Author’s response to reviews

Title: STAT3 polymorphism and Helicobacter pylori CagA strains with higher number of EPIYA-C segments independently increase the risk of gastric cancer

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Author’s response to reviews: see over
Ref: MS9091228601628465
We are submitting the modified version of the manuscript: 2052865147035 “STAT3 polymorphism and Helicobacter pylori CagA strains with higher number of EPIYA-C segments independently increase the risk of gastric cancer”. Gifone A Rocha, Andreia MC Rocha, Adriana D Gomes, César LLF Jr, Fabricio F Melo, Sérgio A Batista, Viviane C Fernandes, Nathália BF Almeida, Kádima N Teixeira, Kátia S Brito, Dulciene Maria Magalhães Queiroz.

The manuscript was revised according to your instructions and comments of the reviewers. We are grateful to you and to the reviewers by comments and suggestions we think contributed to improve the manuscript.

You will also find attached the responses to all issues raised by the reviewers and a copy of Ethics Committees approval.

The changes we made in the text were underlined, except the tables to avoid confusion. Best regards,

Dulciene Queiroz, MD

Response to Reviewers' comments

Associate Editor

We thank the Associate Editor for the kind comments

Reviewer 1
We thank the reviewer for his comments
Reviewer's report:
Major Compulsory Revisions:

1. For the background section where the previous studies on rs744166 are discussed, please define the polymorphism in this section; explain to the reader the type of polymorphism that has been found at the locus. Additionally, it might be useful for the reader to include some of the other references that look at the association of this polymorphism specifically in relation to cancer.

   A- We defined the polymorphism and the type of polymorphism found at the locus as you suggested. As we had stated in the “Background” section of the manuscript, to date there is only one study investigating association between STAT3 rs744166 and gastric cancer, which was conducted in China (Yuan et al, Ref 23, line 448 of the manuscript). We have now included further references related to STAT3 rs744166 polymorphism and other cancers (Ryan et al. An analysis of genetic factors related to risk of inflammatory bowel disease and colon cancer. Cancer Epidemiol, 2014; 38:583-90; Jiang et al., STAT3 gene polymorphisms and susceptibility to non-small cell lung cancer, Genet Mole Res 2011:1856-63). Also as stated in the lines 152-156: “The STAT3 single nucleotide polymorphism (SNP) rs744166 was chosen to be investigated because in genome wide studies the G allele was shown to be a protector factor for inflammatory bowel disease, which might be attributed to a higher production of STAT3, well known as protector of intestinal mucosa”.

2. Please clearly state what the data in Figure 1 is relative to. I assume that it is relative to the unstimulated control. However, if this is not the case, please add the control date to the figure.

   A- The data in Figure 1 are relative to the unstimulated PBMCs (control). We added the information to the legend of figure 1. We also included in the figure the results of the AG genotype.

3. Please add a more detailed description of how the calibration was done for the qRT-PCR studies. Please clarify what the remaining calibrators on line 189 refer to. Was a negative control reaction, such as one lacking reverse transcriptase, conducted to ensure that there was no genomic DNA contamination?

   A- Gene expression was normalized to the expression of a reference gene, glyceraldehyde 3-phosphatase dehydrogenase (GADPH). The qRT-PCR was performed with thermal cycling conditions of 60°C for 1 min, 95°C for 10 min, followed by 50 cycles (95°C for 15 sec and 60°C for 1.5 min) and 60°C for 1 min. To ensure that there was no genomic contamination, distilled water was used as a negative control. All reactions were performed in triplicate using cDNA of stimulated and unstimulated (control) fresh PBMC obtained from each healthy subject in five different days. We added this information to the text.

4. Please add a primer table that contains the primers used for genotyping of the STAT3 polymorphisms and the cagA EPIYA segments, as well as the primer and probe used for STAT3 qRT-PCR. Additionally, list any other primers used in the study.
A- We added to “Patients and Methods” section of the manuscript a table (Table 1) containing the primers used to genotype cagA and EPIYA region as well as the conditions of the reactions. We also added to the table the primers used in qRT-PCR to evaluate STAT3 polymorphism and mRNA expression.

