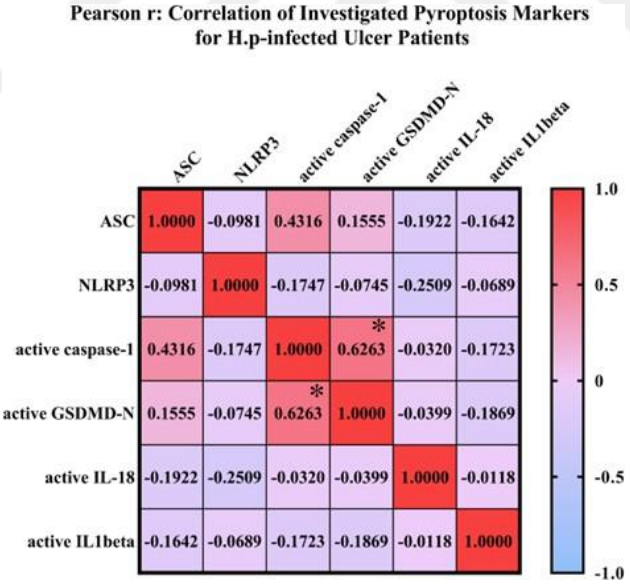


Figure 12 (cont'd). Heatmap graphics created to examine simultaneous changes in the expression of pyroptosis markers in different patient groups. The Pearson r coefficient and p values are shown in the figure as A. in uninfected gastritis patients, B. in uninfected ulcer patients, C. in H.p-infected gastritis patients and D. in H.p-infected ulcer patients. (The symbol * represent $p<0.05$.)

In addition, the relationship between pro and active forms of target markers was examined; graphs showing the balance between active and pro-forms of target markers in each patient sample are given in Figure 13. Graphs were created using GraphPad Prism (9.0.0).

A.

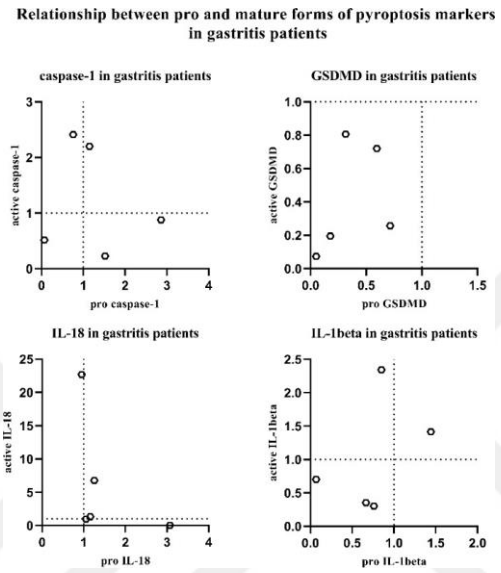
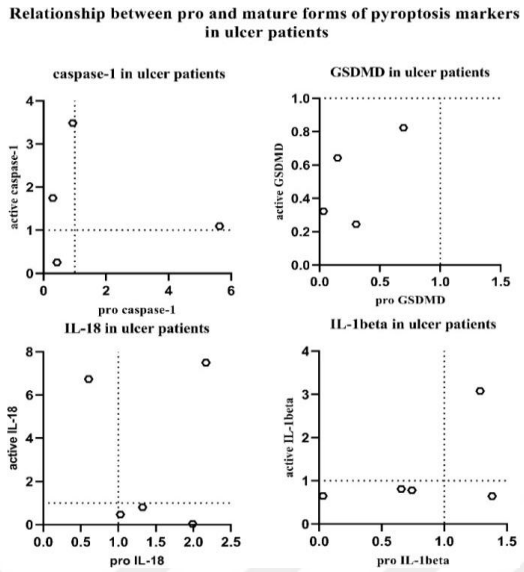


Figure 13. Graphs showing the relationship between the production and activation of pro forms of markers, obtained using the data obtained to examine how many times the pro and mature forms of target markers increased in different patient groups compared to the control patient group. A. Uninfected gastritis patients B. Uninfected ulcer patient C. H. p-infected gastritis patients, D. H.p-infected ulcer patients

B.



C.

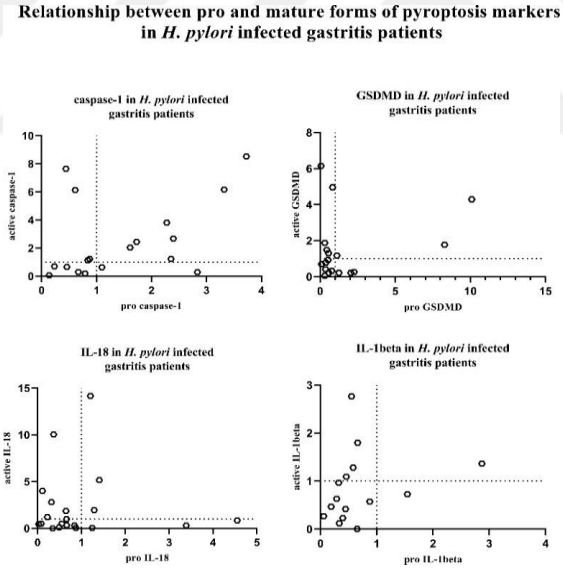


Figure 13 (cont'd). Graphs showing the relationship between the production and activation of pro forms of markers, obtained using the data obtained to examine how many times the pro and mature forms of target markers increased in different patient groups compared to the control patient group. A. Uninfected gastritis patients B. Uninfected ulcer patient C. H. p-infected gastritis patients, D. H.p-infected ulcer patients

D.

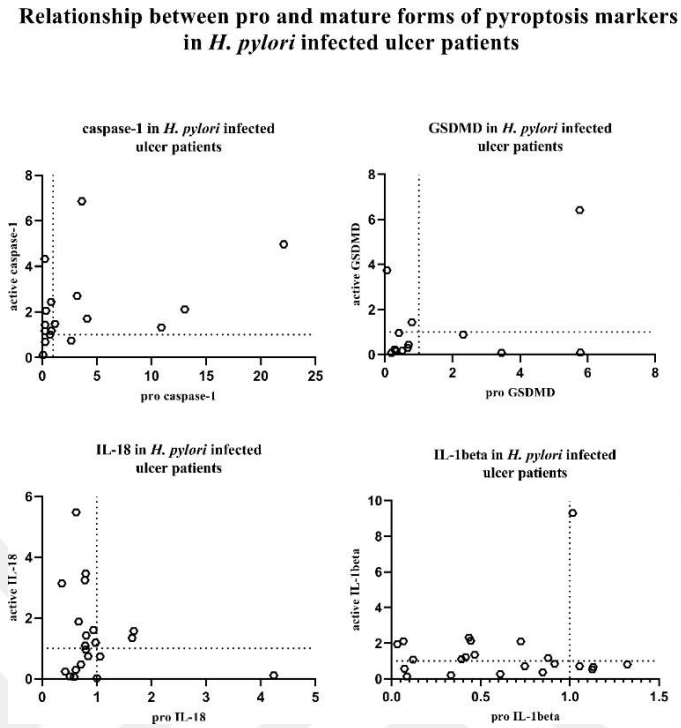


Figure 13 (cont'd). Graphs showing the relationship between the production and activation of pro forms of markers, obtained using the data obtained to examine how many times the pro and mature forms of target markers increased in different patient groups compared to the control patient group. A. Uninfected gastritis patients B. Uninfected ulcer patient C. H. p-infected gastritis patients, D. H.p-infected ulcer patients

In patients with gastritis (72,2%) and ulcers (84,6%) infected with *H. pylori*, concomitance between pro and active forms of caspase-1 is more common than in patients with gastritis (40%) and ulcers (50%) without infection. While the expression of pro and mature forms of caspase-1 is increased in 44,4% of infected gastritis patients compared to the control group, the incidence of this situation in infected ulcer patients is 41,2%. In addition to all these, in 4 infected gastritis patients (22,2% of the population) and 6 infected ulcer patients (35,3% of the population), caspase-1 expression was lower in the pro-form compared to the control group, while the expression level was increased in the active form.

The expression of the active and pro-forms of GSDMD was generally decreased compared to the control group, and the relationship between the pro and active forms was completely simultaneous in uninfected gastritis and ulcer patients. In the infected patient groups, GSDMD expression changed simultaneously in 66.7% of the population in the presence of gastritis and synchronously in 80% of ulcer patients. Expression of both forms of GSDMD was decreased in 38.9% of infected gastritis patients, but this was seen in 70% of infected ulcer patients. Uninfected patients did not show highly mature GSDMD-N and less pro-GSDMD expression, as was the case in 27.8% of infected gastritis patients and 10% of infected ulcer patients.

IL-18 expression is frequently altered simultaneously in infected gastritis patients (60%). Relative fold change values were found to be less than 1 in 45% of this patient group. In patients with pathogen-infected ulcers, this ratio is 38.1%. Also, increased mature IL-18 expression and low pro-IL-18 levels were the most common in the infected ulcer population (42.9% of the population). Similarly, while mature IL-18 was upregulated, infected gastritis patients in whom the pro form was downregulated (20% of the population) were more common than when the two forms were upregulated together.

Expression of IL-1β tended to be simultaneous when down-regulated in uninfected patient groups, but expression of IL-1β was not commonly found ininfected patient groups. Simultaneity was observed in 66.7% of infected gastritis patients, whereas only 36.4% of infected ulcer patients were similarly identified. In *H.pylori*-infected ulcer patients, upregulation of active IL-1β and downregulation of pro-IL-1β was seen in 45.5% of the population, compared with 26,7% of infected gastritis patients.

In order to examine the relationship between the increased expression of pyroptosis markers and the virulence factors of *H. pylori*, the presence of virulence factors in patient tissues where each marker was upregulated was investigated. The incidences of each virulence gene among the upregulated patient groups are given in Table 28.

Table 28. Percentage of bacterial virulence factors in patients with increased expression of pyroptosis marker

	In whole patient population (%)		In the patient population with increased target pyroptosis markers (%)											
			ASC		NLRP3		caspase-1		GSDMD-N		IL-18		IL-1β	
	G	U	G	U	G	U	G	U	G	U	G	U	G	U
oipA	68	82	44	75	54	67	58	81	66	80	63	91	86	64
ureA	100	100	100	100	100	100	100	100	100	100	100	100	100	100
ureB	91	100	89	100	85	100	92	92	78	100	88	89	100	100
cagA	46	59	56	75	54	67	58	56	44	60	50	55	71	55
vacA m1	46	46	78	50	62	44	42	44	33	60	50	55	71	55
vacA m2	50	55	11	50	31	56	50	56	56	40	25	64	71	50
vacA s1	77	86	89	86	85	89	83	87	56	100	63	91	100	82
vacA s2	14	14	0	14	0	11	8	13	22	0	25	9	0	18
sabA	91	96	89	100	85	89	83	56	89	100	75	100	100	91
sabB	91	91	89	100	85	100	92	94	78	100	88	82	100	91
babA	18	9	33	0	23	0	25	6	0	0	25	9	29	9
babB	36	36	44	50	39	44	71	38	44	80	63	55	86	55
babC	68	64	56	63	54	11	75	69	67	60	63	55	86	55
hopQ	96	100	89	100	92	100	92	100	89	100	88	100	100	100
hopZ	91	100	89	100	85	100	92	100	89	100	88	100	100	100
alpA	96	100	89	100	92	100	92	100	89	100	88	100	100	100
alpB	86	96	78	100	85	100	92	100	78	100	88	100	100	100
labA	50	32	78	25	54	67	50	25	33	20	30	38	57	9

Percentages of the *cagA* and *babB* gene and the *vacA s1* alleles were commonly expressed more frequently in patient groups with increased expression of target markers. In contrast, the incidence of important virulence genes of bacteria such as *oipA, ureB, sabA* and *vacA s2* allele was lower in patient groups expressing increased pyroptosis markers. While the *babA2* gene is frequently increased in gastritis patients

with increased pyroptosis response, its incidence is decreased in ulcer patients. No remarkable change was detected between the other virulence factors of the bacteria and the upregulated pyroptosis markers.

