SHORT COMMUNICATION

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# *Helicobacter pylori* and Epstein-Barr Virus Co-Infection in Polish Patients with Gastric Cancer – A Pilot Study

MAGDALENA DZIKOWIEC[1]*, PRZEMYSŁAW LIK[2], JUSTYNA KISZAŁKIEWICZ[1], ALEKSANDRA KUCZYŃSKA[3], MAREK MORDALSKI[4], DARIUSZ NEJC[2], JANUSZ PIEKARSKI[2], EWA BRZEZIAŃSKA-LASOTA[1] and DOROTA PASTUSZAK-LEWANDOSKA[3]

[1] Department of Biomedicine and Genetics, Chair of Biology and Medical Microbiology, Medical University of Lodz, Lodz, Poland
[2] 2nd Oncological Surgery Ward – Oncologic Surgery Clinic, Provincial Multispeciality Center of Oncology and Traumatology named after M. Kopernik in Lodz, Lodz, Poland
[3] Department of Microbiology and Laboratory Medical Immunology, Chair of Biology and Medical Microbiology, Medical University of Lodz, Lodz, Poland
[4] Central Clinical Hospital of the Medical University of Lodz, Lodz, Poland

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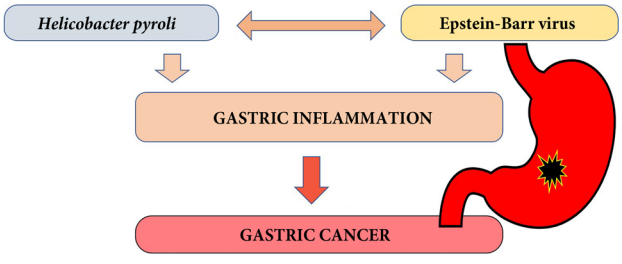
Abstract

The infectious agents may be the etiological factor of up to 15–20% of cancers. In stomach cancer, attention is paid to *Helicobacter pylori* and Epstein-Barr virus, both of which cause gastritis and can lead to tumor development. In co-infection, the inflammatory process is much more intense. We assessed the seroprevalence towards *H. pylori* and EBV in 32 patients with diagnosed gastric cancer. *H. pylori* antibodies were found in 69% patients, and anti-EBV – in all of them. The study confirmed that co-infection of *H. pylori* and EBV seems to be important in etiopathology of gastric cancer.




K e y w o r d s: co-infection, EBV, gastric cancer, *Helicobacter pylori* prevalence, infection agents in cancer

There has been tremendous progress in science and medicine in recent years, but despite this, cancer is the second leading cause of death after cardiovascular disease and is undeniably one of the most critical public health challenges. Neoplastic cells are characterized by reduced growth control, invasiveness to the tissues in which they occur, and the ability to spread. More and more often, attention is paid to the contribution to neoplasms formation by broadly understood infectious agents (Fol and Jachowicz 2016; Masrour-Roudsari and Ebrahimpour 2017; Palrasu et al. 2021). It is estimated that they could cause up to 20% of cancers. Infectious agents can promote tumor development by affecting cell growth, destabilizing the host's immune system, or leading to changes in cells resulting from long-term infection. The neoplastic process can be a consequence of infections caused by certain viruses (most often), bacteria, or even parasites (Fol and Jachowicz 2016). According to the World Health Organization, in 2020, about 10 million people were diagnosed with new cases of cancer, and stomach cancer was in the sixth place (1.09 million), while in terms of the number of deaths, it was in the fourth place (769 thousand), after lung, colon, and liver cancer (WHO 2021). Stomach cancer incidences and mortality vary greatly depending on environmental factors such as diet, alcohol consumption, smoking, cancer stage at diagnosis, and genetic burden. *Helicobacter pylori* and the Epstein-Barr virus

* Corresponding author: M. Dzikowiec, Department of Biomedicine and Genetics, Chair of Biology and Medical Microbiology, Medical University of Lodz, Lodz, Poland; **e-mail:magdalena.dzikowiec@umed.lodz.pl**

are mentioned as significant risk factors for developing gastric cancer (Fasciana et al. 2019a; Machlowska et al. 2020; Sexton et al. 2020; Palrasu et al. 2021).

