Answers to the Reviewers

Reviewer #1

Specific Comments to Authors: This is an interesting study. However, some issues must be clarified.

1. The title of the manuscript is very long and confusing.

1. The title was revised as "**Association between HLA gene polymorphisms and multiple EPIYA-C repeats in gastrointestinal disorders**". There is alimitation of 12 words fort he title and this was fit the best in these conditions.

2. The control group of the study is questionable. The group would give control if they were blood donors, for example.

2. This is a reasonable comment. **It was not possible and ethical to convince blood donors for taking gastric biopsy specimens and the most important point in our study design was the presence of *H. pylori* as to be the dependent variable.** Initially, we aimed to investigate the effect of *H. pylori* both in gastric disorders and human HLA alleles. Our first design was to obtain people with asymptomatic *H. pylori* as a control group. But, it was very difficult to decide a person whether healthy or not with a *H. pylori* presence in their stomachs. Therefore, we eliminated most of the asymptomatic individuals after the evaluation of their pathology reports. We obtained a few asymptomatic individuals as 36 during approximately 4 years. We carefully folllowed up our non-ulser dyspepsia patients and they were recovered in their following visits to the gastroenterology clinics. In Turkey, treatment of *H. pylori* infections is not a first option and our clinicians hardy choose treatment for *H. pylori* infections especially in non-ulcer dyspepsia cases. After our first results obtained, we noticed the virulence of *H. pylori* strains in non-ulcer dyspepsia group was low (the absence of cagA and multiple EPIYA-C repeats in these H. pylori strains. We decided to use NUD and asymptomatic *H. pylori* as a control group.

On the other hand, these were the first steps of this study and our main goal was to find out the assaociation between HLA alleles and multiple EPIYA-c repeats. Our main comparisons were made between gastric cancer and duodenal ulcer subgroups and our main conclusion was made due to this comparisons. We tried to show every step of our study and included initial steps with a questionable control group for this reason. We aimed to explore the association of the virulence factor multiple EPIYA repeats of H. pylori and HLA alleles and gastric pathologies not the presence of H. pylori.

Also, we used this combination of control group in our 2015 publication in *journal of Medical Microbiology* as " **Patterns of EPIYA motifs among cagA-positive *Helicobacter pylori* strains: a**

case-control study in a Turkish population with Eurasian geographical features. J Med Microbiol. 2015 Oct;64(10):1117-23. doi: 10.1099/jmm.0.000141. Epub 2015 Jul 21. PMID: 26198695".

3. The authors do not cite IL-17 (and related cytokines) as important for gastric carcinogenesis.

3. We are agree with the reviewer and added the following paragraphs with the new references to the Discussion part of the manuscript " **A commonly seen (90%) type of GC is adenocarcinoma, which is known to originate from the epithelial cells in chronic inflammation states[28]. Moreover, cytokines, which are the effector cells of inflammatory responses, may regulate a variety of immunologic events, including the inflammation, proliferation, and differentiation of epithelial cells. In the progression of gastric carcinogenesis, initially, *H. pylori* strongly induces specific cytokines, but the immune response generally is not sufficient to clear the *H. pylori* infection completely from the human epithelial cells. As a result, chronic inflammation may occur[29].**

Consequently, the tissue damage increases along with parietal cell atrophy and may progress to dysplasia and GC through the combined effects of the various factors of the host and the environment. A subtype of T cells, Th17 cells, and their associated inflammatory cytokines, interleukin (IL)‑17A, IL‑23, and IL‑1β, have important roles in the development of GC, colorectal cancer, ovarian cancer, and hepatocellular carcinoma. Cytokine IL-23 plays a major role in the primary activation of IL-17A. Th17 responses are reported to be increased during *H pylori* infections. The IL-17/IL-23 axis is believed to have an important role in the progression of chronic inflammation and related pathologies like gastric neoplasms[30].".

4. The authors should cite studies evaluating epiya and relatives of patients with gastric cancer.

4. We are also agree with this comment of the reviewer and added the following paragraphs to the Discussion part of the manuscript with new references "**Several studies suggest that atrophic gastritis and GC risk are increased by CagA-positive H. pylori strains. An association has been reported between multiple EPIYA-C phosphorylation sites and GC. In a recent meta-analysis including 23 studies, Li et al.[8] evaluated the association of EPIYA motifs and gastroduodenal pathologies. They concluded that the EPIYA-D motif was significantly related to GC risk, and multiple EPIYA-C motifs were related to PU and DU in Asia countries.**

Conversely, in the United States and Europe, multiple EPIYA-C motifs were commonly associated with GC risk. Multiple EPIYA-C repeats cause stronger binding of cagA to SHP-2 than a single EPIYA-C. Multiple EPIYA C repeats are associated with a higher risk of GC[34,35]. The functionality of cagA is increased with multiple EPIYA-C phosphorylation sites

and is involved in cellular phenotypic changes. Therefore, *H. pylori* strains with multiple EPIYA-C sites are related to the risk of GC.".