5. Given that the majority of this paper involves complex statistical analysis, it is crucial that the methods used for each statistical test are very clear. Please add more specific details for the analyses that were conducted. For example, please include the Hardy-Weinberg analysis that was done as well as the adjustments that were made for age, gender and cagA status. Additionally, it is unclear to the reviewer how cagA status was adjusted for when comparing gastric cancer patients to blood donors (line 234-235). The reason for the confusion is because the authors apparently next assess the association for cagA status and gastric cancer (line 237-239). How can you assess the status of something that you've adjusted for? Is there a specific reason why the Hosmer-Lemeshow goodness-of-fit test was chosen?

A. We added to the text the Hardy-Weinberg analysis. Concerning CagA, you are right. The age and gender were also included in the logistic regression models and the results were also added to the tables. All variables remained independently associated with gastric cancer. We amended the text. In respect to Hosmer-Lemeshow, we used it to assess the fitness of the models overall departure from the observed data. In our experience, the test works well and we have been using it in the publications of our group when logistic regression models are used (see references in the response to the question 8).

6. Please reformat table 1. Since the healthy blood donors are discussed first, organize the table by healthy donors, gastritis patients, and gastric cancer from left to right. In addition to the total number of patients in each category, please provide the breakdown by male and female and provide the complete data that shows the distribution of genotypes across the group and sex. Also, please break the blood donors into two groups, Hp+ and Hp- so that we can see distributions across both derivations.

A- According to your suggestion, we broke the table 1 in three tables showing the distribution of genotypes according to sex in each patient category as well as the distribution of Hp+ and Hp− and CagA-positive and –negative in the blood donors.

7. Since the authors have the histology for gastritis and gastric cancer patients, it would be nice to see these results as figure 2. Examples of difference in inflammation between the various STAT3 polymorphism groups and the different EPIYA types would be interesting.

A- According to your suggestion, we showed the results of histology as figure (Figure 1).

8. Tables 2 and 3 should be Breurec et al. 2011 (Clinical Microbiology and Infectious Disease reformatted. Good examples to follow can be found in Table 2 from) and Table 1 from Panayotopoulou et al. 2010 (J. Clin. Micro.).
Tables 2 and 3 were reformatted as you suggested. We added to the tables the results observed with age and gender. Both variables are associated with gastric cancer.

9. I appreciate the fact that the authors attempted to increase the breath of their study by adding some form of functional assay analysis to the manuscript. However, since samples from only 3 patients within each group were used, it’s clear that this section could benefit from further studies using more samples. Furthermore, it’s not clear to me what is meant by the statement that the data indicate 5 independent measurements. Does this mean that each patient blood sample was independently stimulated 5 different times? If yes, was that blood from a single draw, or multiple draws. More details are clearly needed.

It is important to note that this part of the study was conducted in control healthy subjects and not in patients. In order to evaluate the effect of the STAT3 rs744166 different genotypes on the mRNA expression, we used PBMCs from nine healthy subjects (three AA, three AG and three GG) of the laboratory team who were H. pylori negative, as determined by 13C-urea breath test. Fresh PBMCs were obtained from each healthy subject after 8-hour fast in five different days and were independently assayed. Thus, the results represent five different experiments of each h performed in triplicate. We clarified this issue in the manuscript. As you can see, we added the results of the AG genotype to Figure 1.

10. In the discussion please further elaborate on the differences between the findings of the Chinese study and this study. Is there a difference in the G and A allele frequencies in these populations? Could the predominant EPIYA type in these populations play a role in the differences observed between the studies? This could be an important point for discussion. The authors go into great detail about other studies and roles of STAT3 in disease or protection from disease but do not relate these other studies back to their own findings. Please add more detail about the findings of this study, and a model of how this data is related to the other studies (lines 287-303). Additionally, in lines 155 and 291 the authors state a protective role of STAT3, please add a citation for this statement, and elaborate on how the polymorphism and their data relate to this protective role.