*H. pylori* was first isolated from the stomach in 1983, and already in 1994, the International Agency for Research on Cancer (IARC) classified *H. pylori* as a class 1 carcinogen (Santacroce et al. 2008; Fasciana et al. 2019b; Palrasu et al. 2021). In most cases, the infection is asymptomatic, but 1–2% of people develop stomach cancer. It has been shown that these bacteria are a strong risk factor for stomach cancer; infection can lead to chronic inflammation, followed by intestinal metaplasia, dysplasia, and gastric cancer (Correa 1992; Santacroce et al. 2008). It is estimated that in 2015 there were approximately 4.4 billion individuals with *H. pylori* infection worldwide. A substantial variation in *H. pylori* occurrence of bacteria depends on the region of the world and the country. The prevalence is very high in most developing countries and is mainly related to socioeconomic status and hygiene levels. The highest prevalence is in Africa, South America, and West Asia; the lowest in Oceania, Western Europe, and North America. In Europe, the lowest prevalence was recorded in Switzerland (18.9%), and the highest in Portugal, 86.4%; in Poland, it is about 66.6% (Hooi et al. 2017).

The Epstein-Barr virus (EBV) is ubiquitous globally, affecting over 90% of people, and is associated with certain cancers such as post-transplant lymphoproliferative diseases, nasopharyngeal cancer, Hodgkin's lymphoma, and gastric cancer (Shinozaki-Ushiku et al. 2015; Smatti et al. 2018). According to the molecular classification of gastric cancer, Epstein-Barr virus-associated gastric carcinoma (EBVaGC) is a distinct subtype in terms of oncogenesis and molecular features and accounts for approximately 10% of cases (Cancer Genome Atlas Research Network 2014; Shinozaki-Ushiku et al. 2015; Yang et al. 2020). EBV has long been linked to some undifferentiated gastric carcinoma, thus indicating that focal EBV infection occurs before oncogenic transformation (Shibata et al. 1991). Interestingly, EBV-positive carcinomas have a better prognosis and a lower percentage of lymph node metastases than EBV-negative carcinomas, as demonstrated by clinical-pathological studies (Kobayashi et al. 2019).

More and more attention is paid to research on the coexistence of *H. pylori* and EBV in gastric diseases and cancer (Morales-Sanchez and Fuentes-Panana 2017; Polakovicova et al. 2018; Dávila-Collado et al. 2020; Rihane et al. 2020). Although less is known on EBV participation in chronic gastric inflammation, many studies recognize both pathogens as etiological agents of chronic gastritis, and in the case of co-infections, the inflammatory process is significantly increased (Cárdenas-Mondragón et al. 2015; Morales-Sanchez and Fuentes-Panana 2017).

The research performed by Santacroce et al. (2000) has indicated a significant relationship between *H. pylori*-positivity and the presence of mast cells in gastritis. It has been shown that neutrophils, eosinophils, mast cells, and dendritic cells can directly infiltrate the gastric epithelium during *H. pylori* infection (Ieni et al. 2016). That is a way these innate immune cells occupy strategic positions and make it easy to initiate chronic active inflammation. Virulent strains of *H. pylori* stop the correct phagosome maturation process in macrophages and dendritic cells and generate large autophagosomes, in which the bacteria can multiply, weakening the host's immune defense. Eventually, apoptosis or functional depletion of macrophages and dendritic cells can be observed in chronic *H. pylori* infection (Ieni et al. 2016). Also, MALT lymphomas, the most frequent lymphoid neoplasms of the digestive tract, are strongly associated with *H. pylori* infection (Santacroce et al. 2008; Violeta Filip et al. 2018). The pathogenesis of primary gastric lymphoma may also be related to other risk factors, including the EBV virus. Interestingly, *H. pylori* and EBV infections alter the expression of the host miRNA, which modulates the host's inflammatory immune response, favoring the survival of bacteria in the gastric mucosa and inhibiting apoptosis in EBV-positive cells (Morales-Sanchez and Fuentes-Panana 2017; Polakovicova et al. 2018).

The aim of the research was to determine the prevalence of *H. pylori* and EBV and their possible co-infections in patients with gastric cancer.

The admission criterion for the research study was the diagnosis of gastric cancer in an adult patient with no history of other neoplasms. After complete gastrectomy, blood serum was obtained from 32 patients diagnosed with gastric cancer (adenocarcinoma). Patients were treated in 2018–2020 at the 2nd Oncological Surgery Ward – Oncologic Surgery Clinic, Provincial Multispeciality Center of Oncology and Traumatology named after M. Kopernik in Lodz and the Central Clinical Hospital of the Medical University of Lodz. The patient group consisted of 20 men and 15 women, aged 36–93 years (mean age 68). The study was approved by the Bioethics Committee at the Medical University of Lodz (No. RNN/206/19/KE). The reference material was serum obtained from people without neoplastic diseases.