According to the presence and absence of bacterial virulence genes, the patient population was divided into two groups, and Kruskal Wallis analysis was performed to examine the relationship between the expression levels of pyroptosis markers in these groups. The alpha value of 0.05 was chosen. It was found to be statistically significant when $p < 0.05$. Graphs showing the relative fold change levels of mature forms of target markers for each bacterial virulence gene investigated are given in Appendixes 2 and 3.

In gastritis patients, it was observed that NLRP3, caspase-1, ASC, GSDMD-N, and IL-18 were expressed higher in the patient groups that did not express the *hopQ* gene than in the patients who expressed it. Similarly, NLRP3, caspase-1, GSDMD-N, and IL-18 responses were found to be higher than the other groups in patients who did not express the *sabA* gene. Especially in the presence of the *vacA m2* allele, the ASC gene was found to be significantly downregulated compared to the uninfected gastritis patient group. Additionally, compared to the control group, the expression of the ASC gene was considerably lower among patients with gastritis who did not express the labA gene.

When infected ulcer patients were examined, it was observed that the highest values of all pyroptosis markers examined were in the presence of the *babA* gene, compared to the control group and the group that did not express the virulence gene. Especially in the presence of *babA*, GSDMD-N expression was found to be significantly upregulated compared to its absence of this genes. Similarly, ASC, NLRP3, caspase-1, IL-18, and IL-β expressions were slightly increased in the presence of the *hopQ* gene. In the same context, it was observed that the expressions of ASC, NLRP3, caspase-1, and IL-18 were slightly increased in the presence of the *sabA* gene, but this was not found to be statistically significant. Except for IL-1β, other pyroptosis markers were found to be upregulated in the presence of the *vacA m2* allele. In

particular, the expression of the ASC gene was significantly increased compared to uninfected ulcer patient.

5 DISCUSSION

In the pathogenesis of *H. pylori* infection, environmental factors and bacterial virulence genes are effective, along with host-specific factors (168). Virulence factors play an active role in the bacteria's reaching the gastric epithelium, attachment, and initiation of colonization. While the urease activity of the bacteria supports its survival in the acidic environment of the stomach, the virulence factors CagA and VacA have been shown to play an active role in the development of pathogen-related diseases 44,54). Bacterial outer membrane proteins interact with cellular receptors to protect the bacteria from mechanisms like acidic pH, mucus, and exfoliation of the stomach 167,170). The HOP family, which is the largest family of bacteria's outer membrane proteins, has been studied relatively extensively in the literature. In the presence of AlpA, AlpB, and BabA proteins, an increased inflammatory response was observed 61,68), and BabB and HopQ proteins were found to support the formation of gastric lesions (64,82). In addition, the presence of BabA, SabA, and OipA proteins was evaluated as a biomarker for gastric cancer (73). Although it is known that the outer membrane proteins of the bacteria are very effective in the pathogenesis of the bacteria, it has not yet been clarified which mechanisms they affect in the development of diseases.

Microbial infection is the main cause of pyroptosis, a kind of controlled cell death, and the relationship between *H. pylori* infection and pyroptosis is very important in understanding the pathogenesis of pathogen-induced diseases. In the literature, *H. pylori* infection increased the expression of IL-18 at the mRNA level (152). In addition, chronic *H. pylori* infection in humans was thought to regulate inflammation activation and pyroptosis for bacterial persistence by increasing IL-1β activation (153). Moreover, it was observed that the expression of pyroptosis markers was higher in patients with gastric cancer infected with *H. pylori* (152). In a study by Zhang, it was shown to induce NLRP3 inflammation due to CagPAI (157). At the same time, in infection experiments using CagA+ and CagA- *H. pylori* strains in 2021, the expression levels of NLRP3, ASC, IL-1β and IL-18 were higher as a result of CagA+ *H. pylori* infection compared to CagA- strains (157). Similarly, infection with CagA+

strains has been shown to increase the NLRP3 inflammatory response and increase the potential for cancer and cell migration in the stomach by stimulating ROS generation (157). Despite all these studies in the literature, there is no study examining the relationship between pyroptosis and *H. pylori* outer membrane proteins in terms of pathogen-related diseases, and this thesis is aimed at eliminating this deficiency in the literature.

In this thesis, DNA, RNA, and protein were isolated from a tissue piece taken during endoscopy from the antrum region of the stomach of *H. pylori*-infected gastritis and ulcer patients and uninfected control group patients. In DNA samples, the expression of bacterial outer membrane virulence factors was investigated at the gene level. In addition, expression levels of ASC and NLRP3, which are major canonical pyroptosis pathway markers, were determined by qRT-PCR in RNA samples. Protein samples were studied by the Western Blot method following the SDS page, and expression levels of pro- and active forms of target pyroptosis markers were compared. In the study, simultaneous examination of bacterial virulence factors and pyroptosis markers in a single tissue piece provided an opportunity to examine the relationship between pathogen characteristics and controlled cell death in *H. pylori*-associated diseases.

Gastric tissue samples were obtained from 128 patients by endoscopy to be used in this thesis study. In a previous study, it was suggested that the prevalence of *H. pylori* in Turkey was more than 80% (11), but the prevalence of *H. pylori* was found to be 41,2% in the patients included in this study. Since it is known that the prevalence of *H. pylori* decreases with socioeconomic development, choosing a private hospital in Istanbul may be the reason for the lower prevalence of the pathogen.

The expression of the outer membrane virulence genes of *H. pylori* in patient samples was investigated by conventional PCR. The *ureA* gene, which is known to be effective in the adaptation of the bacteria to the acidic stomach environment, was observed in all patients, while the *ureB*, *hopZ*, *hopQ*, and *alpA* genes were observed in all ulcer patients, unlike gastritis patients. No change was observed in the expression

of the sabB gene in gastritis and ulcer patients, which is consistent with data from a previous Dutch study (75). Although the expression of bab paralog alleles is often considered a risk for the progression of pathogen-related diseases in the literature (59,171), in this study, it was found that *babA2, babB, babC*, and *labA* genes were found to be more common in gastritis patients than in ulcer patients. And also; *ureB, cagA, oipA, sabA, alpA, alpB, hopZ,* and *hopQ* genes were seen more frequently in ulcer patients than in gastritis patients. In addition, *s1* and *m2* alleles of the *vacA* gene were found to be more common in ulcer patients.

Fisher's Excact Chi-Square test was applied to examine the expression of bacterial virulence genes in patients with gastritis and ulcer infected with *H. pylori*, and *p* values and relative risk factors were calculated for each gene. The relative risk value for *ureB, cagA, vacA s1 and m1, sabA, sabB, hopQ, hopZ, babB, alpA, alpB* and, *oipA* which is expressed as higher in ulcer patients, was found to be higher than 1, and it was evaluated as an ulcer development potential. These results are consistent with studies in the literature showing that the expression of *oipA, sabA, hopQ, cagA, vacA s1* and *m2* alleles may be associated with gastric ulcer development. (83,172-174).

Expression levels of pyroptosis markers ASC and NLRP3 were examined by qRT-PCR in all patient groups and the changes in ASC and NLRP3 expressions were not significant for any patient group. In a clinical study published in the literature in 2023, the ASC gene was found to be relatively more expressed in gastritis patients than in ulcer patients, and higher expression was observed in *H. pylori*-infected ulcer patients compared to uninfected ulcer patients (175). It was seen that the data related to the expression of the ASC and NLRP3 genes obtained in this thesis study and the aforementioned study support each other. However, the obtained data were not found to be statistically significant.

The highest expression level for both the active and pro form of caspase-1 was found in the infected gastritis patient group, and caspase expression was found to increase with *H. pylori* infection. These results are consistent with the study in the

literature showing that the presence of caspase-1 increased after infection of C57BL/6 mice with *H. pylori (*176).

The amount of pro-GSDMD was significantly increased in infected gastritis patients compared to uninfected patients. A similar relationship is also valid for active GSDMD, but it was not found to be statistically significant. In addition, ulcer patients were found to express GSDMD at a higher rate. There is no study in the literature examining the expression of GSDMD in patient samples.

Expression of Pro-IL-1β was increased in patients with gastritis in the presence of infection and decreased in patients with ulcers, but its active form was found to be expressed at a higher rate in both infected gastritis and infected ulcer patients. In the literature, a clinical study conducted by Milic et al., higher IL-1β levels were detected in infected ulcer patients than in uninfected patients (177). This situation suggested that *H. pylori* infection stimulates IL-1β at the beginning of infection and may be effective in its activation in pathogen-related diseases.

Significant higher IL-18 expression was seen in uninfected gastritis patients compared to infected gastritis patients. Presence of *H. pylori* infection and expression of IL-18 varied inversely. It has previously been shown that IL-18 expression is increased in the case of *H. pylori* infection, which is inconsistent with the data obtained in this study (176). However, in a study conducted on Mongolian gerbils, IL-18 and IL-1beta expressions were increased in the gastric mucosa in the first month of *H. pylori* infection, but the expression level decreased to the baseline level at the sixth month of infection (178). This suggested that the expressions of these inflammasome markers may vary depending on the duration of infection. In addition, since the inflammation starts from the antrum region of the stomach and spreads to other parts, the increase in this region does not allow a generalization in terms of the whole stomach.

Statistically significant correlations were observed between some markers in the context of Peearson's r constant and *p* values calculated in the correlation matrices created to examine the correlation between the expression levels of the target markers. When the correlation matrices were evaluated, a significant association was found between increase mature caspase-1 and ASC expressions in patients with uninfected gastritis. Furthermore, higher ASC expression was linked to increased IL-18 and caspase-1 expression. On the other hand, IL-1 expression was shown to be inversely linked with ASC, GSDMD-N, and IL-18 expression. NLRP3 and caspase-1 share a similar interaction. There was a significant association between NLRP3 expression and IL-1 expression in uninfected ulcer patients. Similarly, there is an association between caspase-1 and IL-18 active forms. Furthermore, an adverse correlation was found between the expression levels of the NLRP3 and ASC genes. The correlation between expression levels of mature caspase-1 and mature-IL 18 was the only significant association detected in *H. pylori*-infected gastritis patients. Similarly, in *H. pylori*-infected ulcer patients, activated caspase-1 and GSDMD-N levels have been shown to be significantly associated. Since GSDMD-N and active forms of IL-18 and IL-1beta increased following the activation of caspase-1 in the canonical pyroptosis pathway, the significant relationship between caspase-1 and these markers in different patient groups showed the competence of the method used to detect pyroptosis.

It has been observed that the expression levels of pyroptosis markers can vary in all patient groups; these changes are not statistically significant except in some cases, in which case the size of the population is a limiting factor. In addition, the possibility that different patient samples represent different stages of disease and inflammation may have prevented us from fully examining the relationship between target markers. Therefore, a closer look at the patient population was required.

The relationship between the regulation of the pro and mature forms of the target markers in the patient groups whose fold change values of the expression levels were calculated compared to the control patient group was examined. Significant data could not be obtained in non-infected patient groups due to the size of population. In *H. pylori*-infected patient groups, upregulation of active and pro-forms of caspase-1 was

commonly seen to be simultaneous; however, in GSDMD, IL-18, and IL-1β, synchrony between forms was found to be more common in the down-regulation condition. This indicated that there were patient samples in which the target marker was not synthesized and could not be activated naturally. However, for caspase-1, IL-18, and IL-1β, a significant portion of the population was found to have an upregulated mature form and a downregulated pro form. It was thought that down-regulation of pro-forms was observed in some patients due to activation after expression, and up-regulation of the mature form was increased as described in the canonical pyroptosis pathway (123).