We added the issue you suggested to the “Discussion” section of the manuscript. We also added the reference of the protective role of STAT3 for intestinal mucosa (Hruz et al., Current Opin in Gastroenterol 2015; 16:109-115 – a review study). As we stated in the first paragraph of the discussion, differences in regard to host polymorphism and risk of gastric cancer between Western and Eastern countries have previously been demonstrated, including in genes encoding IL1B-511 and IL1RN2. In respect to STAT3 rs744166 polymorphism, although the authors of the Chinese study did not evaluate whether CagA EPIYA was associated with gastric cancer, it is well known that in China, most of H. pylori strains are CagA-positive and most of them possess EPIYA-D motif, instead of EPIYA-C. EPIYA-D is recognized as the major bacterium risk factor for gastric cancer (Chen et al. J Dig Dis. 2013; 7:358-65) in East Asia population. It is possible that EPIYA-D is more relevant as a risk factor for gastric cancer than STAT3 polymorphism in those populations. According to your suggestion this may justify the differences between the studies. We discussed this point in the paper.
11. The authors use the word “concurrent” on line 306 which suggests that individuals with GG and EPIYA with multiple Cs are at an increased risk for gastric cancer. Please show the breakdown of the number of individuals with AA, AG, and GG and their EPIYA type. This could potentially be done in a table or as part of table 1.

A- The data were added to a new table.

Minor Essential Revisions:

There are numerous concerns with the English usage within the manuscript. I strongly encourage the authors to have the work reviewed by a native English speaker. For example:

1. Line 39: “Using” would be more appropriate than “in” when referring to logistic regression

A- We amended the text

2. Line 51: please clarify that “both” refers to the two different stimuli used. It is unclear what is meant unless you have already read the entire paper.

A- You are right. We re-wrote the sentence

3. Line 63: Gastric cancer is now the third leading cause, please see Ferlay et. al GLOBOCAN 2012 v 1.0, Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 11, International Agency for Research on Cancer, 2013.

A- We amended the text and added the IARC reference.

4. Line 110: Starting with “To the best of our knowledge” would read more smoothly.

A- We smoothed the statement.

5. Lines 114 and 204: It would be more accurate to use the term “logistic regression model” then just “logistic model.”

A- We corrected the term in line 114 and 204 and used the terms “logistic regression model” throughout the paper.

6. Line 114: please define what the major point is

A- "Major point" means that, to define the variables that are independently associated with gastric cancer without bias, the data has to be analyzed in regression models as is the case of this study, in which we used a binary logistic regression. We re-wrote the sentence

7. Line 119 and 310: The authors refer to increased “secretion” of mRNA, secretion seems inappropriate and perhaps the authors mean expression?
A- We amended the text.

8. Line 126: “from” would be a better word choice than “among”

A- We amended the sentence.

9. Line 184+185: Manufacturer and details are needed for the spectrophotometer

A- The DNA concentration was determined by spectrophotometry using NanoDrop 2000 (Thermo Scientific, Wilmington, NC). We added the information to the text.

10. Line 220: please add a heading such as “demographic data and rs744166 STAT3 polymorphism” for the first section. In this section please add lines 152-156 from the STAT3 polymorphisms method section, define the minor allele, and SNPs and include line 228 with the Hardy-Weinberg statistics.

A- We added “Demographic data and rs744166 STAT3 polymorphism” as a heading to the first part of the results section. We also included the lines 152-156 from the STAT3 polymorphism method section.

11. Lines 249-252 may fit better above line 240, since they focus on cagA presence in the healthy donors, and section 240 moves into EPIYA type.

A- We agree with you and modified the text.