In patients with adenocarcinoma, after complete gastrectomy, the primary tumor size and its' invasion into adjacent tissues (T), the state of regional lymph nodes (N), and the presence or absence of distant metastases (M) were analyzed histopathologically (TNM classification) (Rosen and Sapra 2021).

Detection of anti-cagA antibodies against *H. pylori* was performed using ELISA kits (EIA Helicobacter MONO IgM, EIA Helicobacter MONO IgG, EIA

Table I
The number of tumor specimens (n) classified according to TNM classification and AJCC staging.

| | TNM classification | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | T1 | T2 | T3 | T4 | N0 | N1 | N2 | N3 | M0 |
| n | 7 | 3 | 18 | 4 | 11 | 5 | 7 | 9 | 32 |
| AJCC staging | | | | | | | | | |
| | stage I (localized cancer) | | stage II (locally advanced cancer, early stages) | | | | stage III (locally advanced cancer, late stages) | | |
| n | 8 | | 9 | | | | 15 | | |

Helicobacter MONO IgA, TestLine Clinical Diagnostics s.r.o.). In the case of EBV, the presence of antibodies against viral capsid, early and nuclear antigens were analyzed, for which the indirect immunofluorescence method (IIFT) was used. The IIFT method is considered the gold standard in EBV diagnostics (IIFT BIOCHIP EBV, Euroimmun).

Statistical analysis was performed using Statistica StatSoft Inc. The significance of differences between the control and study groups was assessed using the Kruskal-Wallis test, with the significance level $p < 0.05$. Correlations of the presence of antibodies to *H. pylori* infection with demographic factors (age, sex) and the degree of tumor development (TNM classification, AJCC staging) were determined using the Spearman Rank Correlation, with the significance level $p < 0.05$.

The results of histopathological analysis are collected in Table I.

In search for *H. pylori*, IgM, IgG, and IgA antibodies were assessed to obtain the most reliable results, excluding false-negative results. The prevalence of infection among patients was 69%. IgG antibodies were detected in nine patients only, IgG and IgA antibodies were present in 12 others, and one patient had only IgA antibodies against *H. pylori*. The obtained results indicated the past infection, as no IgM antibodies were detected in any of the patients, which would point to a recent infection. Antibody assays in the control group showed the presence of infection in 42% of subjects – in one case, the presence of IgM was detected, which indicated a recent infection. In the remaining cases, the infection occurred in the past; in seven persons, IgG and IgA antibodies were present, in five – only IgG antibodies, and in one case, only IgA antibodies were detected.

Statistical analysis showed a positive correlation (Spearman rank correlation, $p < 0.05$) between a tumor stage and the presence of IgG against *H. pylori* (Fig. 1), and between the size of the primary tumor and the presence of IgG against *H. pylori* (Fig. 2). There was no statistically significant difference between the patients' age and the cancer stage and between the patients' age and the prevalence of antibodies to *H. pylori*.

Serological analysis of anti-EBV antibodies included IgM and IgG antibodies to capsid antigens (CA), early antigens (EA), and nuclear antigens (EBNA). The avidity of IgG antibodies was also assayed. A positive