When the incidence of bacterial virulence genes in patient groups with upregulated pyroptosis markers is examined, the incidence of *cagA* and *babB* genes and *vacA s1* alleles tends to increase, and the opposite is true for *oipA, ureB, sabA,* and *vacA s2* alleles. It is very important to detect such a relationship between the increase in the presence of the *cagA* and *vacA s1* alleles, which are known to support ulceration (53) and are the most important features of the virulence of the bacteria, and the pyroptosis response. In the literature, the presence of OipA (89) and SabA (74) has been associated with the development of peptic ulcers, but in this study, it was seen that the expression of these proteins at the gene level was inversely proportional to the increased pyroptosis response. OipA and SabA proteins can be produced specifically with ("on" status) or without ("off" status) functionality (70, 89), so the existence of these virulence genes at the gene level may not mean that they can be produced functionally, so looking at their expression at the gene level may have been insufficient to examine their effects on pyroptosis. While the *babA2* gene is more common with an increased pyroptotic response in gastritis patients, its incidence is decreased in ulcer patients. On the other hand, the production of BabA has been associated with the development of peptic ulcers and gastric cancer in the literature (58,59).

The Kruskal-Wallis test was used to examine changes related to the virulence genes of *H. pylori* in patient groups in which pyroptosis markers were upregulated. Especially in the presence of *hopQ* and *sabA* genes, pyroptosis markers were frequently upregulated than in uninfected control patient groups. Since *hopQ* (82) and

sabA (74) expressions were previously associated with ulcer development in the literature, these results suggested that they may have induced pyroptosis while stimulating tissue damage caused by infection. While the incidence of the *sabA* gene decreased in patients with pyroptosis-induced, an increased pyroptosis response was observed in patients expressing the *sabA* gene, suggesting that this gene is not seen frequently, but if it is present, it plays an active role in pyroptosis. The small size of the population has been a limiting factor in better understanding this relationship.

In addition, GSDMD-N expression is significantly higher in infected ulcer patients in whom the *babB* gene is expressed than in patients who do not express this gene. Moreover, in gastritis patients without the *labA* gene, the expression of the ASC gene was significantly decreased compared to the control group. Although there is not much information about *labA* and *babB* in the literature, the relationship between these genes and increased pyroptosis response should be examined in more detail.

The changes in the amount of ASC due to the absence of the *vacA m2* allele in infected gastritis patients and the presence of the same allele in infected ulcer patients were found to be statistically significant. This showed that the *vacA m2* allele, which is known to have an active effect on ulcer development, may support the ulceration potential by increasing the amount of ASC. This situation suggested that pyroptosis might be the effective mechanism in bacterial gastric ulcer formation and that the *vacA m2* allele might play an active role in the process.

Combining the data, the *vacA m2* allele, which elicits an opposite and significant ASC response in patients with gastritis and ulcer, and the *babB* gene, with its increased incidence, and upregulation of GSDMD in ulcer disease, emerged as two important candidates for future studies.

Although the power analysis technique was used to determine the number of patients to be included in the study, the large number of variables and the size of the population may have prevented the obtained data from being statistically significant. In addition, although the infection in the stomach generally starts in the antrum region,

the inflammatory response in tissue samples taken only from the antrum region may be insufficient to express the general stomach. Moreover, the periodic increase and decrease of markers during pyroptosis, instead of showing a linear increase, was another limiting factor encountered in the study. In addition to these disadvantages, this study is very valuable as the relationship between bacterial virulence genes and pyroptosis has been examined for the first time in this study. It has been observed that the pathogen is effective in pyroptosis, and two virulence factors whose functions have not been fully determined in *H. pylori*-related pathology have the potential to be directly associated with pyroptosis as a result of more extensive studies. Future studies should continue to examine the relationship between the virulence characteristics of bacteria and pyroptosis in *H. pylori*-related diseases.

6 CONCLUSION

In conclusion, it was observed that *H. pylori* infection caused changes in the expression levels of ASC, NLRP3, caspase-1, GSDMD, IL-18, and IL-1β, and it was determined that there was a correlation between pyroptosis markers and pathogen-related diseases. The statistically significant change in the relationship between the increase in the amounts of target markers and active caspase-1 in different patient groups showed that the detection of pyroptosis was successful. The pro and active forms of caspase-1, GSDM-D, IL-18 and IL-1β were examined in the population, and patients thought to have pyroptosis were identified.

The expression of the vacA m2 allele was significantly decreased in gastritis patients, while it was significantly increased in ulcer patients. GSDMD upregulation in ulcer disease and the *babB* gene are prominent for understanding the relationship between the virulence factors of *H. pylori* and pyroptosis in bacteria-associated diseases. These data shed light on a deficiency in the literature.

This thesis study is scientifically important as it allows the expression of bacterial virulence genes and pyroptosis markers to be examined in a single tissue piece in pathogen-related diseases for the first time.

It has been observed that the pathogen is effective in pyroptosis, and bacterial virulence factors in *H. pylori*-related diseases have the potential to be directly associated with pyroptosis as a result of this study. For future aspects, it would be meaningful to expand the study to larger patient populations and include other bacterial-associated patient groups, such as patients with gastric cancer.

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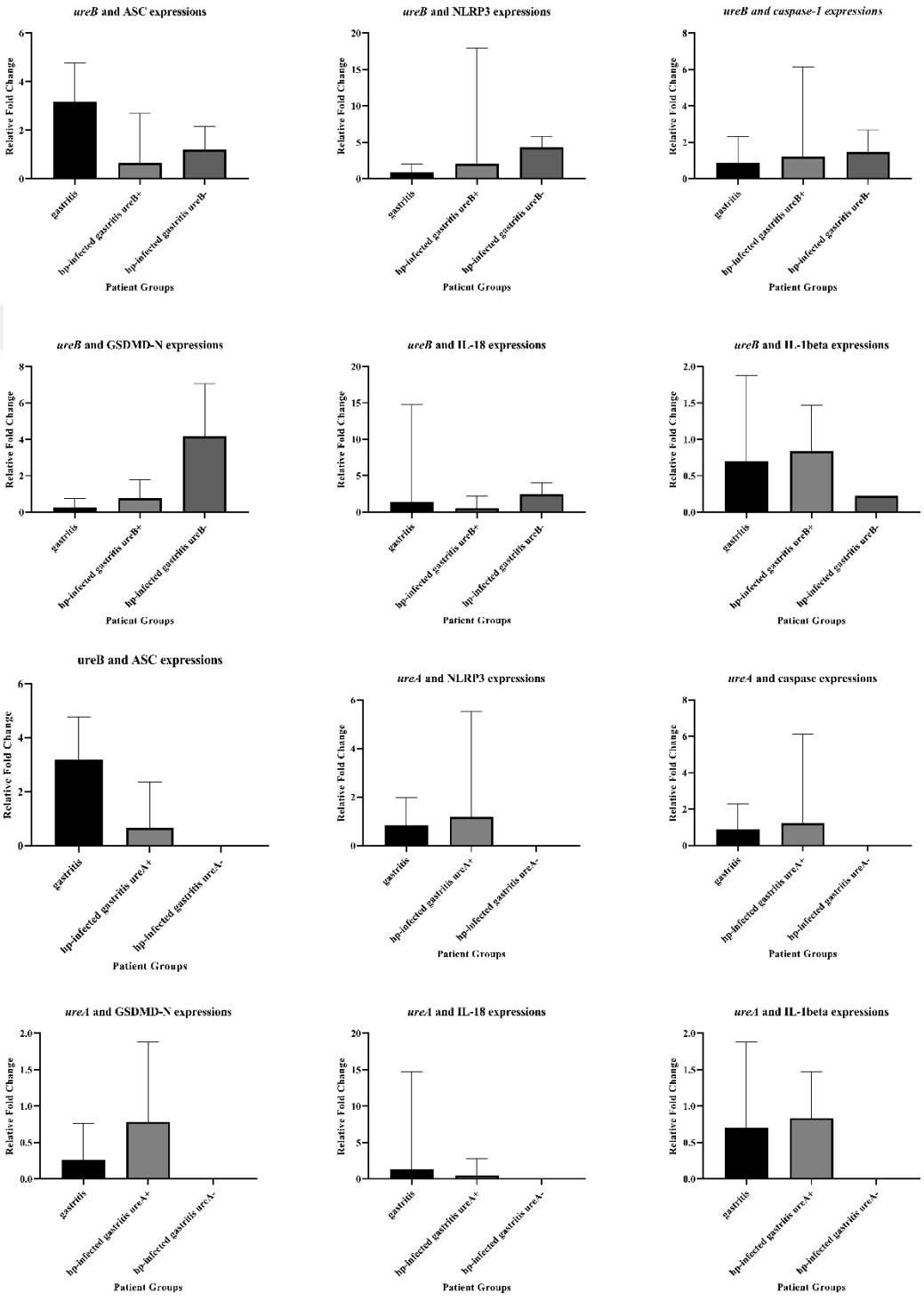
8 APPENDIX

Appendix 1. The approval of the ethics commitie

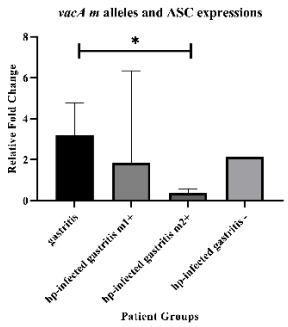
Appendix 1. The approval of the ethics commitie (cont'd)

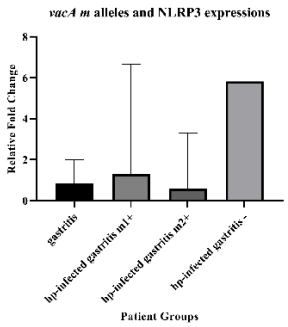
APPENDIX 2

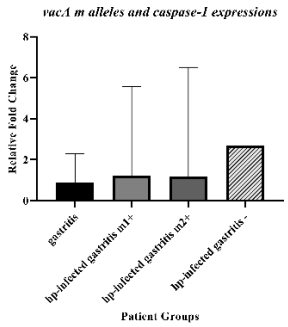
Appendix 2. Graphs showing the distribution of target markers by expression of virulence genesin gastritis patients with upregulated pyroptosis markers

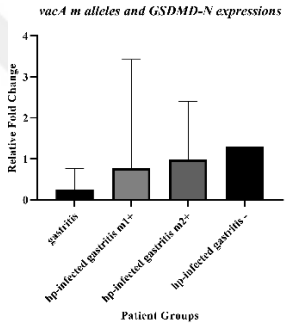


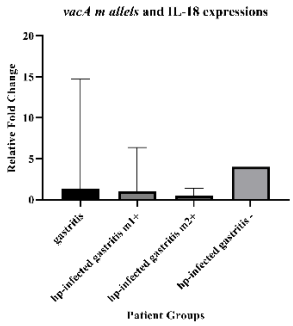
Appendix 2. Graphs showing the distribution of target markers by expression of virulence genesin gastritis patients with upregulated pyroptosis markers (cont'd)