5. In the introduction, I think the authors should cite possible regional and ethnic differences to justify the importance of the study.

5. We are agree with the reviewer and we added the following paragraps to the Introduction part of the nanuscript as " **Reported HLA allele frequencies related to GC pathologies with *H. pylori* positivity have been contradictory among different ethnic groups. The regional and ethnic differences are very important for the association of HLA gene polymorphism and GC risk. These contradictory results may be attributed to factors such as differences in populations, research designs, environmental factors, *H. pylori* virulence factors, and host genetic factors, such as polymorphisms of HLA alleles. For example, the HLA-DQB1*0301 allele was positively associated with GC in Caucasian populations but negatively associated with it in Taiwanese populations[13,16,]. This HLA polymorphism had no effect in Japanese populations[17]. HLA-DQB1*0401 82 and *0602 alleles increase the GC risk in European and Indonesian populations[9, 18].**".

6. In addition, studies that evaluated polymorphisms in genes encoding cytokines and other molecules of the immune response and gastric carcinogenesis could be presented. Some of these studies also look at virulence factors (including epiya).

6. We are thankful for this comment and we added the following paragraphs to the Disccussion part of the manuscript with new references "**Other than their main roles in chronic inflammation in gastric epithelial cells, cytokines also have specific polymorphisms in their genes. Polymorphisms in cytokine genes may modify the effect of gene-environment interaction and increase the degree of cytokine expression in the promoter regions of the genes. Polymorphisms in genes coding various cytokines such as IL-1β, IL-1Ra, IL-8, IL-10, and tumor necrosis factor-α are also suggested to be associated with the risk of GC. The IL1RN2 allele polymorphism is related to the risk of GC[31]. Wu et al.[32] found a relation between IL-17F A7488G and GC, and Felipe at al.[33] also reported a relation between IL-8 (rs4073)–251A/T gene polymorphism and GC development.**

Some virulent factors of H. pylori seem to be associated with GC risk, including vacuolating cytotoxin A, cytotoxin associated antigen A, DU promoting gene protein A, and outer inflammatory protein with blood group antigen binding adhesins. Moreover, the CagA gene of H. pylori strains with multiple EPIYA-C repeats and EPIYA-D motif in its CagA gene are suggested to increase the risk of GC development. However, the role of host polymorphisms and

the virulence factors of *H. pylori* in the risk of GC development varies among regions and ethnicities[31].".

7. Were patients with autoimmune diseases excluded from the study?

7. We were avare of the importance of autoimmune diseases and their association with the HLA polymorphisms in the early design steps of our research and we didn't specifically include the patients and control group with autoimmune diseases in our research. We revised the follwing sentence identifying exclusion criteria for study and control group individuals in material and Methods section of the study as "**We excluded patient and control group individuals with autoimmune diseases and who were under 18 years old, underwent previous gastric surgery and *H. pylori* eradication treatment, or had therapy with antibiotics, antisecretory drugs, bismuth salts, or sucralfate in the month prior to sampling**.".

8. Did the authors sequence the epiya gene from any sample? If so, that should be in the paper.

8. We are also very thankfull for this comment. We had obviosly sequenced the epiya gene and made a new table 4 indicating all EPIYA sequences and added the following sentence to the Results section of the manuscript as "Other EPIYA motifs and their numbers are shown in Table 4." And also the following new table 4 which showing all EPIYA motif distribution for all groups.

Table 3. The distribution of EPIYA motifs for study and control groups.