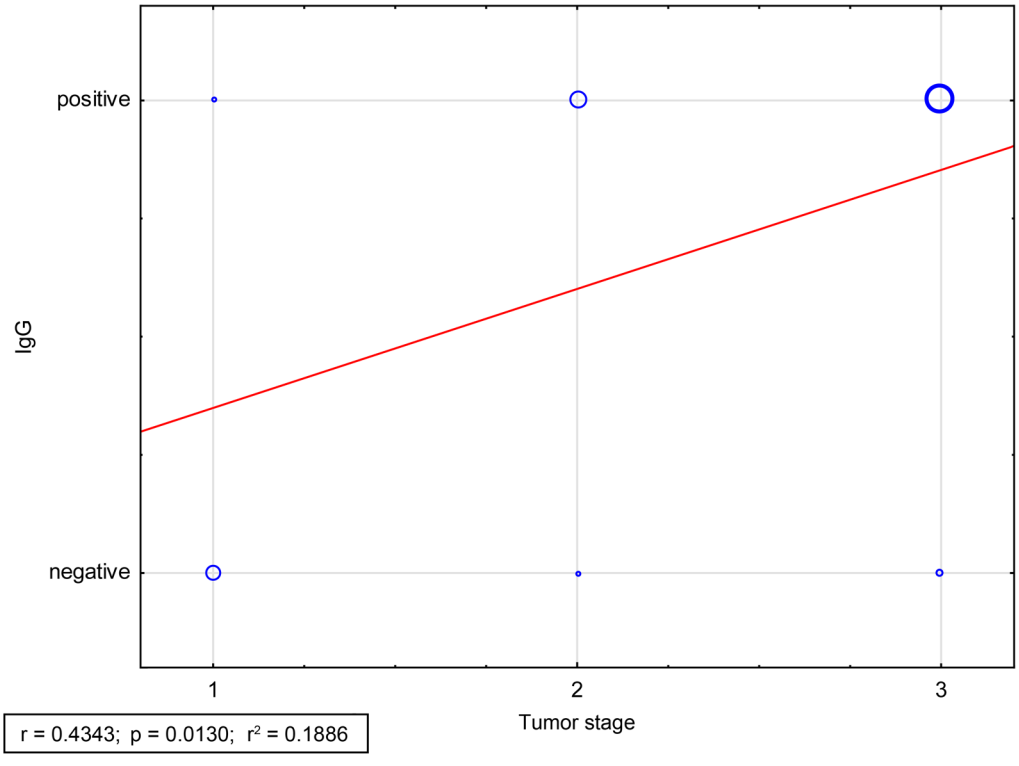



Fig. 1. The correlation between tumor stage and the presence of IgG against *H. pylori*.

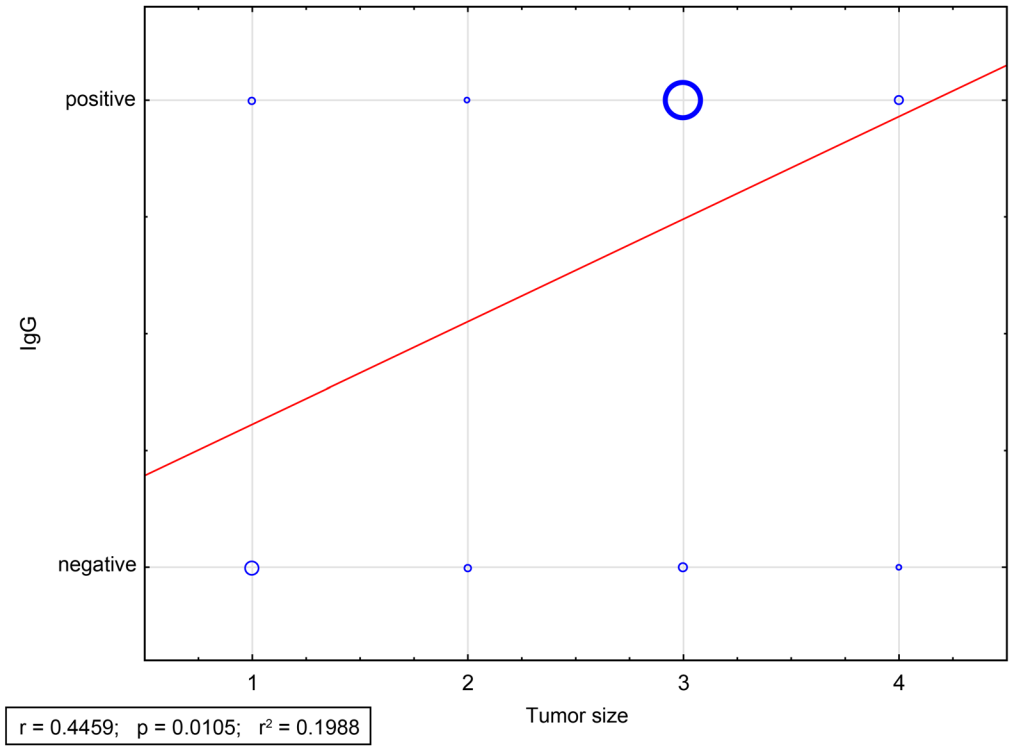



Fig. 2. The correlation between tumor size (TNM classification) and the presence of IgG against *H. pylori*.