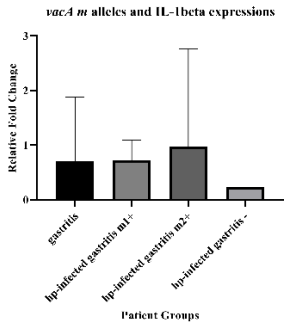


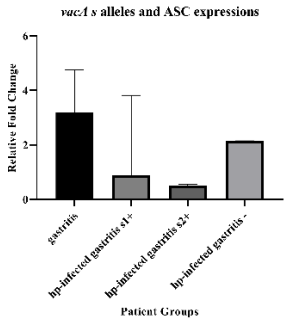


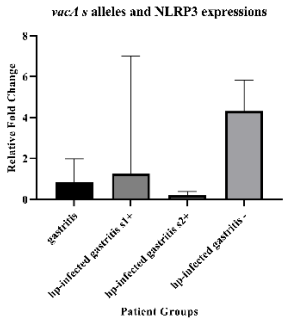


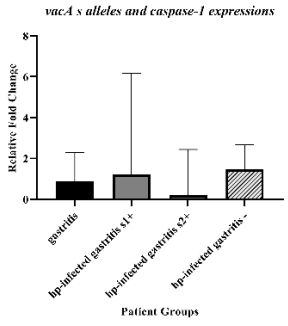


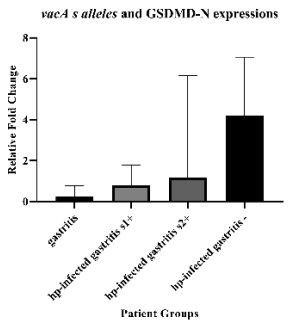


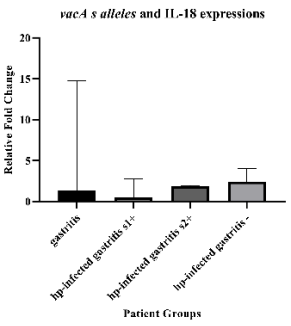


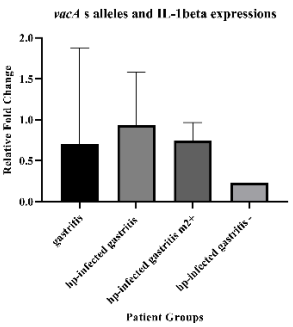




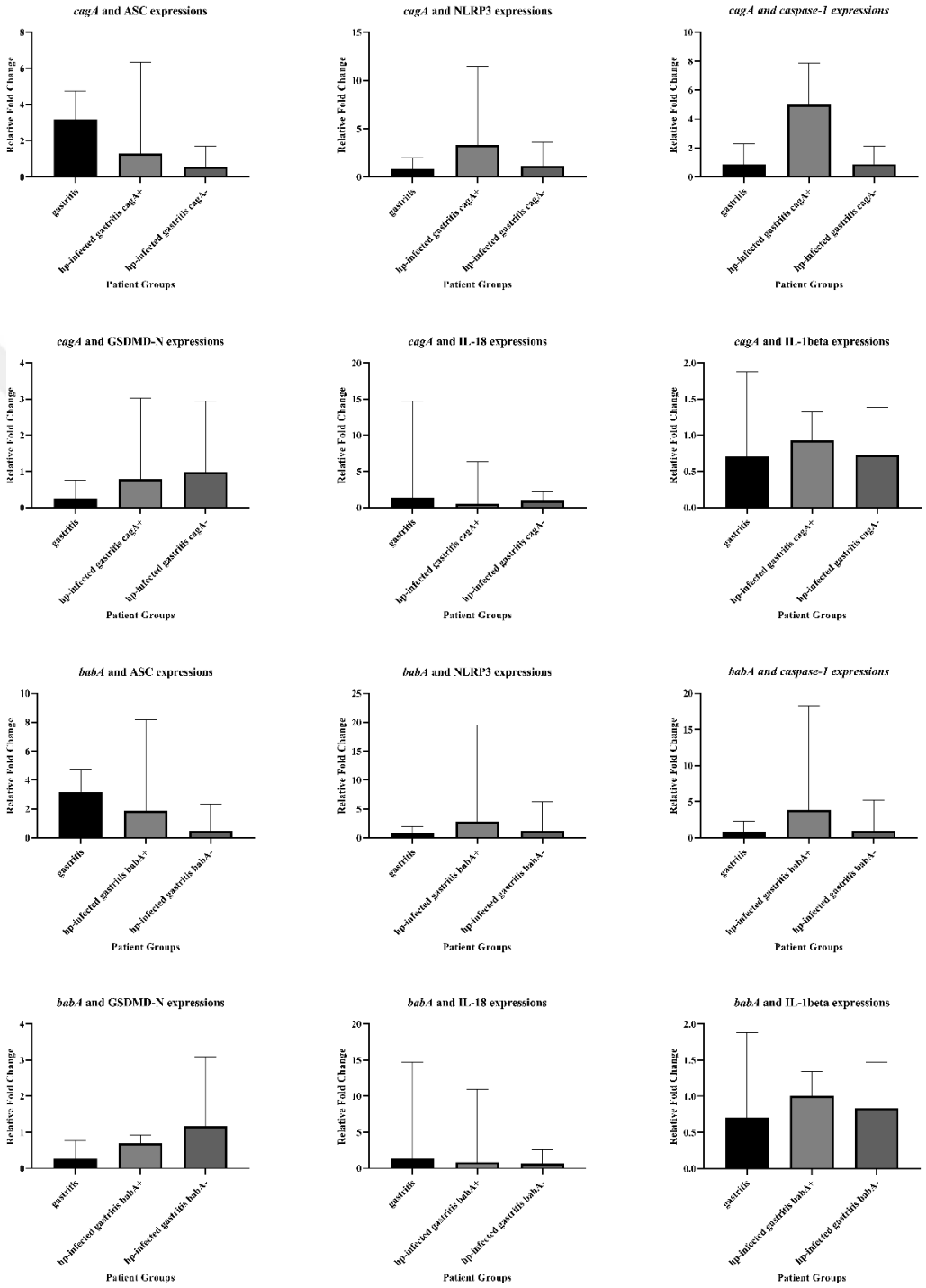




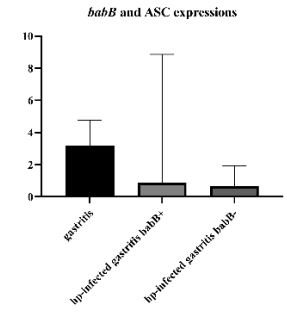


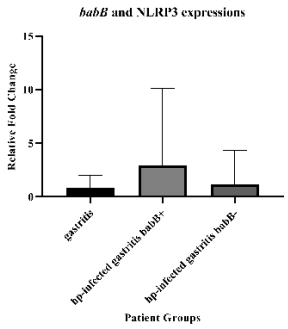


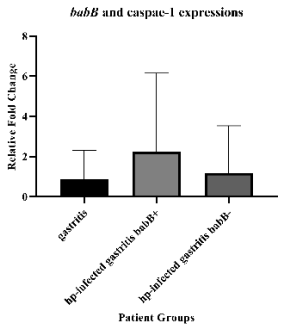
Appendix 2. Graphs showing the distribution of target markers by expression of virulence genesin gastritis patients with upregulated pyroptosis markers (cont'd)

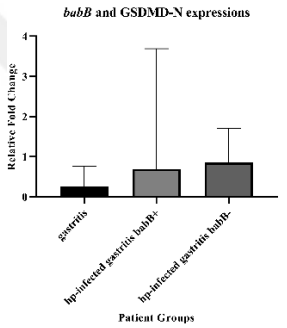


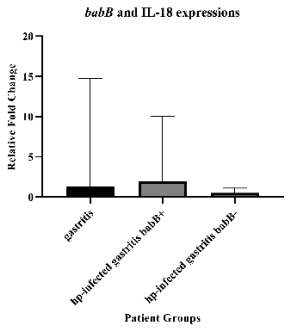
Appendix 2. Graphs showing the distribution of target markers by expression of virulence genesin gastritis patients with upregulated pyroptosis markers (cont'd)