EPIYA-C Repeat Patterns	Patient Group		Control Group		Total (n= 140)
	Gastric Cancer [n (%)]	Duodenal Ulcer [n (%)]	Non-Ulcer Dyspepsia [n (%)]	Individuals with Normal Gastrointestinal System [n (%)]	
ABC	12 27.2	34 68	28 93.3	16 100	90
AC	2 4.5	2 4	-	-	4
BC	2 4.5	-	-	-	2
ABCC	16 36.4	8 16	-	-	24
BCC	2 4.6	2 4	2 6.7	-	6

ACCC	-		-		-		-		-
ABCC	8	18.2	4	8	-		-		12
AB	2	4.6	-		-		-		2
Total	44	100	50	100	30	100	16	100	140

9. The authors did not calculate the Hardy-Weinberg balance of the alleles. What's the reason?

9. Assoc. Prof Dr. Murat Telli from the Department of Biology in Bolu Abant Izzet Baysal University (Turkey) help us fort his comment. He had a post doctoral fellowship from USA in the field of evolitionery biology and population genetics. We also added his name to the acknowledgement section. We added to the statistical analyses section of the manuscript the following paragraph "**Hardy-Weinberg (H-W) equilibrium and Linkage Disequilibrium (LD) were examined for HLA-A, HLA-B, HLA-C,HLA-DR1, HLA-DQA1 and HLA-DQB1 allele polymorphisms[24]. Genepop software version 4.7 was used to calculate the Hardy–Weinberg equilibrium and LD. H–W equilibrium was present for p-values >0.05.**". We also added the following paragraph to the Results section as "**Among GC cases, the genotype frequencies of HLA- B, DQA1 and DQB1 loci were in H-W equilibrium. Among DU cases, all of the genotype frequencies were in H-W equilibrium. Moreover, Among NUD cases, all of the genotype frequencies in H-W equilibrium except HLA-DQB1 cases and in asymptomatic H. pylori control group, all of the genotype frequencies were in H-W equilibrium except HLA-A cases. H-W equilibrium is only valid with a very large sample size and therefore, we suggest that some of our genotype frequencies are not in H-W equilibrium. The deviations from H-W equilibrium is larger at small sample sizes like this study and smaller at large sample sizes.**.".

10. The authors did not assess whether the alleles were linkage disequilibrium.

10. Assoc. Prof Dr. Murat Telli also helped in this topic. We added to the statistical analyses section of the manuscript the following paragraph "**Hardy-Weinberg (H-W) equilibrium and Linkage Disequilibrium (LD) were examined for HLA-A, HLA-B, HLA-C,HLA-DR1, HLA-DQA1 and HLA-DQB1 allele polymorphisms[24]. Genepop software version 4.7 was used to calculate the Hardy–Weinberg equilibrium and LD. H–W equilibrium was present for p-values >0.05.**". We also added the following paragraph to the Results section as "**HLA-DR1*13-HLA-DQA1*01-HLA-DQB1*06 haplotype frequency (10/44, 22% for GC; 2/50, 4% for DU, 2/50, 4% for NUD and 0 for NGIS) showed LD and had an OR value as 6.143 (95%C.I. 1.33-28.31, P = 0.0027) in the comparison of PG and CG cases. Moreover, HLA-DR1*13-HLA-DQA1*01-HLA-DQB1*06**

haplotype frequency compared between GC and DU cases and OR was detected as 10.58 (95%C.I. 2.27-49.34).". We also added the following paragraph to the end of Discussion part of the manuscript as " **Other important result of our study was that HLA-DR1*13-HLA-DQA1*01-HLA-DQB1*06 haplotype frequency was detected significantly higher in our GC subgroup cases more than both DU and control group cases. This results is similar for the locus type but different for HLA allele types from the study of Ando et al.[47] They reported DRB1*04:05-DQA1*03:03-DQB1*04:01 haplotype frequency with 10%–30% in Japanese population and the risk of GC development**.".

Reviewer #2:

Specific Comments to Authors: The following are some recommendations.

1. "The first study in a Turkish population" should be deleted in the title, if the authors do think their results are race-related, "Turkish" should be input somewhere in the title other than at the end.

1. The title was revised as "**Association between HLA gene polymorphisms and multiple EPIYA-C repeats in gastrointestinal disorders**". There is alimitation of 12 words fort he title and this was fit the best in these conditions.

2. The definition of groups in Fig.1&2 are not appropiate, control group are also patients group, as dyspepsia is a kind of GI disease and *H.pylori* infection is a kind of disease according to the *H.pylori* Kyoto consensus.