anti-CA (IgM) results are the classic marker of fresh infection. IgG anti-CA antibodies remain at the same level throughout life. About 6–8 weeks after the infection, antibodies against nuclear antigens (anti-EBNA) are produced, and their presence indicates a past EBV infection. It is helpful to test the avidity, i.e., the binding strength of specific IgG antibodies to the antigen. Thanks to the avidity test, it can be determined whether the positive reaction of IgG antibodies results from a recent or past infection. At first, the immune system responds to infection by producing antibodies in the IgG class with low antigen-binding power (low avidity). As the infection progresses, the avidity of IgG antibodies increases. The late-stage or past infection is suspected if high avidity antibodies are detected. The study on the avidity of anti-CA antibodies in the IgG class in the diagnostics of EBV infections allows for interpretation of the problematic and questionable results. Based on the simultaneous detection of the presence of several specific antibodies, and the assessment of their avidity, it can be estimated whether the antibodies present in the patient's serum are the result of an active infection or the evidence of a past infection. None of the patients had IgM anti-CA antibodies, which would indicate a new infection, but all had high avidity anti-CA IgG antibodies, eight patients had anti-EA IgG antibodies, and five patients did not have anti-EBNA antibodies.

Gastric cancer is one of the most common neoplasms, and since the symptoms of the disease appear at an advanced stage, it is diagnosed very late. It develops due to genetic and environmental factors, such as eating habits, alcohol drinking, cigarette smoking, and excessive body weight. Stomach cancer usually has a poor long-term prognosis; only the five-year survival rate in Japan is relatively good, reaching 90%. In Europe, this value ranges from 10% to 30%. Therefore, it is very essential to understand the etiology and the ability to early diagnose of gastric cancer (Sitarz et al. 2018; Machlowska et al. 2020). Stomach cancer rates increase with age and reach a plateau between 55 and 80. On average, gastric cancer incidence rates are 2 to 3 times higher in men than in women (Machlowska et al. 2020; Thrift and El-Serag 2020). Also, in our study group, the majority of patients were men (20 men versus 12 women), and the age of the patients in most cases ranged from 60 to 80 years.

More and more often, infectious agents are mentioned to trigger cancer development. In the case of gastric cancer, *H. pylori* is regarded as the leading environmental risk factor with proven pathogenicity; however, the contribution of EBV in its development is not fully understood (Cárdenas-Mondragón et al. 2015; Singh and Jha 2017; de Souza et al. 2018; Castaneda et al. 2019; de Martel et al. 2020; Sexton et al. 2020). It has been estimated that approximately 2.2 million cases of infection-related cancers were diagnosed worldwide in 2018, corresponding to an infection-related age-standardized incidence rate (ASIR) of 25.0 cases per 100,000 persons yearly. *H. pylori* appeared in the first place as a cancer-related infectious agent. ASIR

attributed to infection was the highest in eastern Asia – 37.9 cases per 100,000 persons per year, and lowest in northern Europe – 13.6 cases per 100,000 persons per year (de Martel et al. 2020).

To the best of our knowledge, this is the first project about the coexistence of *H. pylori* and EBV in the development of gastric cancer in the Polish population. Both pathogens are involved in the development of inflammation, which may be a consequence of changes in the gastric mucosa that may lead to the development of the neoplastic process (Dávila-Collado et al. 2020; Rihane et al. 2020). The incidence of gastric cancer varies widely geographically. Over 50% of new cases occur in developing countries. Likewise, the prevalence of *H. pylori* is the highest in the developing world, and the infection is widespread in East Asia, strongly associated with the development of gastric cancer (Hooi et al. 2017). In our study, antibodies against *H. pylori* were detected in 69% of patients with gastric cancer, a much higher prevalence than the control group (42%). The obtained results have also confirmed a correlation between *H. pylori* infection and the degree of tumor stage and the correlation between *H. pylori* infection and tumor size, indicating the role of the pathogen in the progression of gastric cancer.

In Poland, little research has been conducted on the epidemiology of *H. pylori*. Multicenter studies on the presence of *H. pylori* in children and adults (6 months-89 years of age) in Poland occurred in 2002–2003. The presence of antibodies was detected in over 58% of the subjects studied: 32% in children and 84.2% in adults. In adults, a statistically significant correlation was found between *H. pylori* seropositivity and the occurrence of gastrointestinal symptoms. In histopathological specimens, inflammation predominated in children, and atrophic gastritis and intestinal metaplasia were common in adults (Iwanczak et al. 2014). An earlier study, performed in 2008, showed a lower infection rate in pediatric patients of approx. 16% (Biernat et al. 2016). However, the later study used the 13C-urea breath test (UBT) for current *H. pylori* infection showed a positive result for 23.6% of cases (Szaflarska-Popławska and Soroczyńska-Wrzyszcz 2019), indicating an increasing trend. In our study, the incidence of *H. pylori* in the entire group (patients and controls) was 55.5%, similar to the results reported in later studies carried out in Poland, although our group was relatively small.