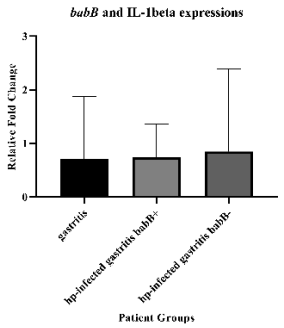


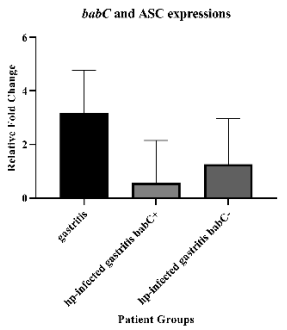


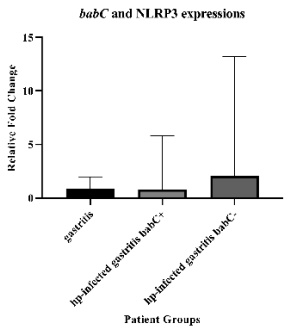


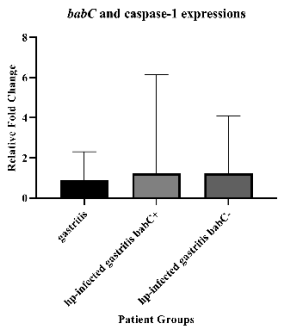


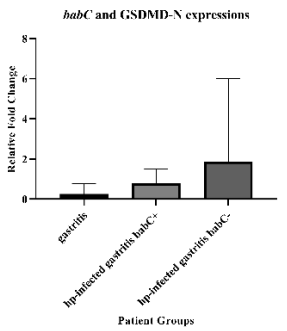


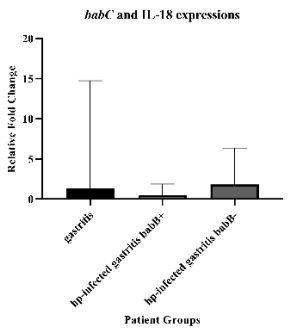


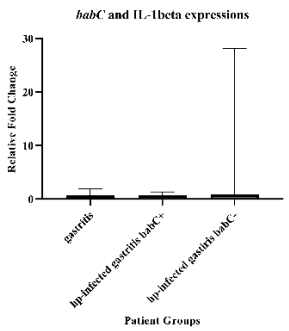




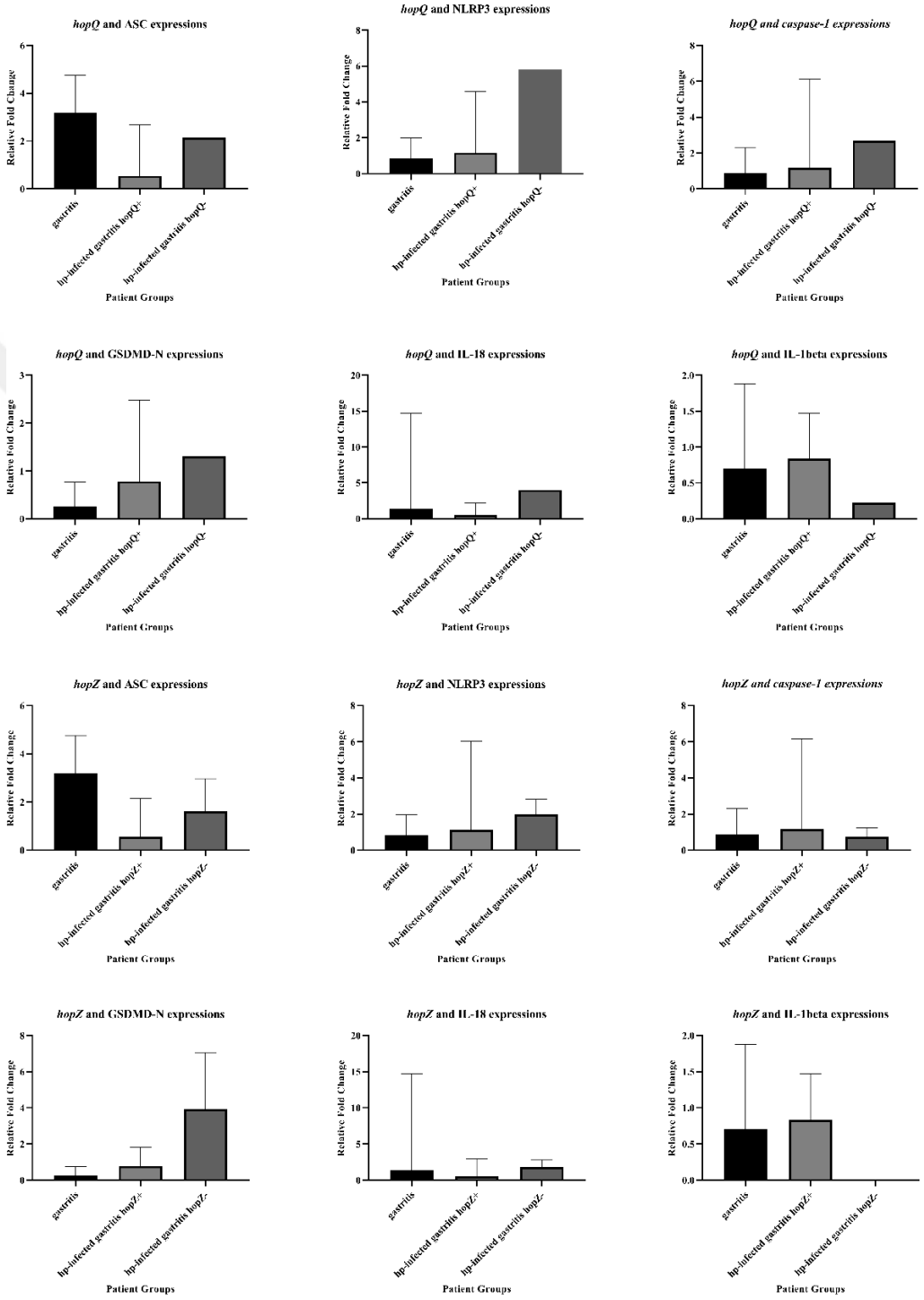




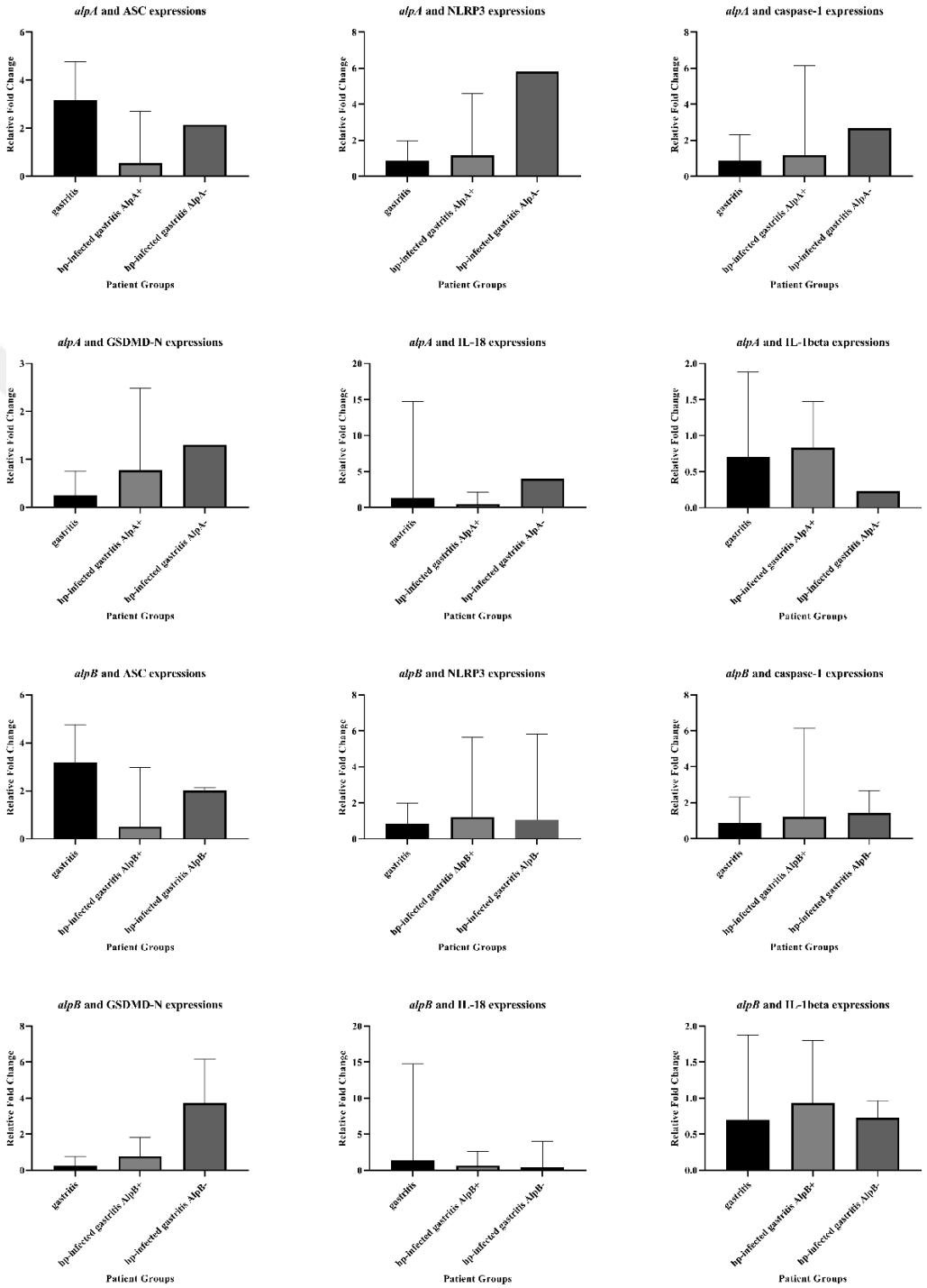




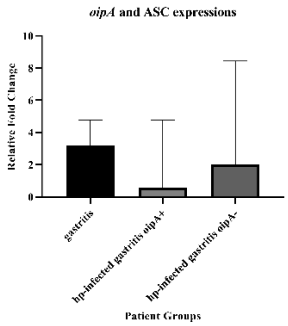
Appendix 2. Graphs showing the distribution of target markers by expression of virulence genesin gastritis patients with upregulated pyroptosis markers (cont'd)

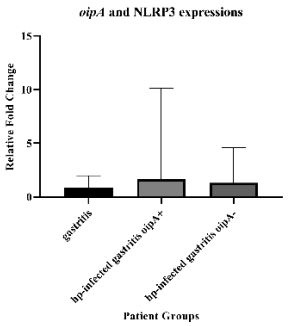


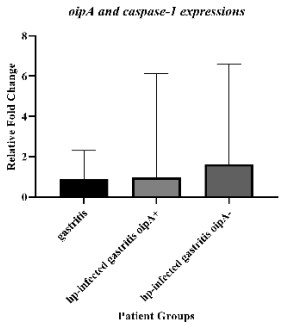
Appendix 2. Graphs showing the distribution of target markers by expression of virulence genesin gastritis patients with upregulated pyroptosis markers (cont'd)

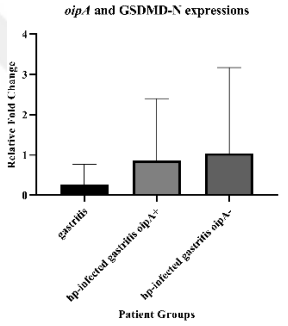


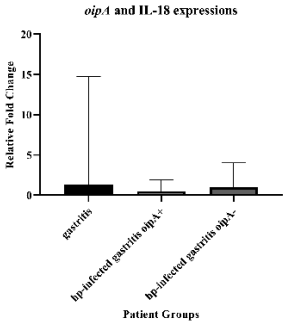
Appendix 2. Graphs showing the distribution of target markers by expression of virulence genesin gastritis patients with upregulated pyroptosis markers (cont'd)