12. Line 94. CagA stands for “cytotoxin-associated gene A” not the designation that is currently listed.

A- In the line 94 we are referring to the protein CagA (cytotoxin-associated antigen) and not to the cagA gene (cytotoxin-associated gene A). We re-wrote the sentence.

13. Line 126 Mention how Hp positivity was determined for healthy controls.

A- We added to the “Patients and Methods” section of the manuscript a more detailed description of the serology used. Briefly in the control group *H. pylori* status was investigated by using a commercial ELISA kit (Cobas Core anti-*H. pylori*, EIA Roche, Basel, Switzerland), which was previously validated for the Brazilian population being 95.4% sensitive and 100% specific [ref 32 of the first version of the paper, and 38 in the new one]. CagA status was investigated by serology using commercial ELISA kit (ELISA kit Helicobacter pylori p120 CagA; Viva Diagnostika, Hürth, Germany), which was also previously validated for the Brazilian population being 97.4% sensitive and 88.9% specific [ref. 33 of the first version of the paper and 39 in the new one]. We added the information to the text.

14. Also define how CagA positivity was determined since it is mentioned in Table 1.
**A-** In the patients, cagA status was determined by PCR using two different set of primers previously described (Peek et al. Clin Microbial 1995,33:28-32. Kelly et al. Gastroenterology 1994,107:1671-1674). The H. pylori strains were considered to be cagA-positive when at least one of the two reactions was positive. In the blood donors CagA status was determined by serology. The information was added to the “Patients and Methods” section of the paper.

15. Additionally, there is no real mention of how the CagA EPIYA typing was performed.

**A-** For the PCR amplification of the 3’ variable region of the cagA gene (that contains the EPIYA sequences), 20 to 100 ng of DNA were added to 1% Taq DNA polymerase buffer solution (KCl 50 mM and Tris-HCl 10 mM), 1.5 mM MgCl2, 100 µM of each oxynucleotide, 1.0 U Platinum Taq DNA polymerase (Invitrogen, São Paulo, Brazil), and 10 pmol of each primer, for a total solution volume of 20 µL. The primers used were previously described by Yamaoka et al. (Yamaoka et al. J Clin Microbiol 1998,36:2258-63) and are listed in table 1. The amplified products were electrophoresed in 1.5% agarose gel that was stained with ethidium bromide, and analyzed in an ultraviolet light transilluminator. The reaction yielded products of 500 to 850 bp according to the number of EPIYA C. This methodology also allows the detection of mixed infection. To confirm the results, the 3’ variable region of the cagA gene was sequenced. Briefly PCR products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, MI) according to the manufacturer’s recommendations. Purified products were sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit in an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequences obtained were aligned using the CAP3 Sequence Assembly Program (available from: http://pbil.univ-lyon1.fr/cap3.php) and compared to sequences deposited into the GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). We added the information to the “Patients and Methods” section of the manuscript.

16. It might be beneficial to discuss the limitation of using serology to determine H. pylori positivity in the healthy controls and culture for the ill patients. Might this affect the results? Is treatment for H. pylori common in this region?

**A-** As answered in the comments 14 and 15, serology was used to determine H. pylori status and CagA in control group. The two commercial tests were previously validated for the Brazilian population showing high accuracy. Because H. pylori infection is predominantly acquired in early childhood and the infection persists lifelong unless treated, a positive specific IgG anti-H. pylori test in adults means current infection. In Brazil, patients receive antimicrobials to eradicate H. pylori in cases of duodenal ulcer, MALT lymphoma, gastric cancer or when the subject with a H. pylori-positive result and a familiar history of gastric cancer is detected. Ethics reason impaired us to use invasive methods to diagnosis H. pylori infection in blood donors. Additionally, as demonstrated previously by our group, none diagnosis method per se has good accuracy for the diagnosis of H. pylori infection in patients with gastric cancer (Queiroz et al. J Med Microbiol. 1999 48:501-6). We believe that the different methods used in the present study did not interfere with the consistency of our results because of their high accuracy.
17. Line 164. Clarify that the 6 patients were divided into two groups, each of which contained 3 individuals.