2. We are gratefull for this comment of the regviewer and we changed the names of the groups as "**Figure 1. The representations of the highest HLA allele frequencies (%) in the gastric cancer+duodenal ulcer patient group when compared to non-ulcer dyspepsis patients+Asymptomatic *H. pylori* control group and Figure 2. The representations of the highest HLA allele frequencies (%) in the (%) in the non-ulcer dyspepsis patients+Asymptomatic *H. pylori* control group when compared gastric cancer+duodenal ulcer patient group**". On the other hand, we couldn't change our groups in the study. On the other hand, the group selection aim of this case-control study was explained to the first reviewer in the second comment. To avoid an unnnecessary repeating, plese refer to also the answer of second cmment of Reviewer 1.

3. We need some pictures of HLA-PCR results to show the realness of the study.

3. Individual HLA-A, -B, -DR, and -DQ tissue typing was performed by low-resolution polymerase chain reaction with sequence-specific oligonucleotides (PCR-SSO) using the Luminex platform (OneLambda Inc., West Hills, CA, USA).We did not use sequence-specific primers (PCR-SSP). This is a closed system and therefore it is not possible to get any pictures of PCR gel. You may check the reality of this answer from Maternal killer-cell immunoglobulin-like receptors and paternal human leukocyte antigen ligands in recurrent pregnancy loss cases in Turkey, doi.org/10.5653/cerm.2019.03223 pISSN 2233-8233 · eISSN 2233-8241 Clin Exp Reprod Med [Epub ahead of print]" and from reference "Bettencourt BF, Pinheiro JP, Bruges-Armas J. Killer-cell immunoglobulin-like receptors genotyping of 127 individuals from Terceira Island, Azores, Portugal. Hum Immunol. 2016;77(12):1113. doi:10.1016/j.humimm.2016.08.002".

One of our writers Assoc. Prof Dr Erkan Yilmaz is the chief of Istanbul University-Cerrahpasa, Cerrahpasa Medical Faculty, Department of Organ Transplantation, HLA Laboratory, Istanbul, Turkey. This is a collaborative study with this laboratory. We have ethical approval and a fund from Istanbul University Research fund. This Project was accepted as an highly important Project and they gave us a great amount of fund. Their condition is a majör publication in a majör journal otherwise we will be banned at least three years. They checked every step of the study and demanded a final report to close the Project. We had some PC outputs of our patients' from the system. I am adding samples of some GC patient output reports, BUT system is not allowed to give Picture and this is true for all researchers studying with PCR-SSO method. These patients were also our routine patients of our university hospital for surgery clinics.

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Patient Information:

Patient Name: ALI OZBEK
Sample ID: 20180730-29
Accession #:
ADS:

Final Typing Assignments:

Loci	Batch	AG1	AG2	Match Type	Merged
HLA-DQB1	20180730 DQ_DQB	03:XX	06:XX	Exact	No
HLA-DQA1	20180730 DQ_DQA	01:XX	06:XX	Exact	No

Batch Information:

Loci	Lot ID	Allele Database Version	Run Date	Comments	Completed By Date	Approved By Date
DQB	10205A 08035B-DQB	3.25	30.07.2018		Lab Supervisor 30.07.2018	Lab Supervisor 30.07.2018
DQA	10205A 08035B-DQA	3.25	30.07.2018		Lab Supervisor 30.07.2018	Lab Supervisor 30.07.2018

Completed By: _____ Date: _____

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Patient Information:

Patient Name: GURBUZ CATAL
Sample ID: 20180730-27
Accession #:
ADS:

Final Typing Assignments:

Loci	Batch	AG1	AG2	Match Type	Merged
HLA-DQB1	20180730 DQ_DQB	03:XX	05:XX	Exact	No
HLA-DQA1	20180730 DQ_DQA	01:XX	05:XX	Exact	No

Batch Information:

Loci	Lot ID	Allele Database Version	Run Date	Comments	Completed By Date	Approved By Date
DQB	10205A 08035B-DQB	3.25	30.07.2018		Lab Supervisor 30.07.2018	Lab Supervisor 30.07.2018
DQA	10205A 08035B-DQA	3.25	30.07.2018		Lab Supervisor 30.07.2018	Lab Supervisor 30.07.2018

Completed By: _____ Date: _____

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Patient Information:

Patient Name: IBRAHIM USTUN
Sample ID: 20180730-33
Accession #:
ADS:

Final Typing Assignments:

Loci	Batch	AG1	AG2	Match Type	Merged
HLA-DQB1	20180730 DQ_DQB	06:XX	06:XX	Exact	No
HLA-DQA1	20180730 DQ_DQA	01:XX	01:XX	Exact	No

Batch Information:

Loci	Lot ID	Allele Database Version	Run Date	Comments	Completed By Date	Approved By Date
DQB	10205A 08035B-DQB	3.25	30.07.2018		Lab Supervisor 30.07.2018	Lab Supervisor 30.07.2018
DQA	10205A 08035B-DQA	3.25	30.07.2018		Lab Supervisor 30.07.2018	Lab Supervisor 30.07.2018

Completed By: _____ Date: _____

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atient Information:

atient Name: MEHMET TOPCU
ample ID: 20180730-34
Accession #:
ADS:

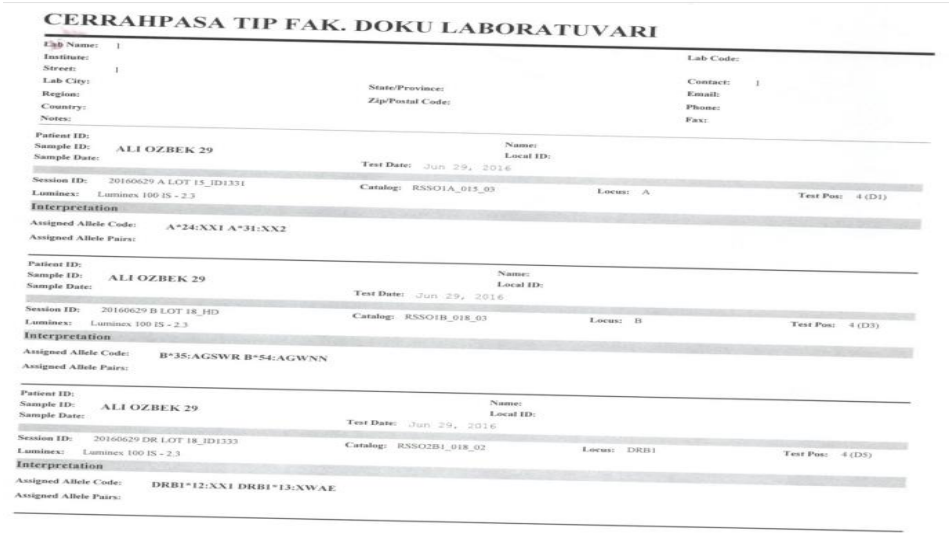
inal Typing Assignments:

Loci	Batch	AG1	AG2	Match Type	Merged
HLA-DQA1	20180730 DQ_DQA	01:XX	01:XX	Exact	No

latch Information:

Loci	Lot ID	Allele Database Version	Run Date	Comments	Completed By Date	Approved By Date
DQA	10205A 08035B-DQA	3.25	30.07.2018		Lab Supervisor 30.07.2018	Lab Supervisor 30.07.2018

Completed By: _____ Date: _____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Zip/Postal Code:
Lab Code:
Contact: 1
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Patient ID:
Sample ID: ALI OZBEK 29
Sample Date:
Name:
Local ID:
Test Date: Jun 29, 2016
Session ID: 20160629 A LOT 15_ID1331
Luminex: Luminex 100 IS - 2.3
Catalog: RSSO1A_015_03
Locus: A
Test Pos: 4 (D1)
Interpretation
Assigned Allele Code: A*24:XX1 A*31:XX2
Assigned Allele Pairs:

Patient ID:
Sample ID: ALI OZBEK 29
Sample Date:
Name:
Local ID:
Test Date: Jun 29, 2016
Session ID: 20160629 B LOT 18_HD
Luminex: Luminex 100 IS - 2.3
Catalog: RSSO1B_018_03
Locus: B
Test Pos: 4 (D3)
Interpretation
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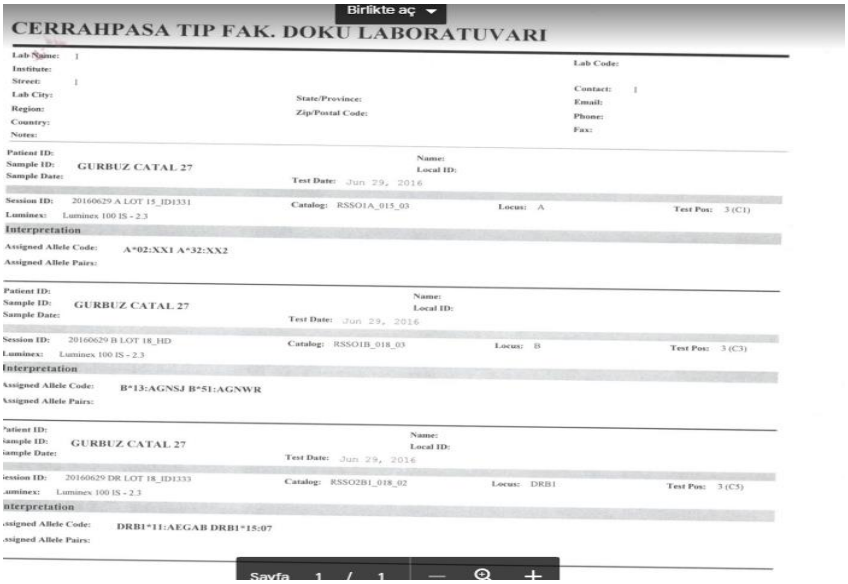
Assigned Allele Pairs:

Patient ID:
Sample ID: ALI OZBEK 29
Sample Date:
Name:
Local ID:
Test Date: Jun 29, 2016
Session ID: 20160629 DR LOT 18_ID1333
Luminex: Luminex 100 IS - 2.3
Catalog: RSSO2B1_018_02
Locus: DRB1
Test Pos: 4 (D5)
Interpretation
Assigned Allele Code: DRB1*12:XX1 DRB1*13:XWAE
Assigned Allele Pairs:


Birlikte aç

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Lab Name: 1
Institute:
Street: 1
Lab City:
Region:
Country:
Notes:
State/Province:
Zip/Postal Code:
Lab Code:
Contact: 1
Email:
Phone:
Fax:

Patient ID:
Sample ID: GURBUZ CATAL 27
Sample Date:
Name:
Local ID:
Test Date: Jun 29, 2016
Session ID: 20160629 A LOT 15_ID1331
Luminex: Luminex 100 IS - 2.3
Catalog: RSSO1A_015_03
Locus: A
Test Pos: 3 (C1)
Interpretation
Assigned Allele Code: A*02:XX1 A*32:XX2
Assigned Allele Pairs:

Patient ID:
Sample ID: GURBUZ CATAL 27
Sample Date:
Name:
Local ID:
Test Date: Jun 29, 2016
Session ID: 20160629 B LOT 18_HD
Luminex: Luminex 100 IS - 2.3
Catalog: RSSO1B_018_03
Locus: B
Test Pos: 3 (C3)
Interpretation
Assigned Allele Code: B*13:AGNSJ B*51:AGNWR
Assigned Allele Pairs:

Patient ID:
Sample ID: GURBUZ CATAL 27
Sample Date:
Name:
Local ID:
Test Date: Jun 29, 2016
Session ID: 20160629 DR LOT 18_ID1333
Luminex: Luminex 100 IS - 2.3
Catalog: RSSO2B1_018_02
Locus: DRB1
Test Pos: 3 (C5)
Interpretation
Assigned Allele Code: DRB1*11:AEGAB DRB1*15:07
Assigned Allele Pairs:

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4. How is the result relevant to the reality? Does that mean HLA type is not associated with *H.pylori*'s outcome of gastric cancer? And the prevalence of gastric caner in Turkey is associated with other factors?

4. Thank again to the Reviewer, we added the following paragraph to the discussion part of the Manuscript as "**We believe that there is an association between HLA allele polymorphisms and gastric pathologies, but this is not a definite reality because of differences between regions and ethnicities and the virulence factors of *H. pylori*. The same HLA alleles sometimes show a positive correlation and sometimes show a negative association in different regions and ethnicities. We suggest that the role of host polymorphisms of the host and *H. pylori* virulence factors in the risk of GC development varies among countries of different regions and different ethnicities**.".

For the prevalence of gastric caner in Turkey is associated with other factors?, we added the following paragraphs to the Discussion part of the Manuscript with a unique (because, unfortunately, there is ony one study in Turkey) reference as "The incidence of GC in Turkey is higher than in Eastern countries and lower than in Western countries at 5.7 and 9.6 cases per 100,000 people for women and men, respectively. The mean age of occurrence is 56 years. It is the second and third leading cause of cancer-related deaths in men and women in Turkey, respectively[25].

The higher incidence in Turkey is mainly associated with dietary factors, and the differences in incidence are especially significant in the central, northeastern, and eastern regions of the country. Salt is commonly used for food preservation, and wood charcoal with dried cow dung is commonly used for cooking in these regions, which are known to have carcinogenic effects on food. Another important factor for the development of GC is H. pylori infections[25].