Relatively recently, attention was paid to the role of EBV as an etiological factor in the development of gastric cancer. The presence of viral DNA in cancer cells was confirmed by molecular methods in 1990. It has even been suggested that focal EBV infection occurs before oncogenic transformation (Shibata et al. 1991).

The incidence of EBV infection in gastric cancer ranges from 2 to 20%, the global average is about 10% (Shinozaki-Ushiku et al. 2015). Researches carried out in various regions of the world has shown that the incidence of gastric cancer associated with EBV is different, and in Europe, it is 13.9%, in America – 12.5%, and in Asia – 7.5% (Camargo et al. 2014). The differentiation in the occurrence of EBVaGC can also be observed, depending on individual countries, e.g., it ranges from 4% in China to 17.9% in Germany, while in Poland, it is about 12.5% (Czopek et al. 2003; Sitarz et al. 2018). In our study, based on serological results, the presence of EBV antibodies in the serum of all gastric cancer patients was demonstrated.

Inflammation of the gastric mucosa is much more severe in *H. pylori* and EBV co-infection than infection with a single pathogen (Cárdenas-Mondragón et al. 2015; Polakovicova et al. 2018; Castaneda et al. 2019). Cardenas-Mondragón et al. (2015) analyzed antibodies against EBV and *H. pylori* in 525 patients with gastric diseases from Mexico and Paraguay. They found that 94.7% of patients had positive antibodies to EBV and 87.7% to *H. pylori*, with a similar incidence in both countries. A relationship has been demonstrated between the coexistence of EBV and *H. pylori* and pre-neoplastic lesions and intestinal gastric carcinoma. Castaneda et al. (2019) studied patients with gastric cancer in Peru, where the incidence of *H. pylori* was 60.8%, EBV – 14.1%, co-infection was found in 7.8% of cases.

Studies on the coexistence of EBV and *H. pylori* have also been conducted in pediatric patients with severe gastritis. Based on the study of antibodies in children with chronic abdominal pain, it has been shown that a single infection is associated with mild inflammation, while co-infection of both pathogens significantly worsens gastric inflammatory state. Even in *H. pylori* $CagA^+$ strain infection, inflammation was not as strong as in co-infections (Cárdenas-Mondragón et al. 2013).

In a study conducted by de Souza et al. (2018), the co-infections with *H. pylori* $CagA^+$ and EBV were correlated with the most advanced stages of cancer. Although only 20% of tumors were positive for EBV, infection with this virus was associated with distant metastases.

Gastric cancer is one of the most common cancers globally, with a very high mortality rate. Due to the lack of characteristic symptoms, it is usually diagnosed late. Therefore, early and effective diagnosis is critical, and the search for non-invasive markers will allow for early cancer detection and effective treatment. In this respect, it is crucial to understand tumor biology, i.e., the mechanisms underlying the neoplastic process. Although many factors contribute to the development of cancer, both genetic and environmental, the increasingly important role is assigned to infectious factors, primarily *H. pylori* and EBV. Both pathogens cause inflammation within the gastric mucosa, and in the case of their co-infection, it is much more intense. The

results of our study show a high prevalence of *H. pylori* and EBV in patients with gastric cancer and the correlation of infection with its progression.

The seroprevalence of *H. pylori* was positively correlated with both tumor size and tumor stage, according to TNM classification and AJCC staging, respectively. The co-presence of anti-EBV antibodies (anti-CA IgG) and IgG against *H. pylori* was confirmed in 69% of patients. This result suggests that co-infection of *H. pylori* and EBV, both harmful biotic agents, could be linked with the advanced stages of gastric cancer and its progression.

The number of patients included in the study is not large. The material was collected from 2018 to 2020. At the time of the announcement of the SARS-CoV2 pandemic, the access to treatment of diseases other than COVID-19 was limited. Hence, the difficulties in obtaining biological material from cancer patients. That is why we describe the study as a pilot study – to share the results obtained so far. We are still collecting material from patients and will continue our research, extending it to other factors related to gastric carcinogenesis.

**ORCID**
Magdalena Dzikowiec https://orcid.org/0000-0002-4044-6424

**Conflict of interest**

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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