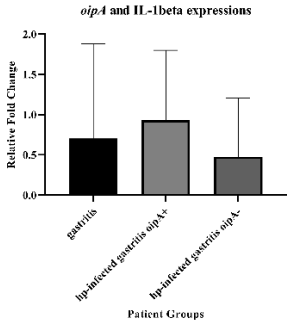


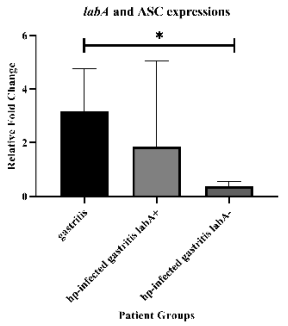


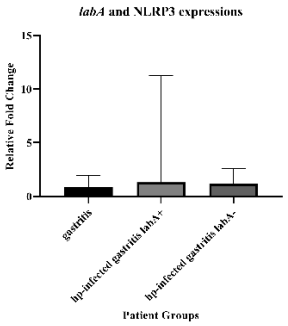


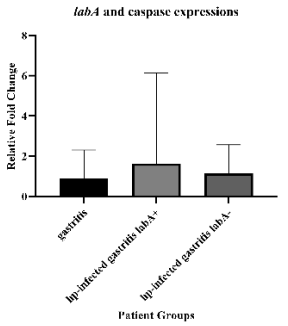


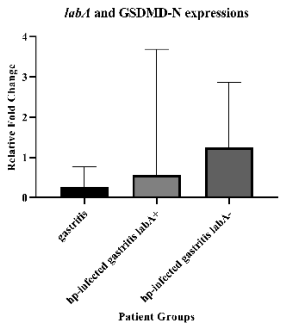


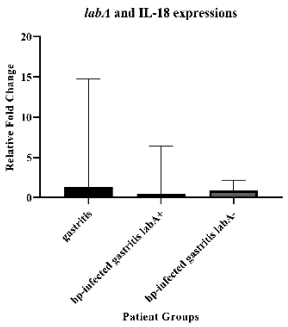


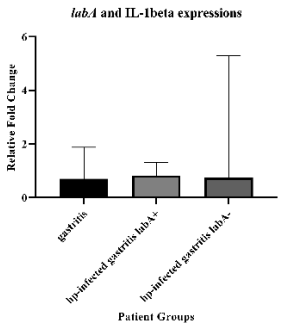




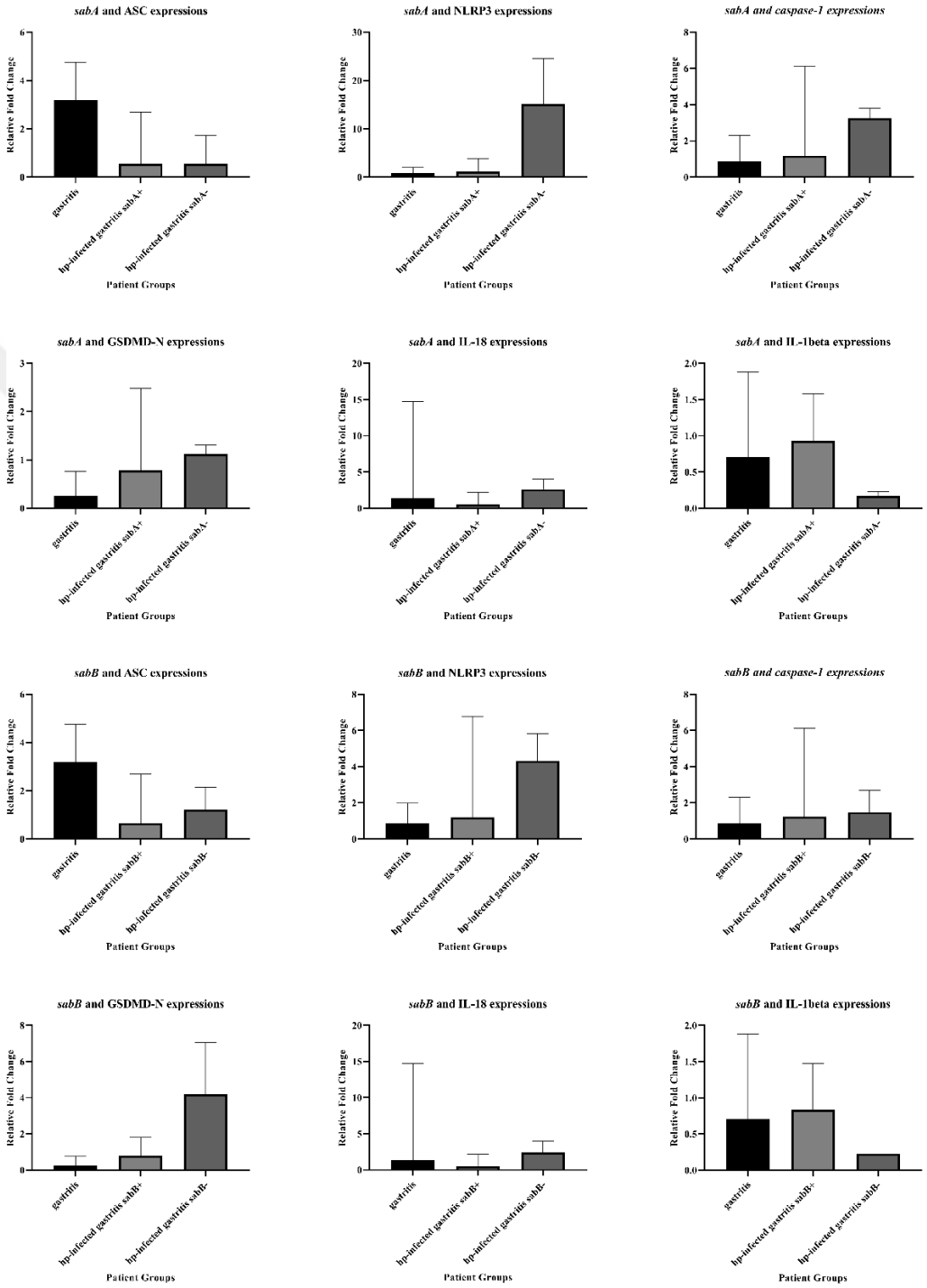






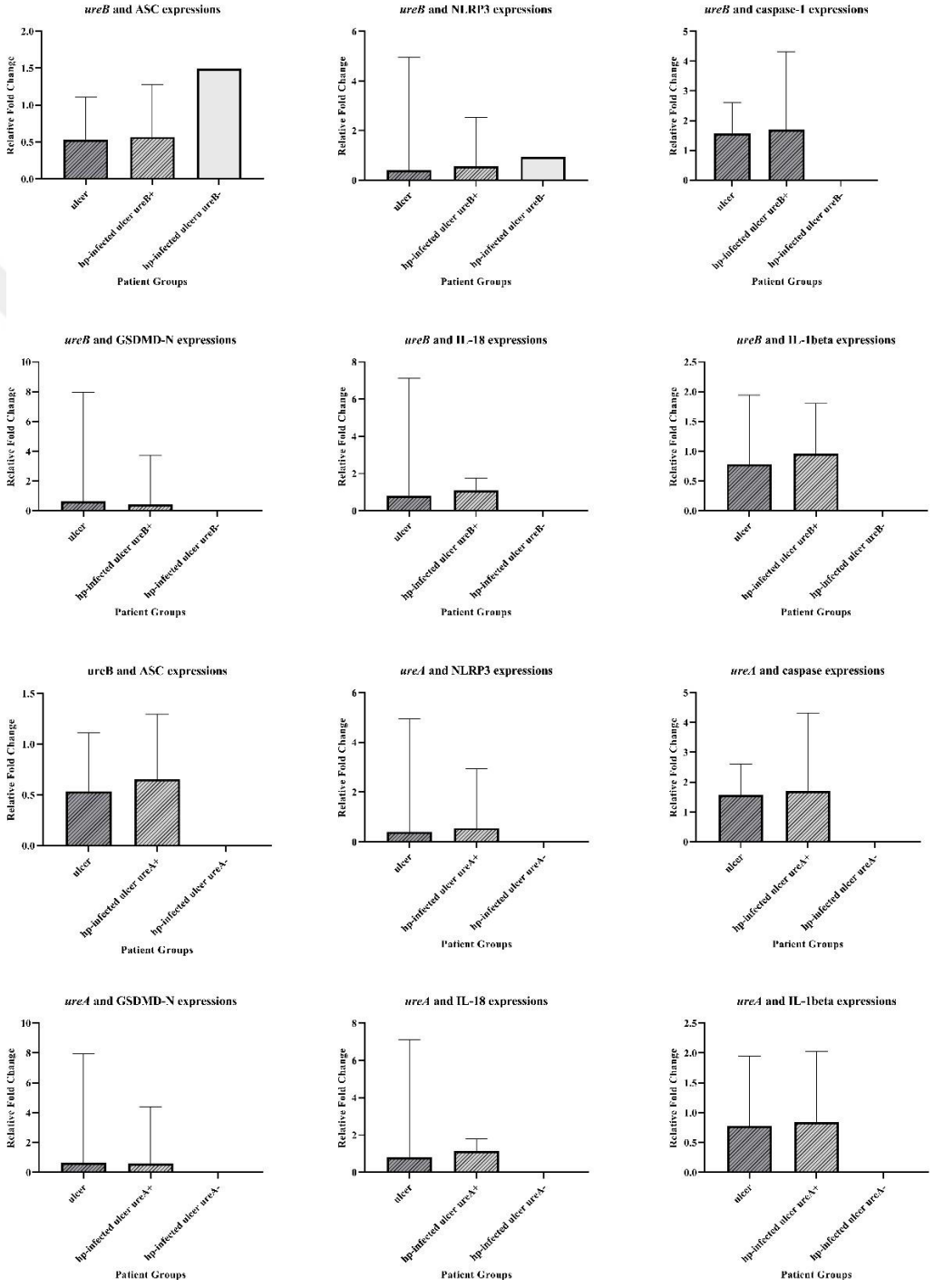


Appendix 2. Graphs showing the distribution of target markers by expression of virulence genesin gastritis patients with upregulated pyroptosis markers (cont'd)

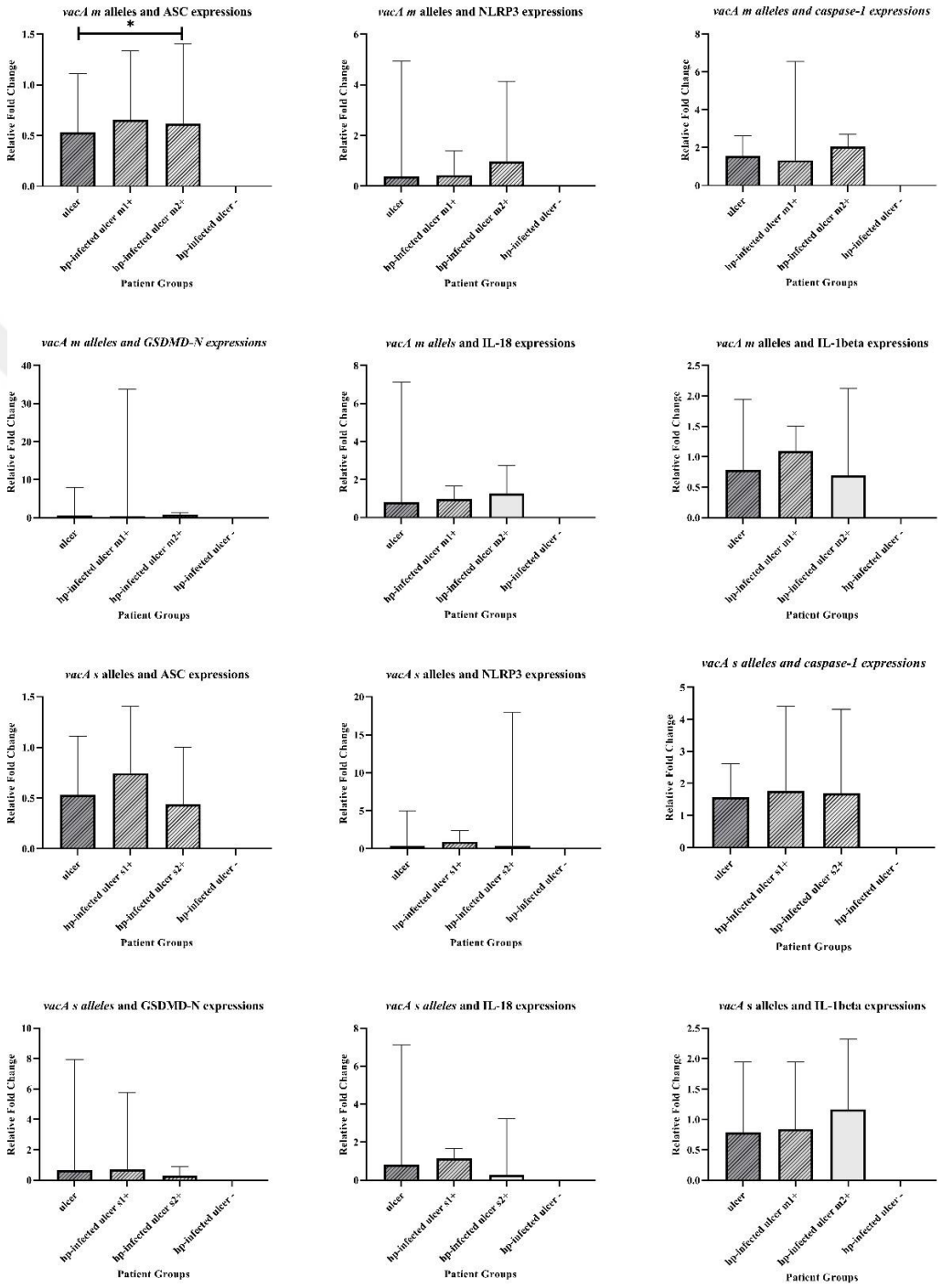


APPENDIX 3

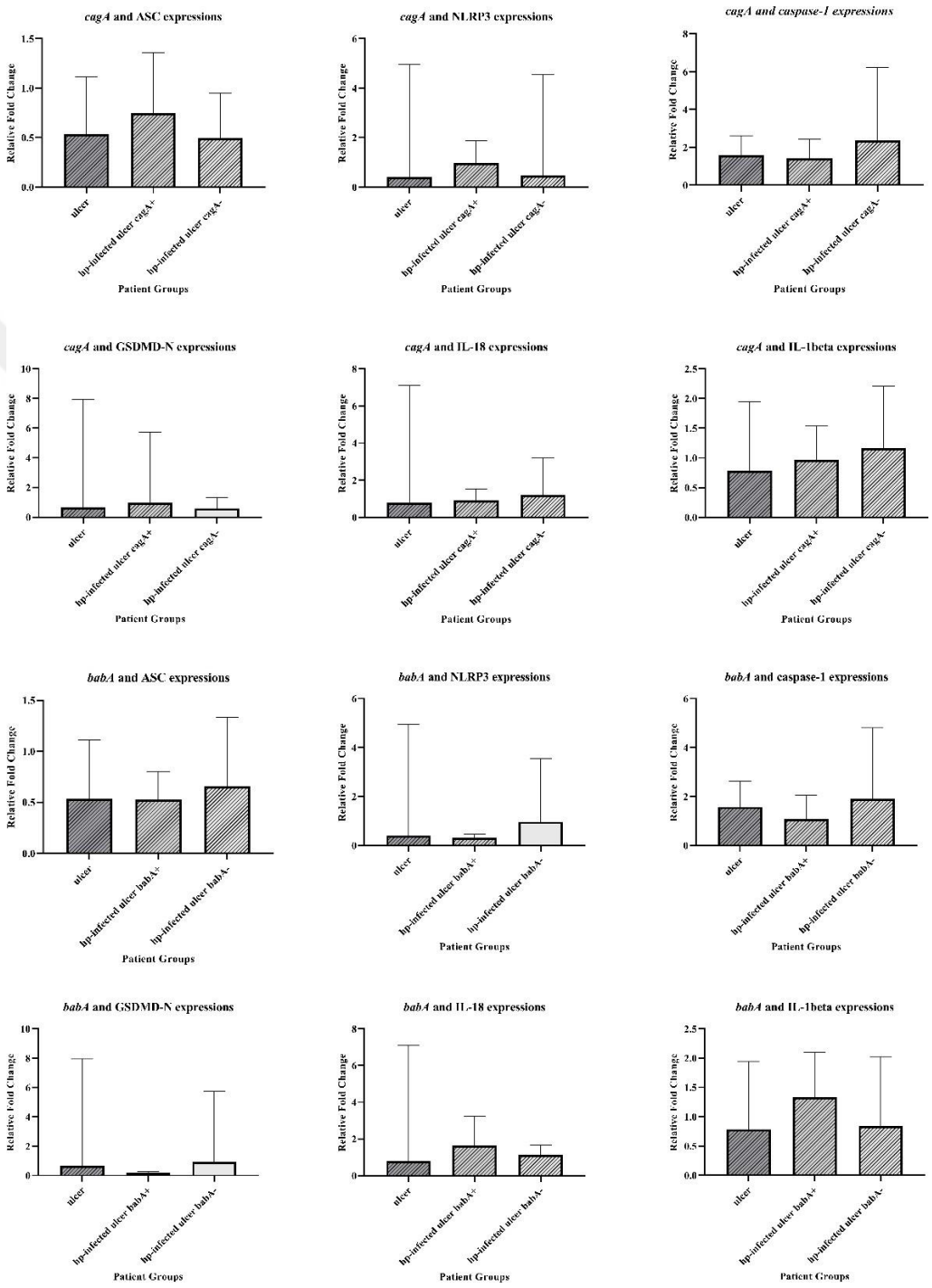
Appendix 3. Graphs showing the distribution of target markers by expression of virulence genesin ulcer patients with upregulated pyroptosis markers



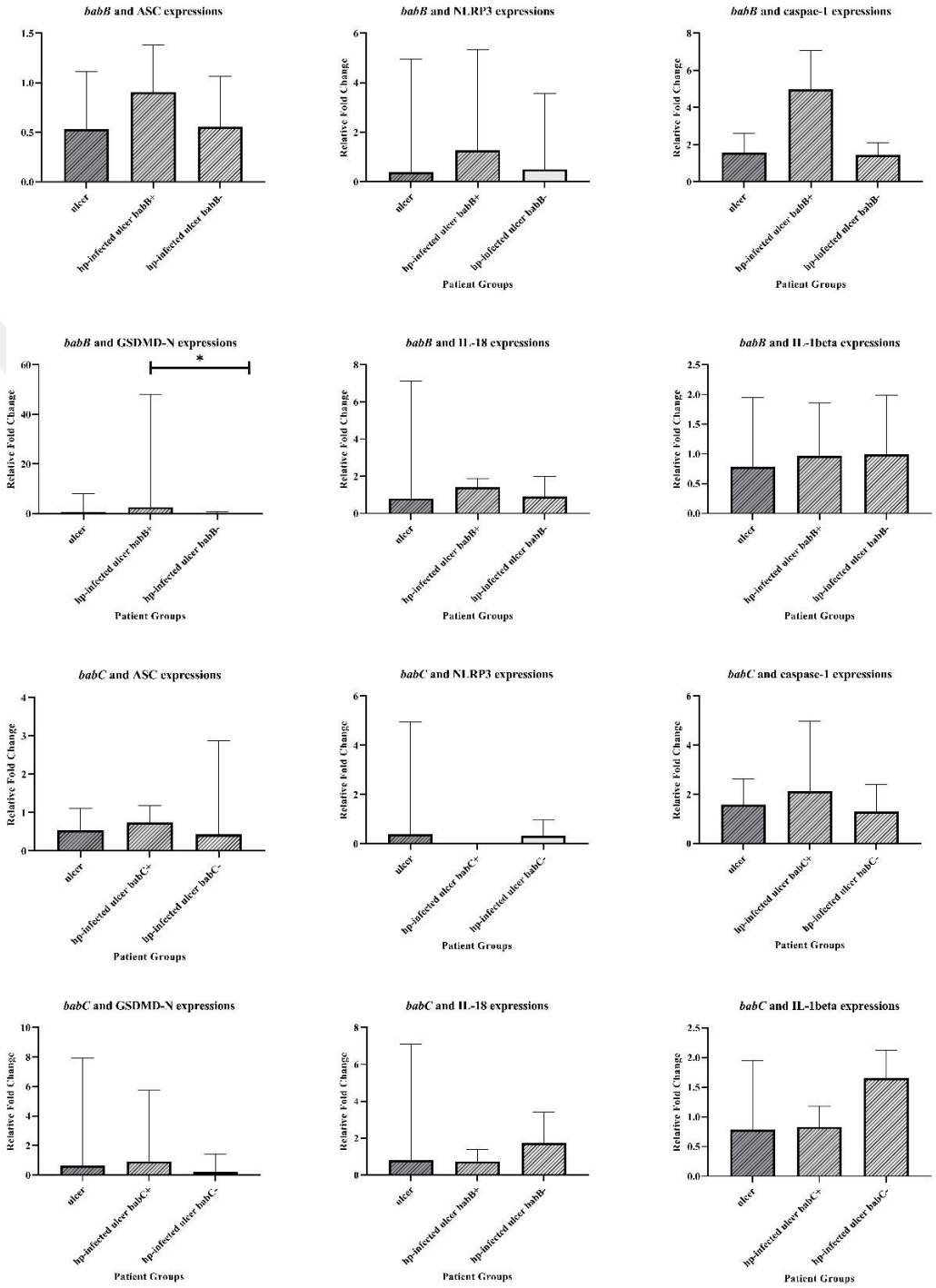
Appendix 3. Graphs showing the distribution of target markers by expression of virulence genes in ulcer patients with upregulated pyroptosis markers (cont'd)



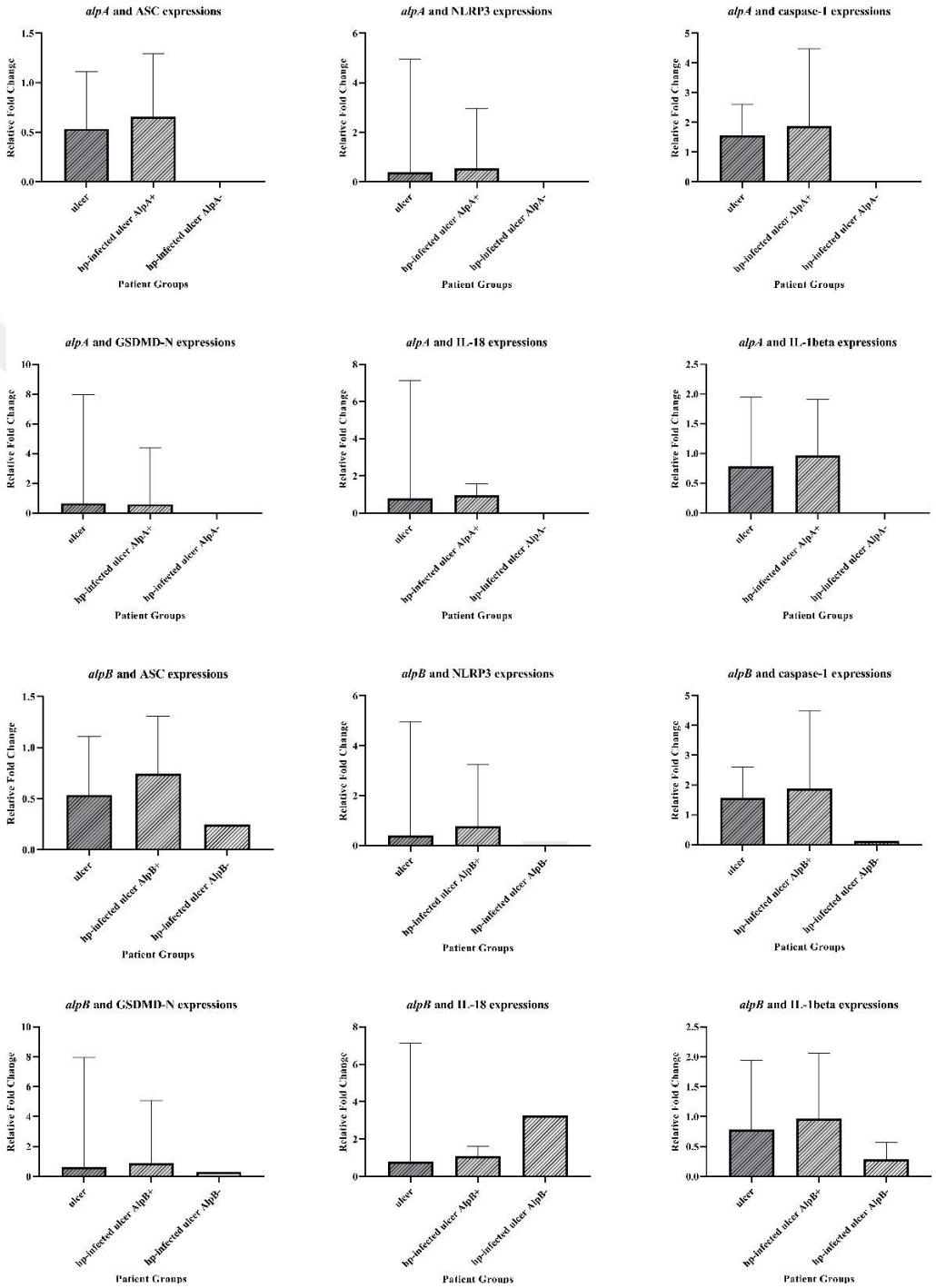
Appendix 3. Graphs showing the distribution of target markers by expression of virulence genes in ulcer patients with upregulated pyroptosis markers (cont'd)



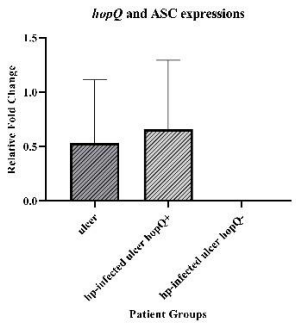
Appendix 3. Graphs showing the distribution of target markers by expression of virulence genes in ulcer patients with upregulated pyroptosis markers (cont'd)

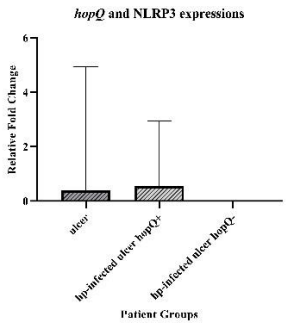


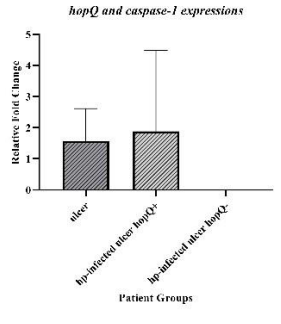
Appendix 3. Graphs showing the distribution of target markers by expression of virulence genes in ulcer patients with upregulated pyroptosis markers (cont'd)

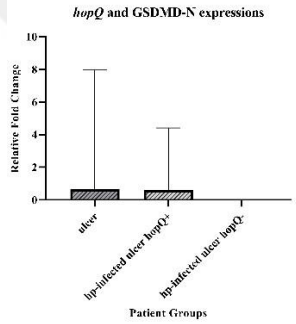


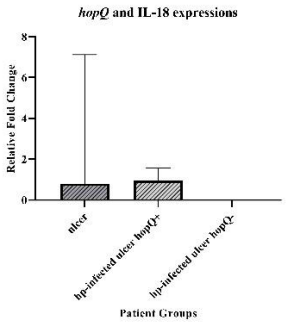
Appendix 3. Graphs showing the distribution of target markers by expression of virulence genes in ulcer patients with upregulated pyroptosis markers (cont'd)

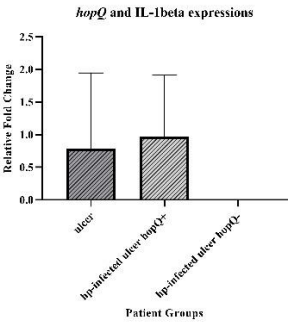


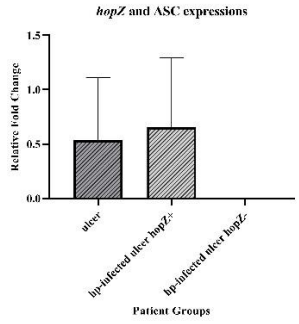


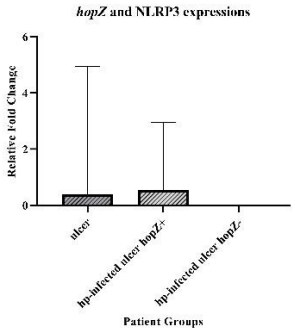


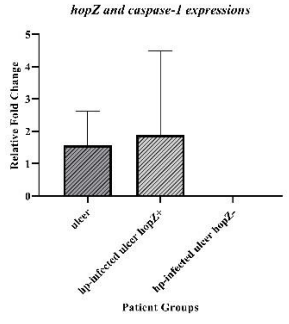


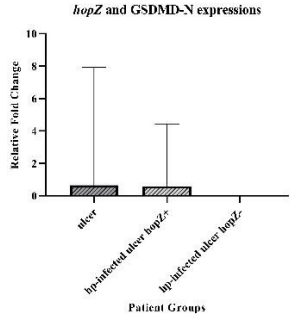


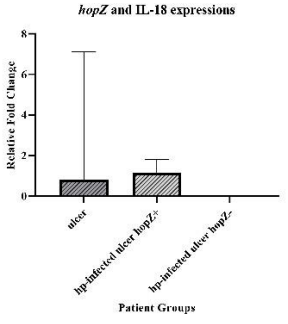


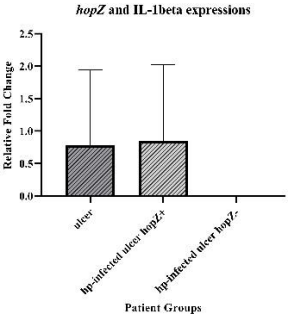




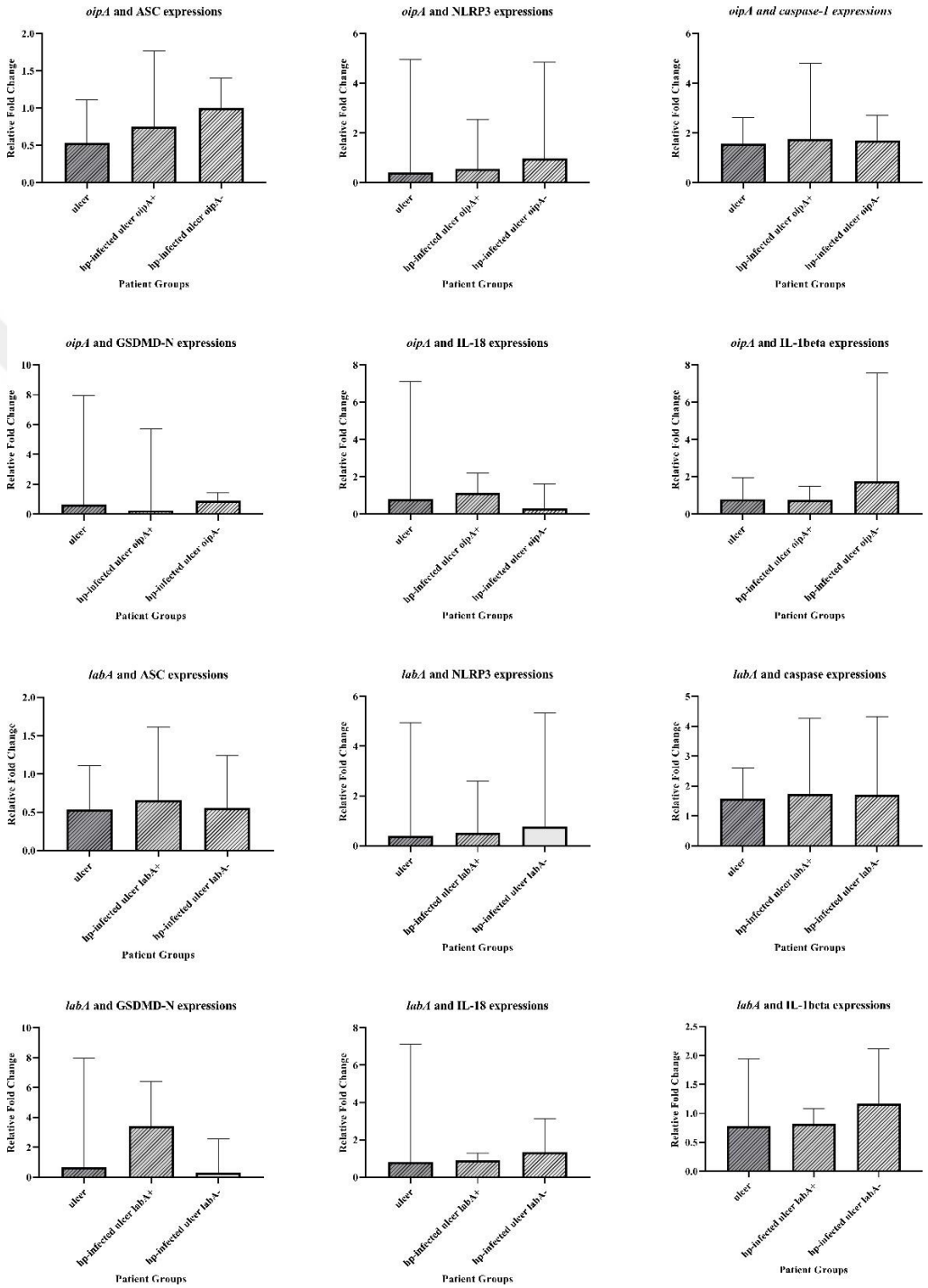




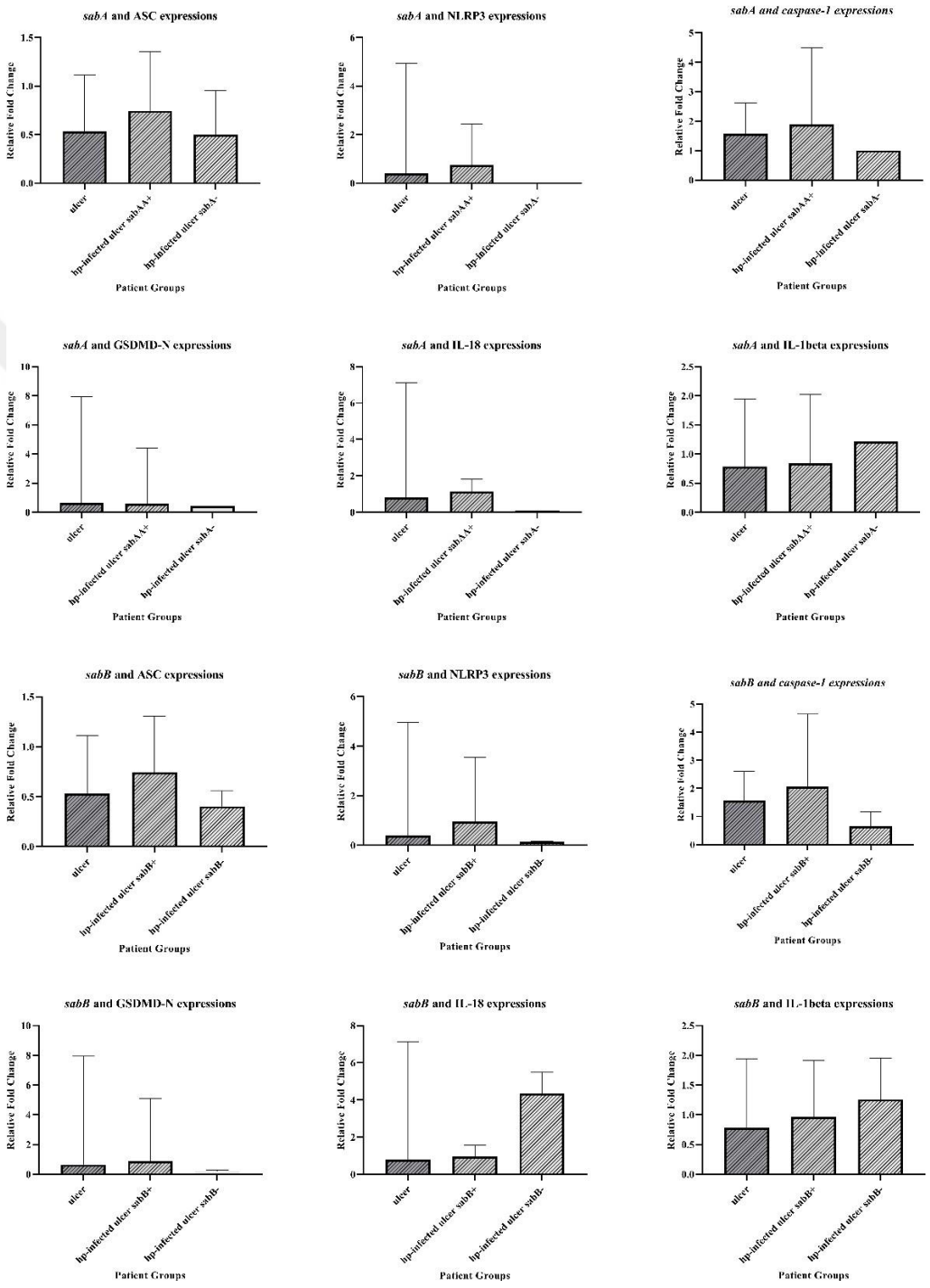




Appendix 3. Graphs showing the distribution of target markers by expression of virulence genes in ulcer patients with upregulated pyroptosis markers (cont'd)



Appendix 3. Graphs showing the distribution of target markers by expression of virulence genes in ulcer patients with upregulated pyroptosis markers (cont'd)



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