**A** - Please, see the response to the question 9 (Major Compulsory Revisions).

Discretionary Revisions:

1. It would be interesting to see analysis of the distribution of AA and GG genotypes within the EPIYA groups for both patients with gastritis and gastric cancer. Since ABC is the most common EPIYA type, is there a difference in AA v. GG between patients with gastric cancer and gastritis? Is the breakdown the same when you look at ABCC and ABCCC?

**A** – We put the data in another table and the results in the text.

2. I assume the authors are thinking about the next steps. For instance, given that the authors are looking at an association with gastric disease and STAT3, it would be nice to see STAT3 expression data from the gastric tumours or gastric biopsies. The authors could stain for STAT3 in tissue sections or look at STAT3 RNA expression in the tissue. Since the authors know the EPIYA type of the infecting H. pylori strain and the STAT3 SNP, it would be interesting to see the results from different combinations, such as a GG polymorphism and EPIYA ABCCC. Furthermore, to show a functional difference in of the STAT3 polymorphism, looking at a downstream gene which STAT3 acts upon in the PBMCs would also ass support to this line of study. Level of interest: An article of importance in its field

**A** - As we suggested we evaluated the protein expression of STAT3 on gastric mucosa. As it is well known that the tissue expression of STAT3 varies according to the stage of the tumor and presence of metastasis (Kim et al. J Gastroenterol Hepatol. 2009 24:646-51; Woo et al. Pathobiology. 2011, 78:295-301), the STAT3 cell number was assessed in formalin-fixed paraffin-embedded sections of the antral and corpus mucosa of gastritis patients harbouring rs744166 AA, AG and GG genotypes, who were randomly selected, by conventional immunohistochemistry. We found that STAT3 expression was significantly higher in the gastric mucosa of patients carrying the GG genotype than in those carrying AG or AA. These results are in accordance the results found in qRT-PCR. We added the results to the manuscript.

**Statistical review:** Yes, and I have assessed the statistics in my report.

**Declaration of competing interests:**

I declare that I have no competing interests crucial that the methods used for each statistical test are very clear.
Response to Reviewers' comments

Associate Editor

We thank the Associate Editor for the kind comments

Reviewer 2

We thank the reviewer for her comments

Reviewer's report:

This study analysed the relationship between STAT3 polymorphism and H. pylori CagA status and the risk of gastric cancer, it has some interests in its field.

Major Compulsory Revisions

1. In this study, all the patients were selected by H. pylori-positive but the voluntary healthy blood donors were not, so the cases and controls were not comparable. The results in Table 2 are not reliable, especially in CagA-positive status.

A- Serology was used to determine H. pylori status and CagA in control group. The two commercial tests were previously validated for the Brazilian population showing high accuracy. Because H. pylori infection is predominantly acquired in early childhood and the infection persists lifelong unless treated, a positive specific IgG anti-H. pylori test in adults means current infection. Ethics reason impaired us to use invasive methods to diagnosis H. pylori infection in blood donors. Additionally, as demonstrated previously by our group, none diagnosis method per se has good accuracy for the diagnosis of H. pylori infection in patients with gastric cancer (Queiroz et al. J Med Microbiol. 1999 48:501-6). Gastric mucosal atrophy that precedes and is present in patients with gastric cancer decreases the bacterial load and then the sensitivity of all tests used to diagnosis H. pylori infection. We believe that the different methods used in the present study did not interfere with the consistency of our results because of their high accuracy. We have been publishing results of similar studies in several Journals, including: (Rocha et al., Haematologica. 2011 96:1560-4), Microbes and Infection (Queiroz et al., Microbes Infect. 2009 12:980-7), Pediatric Research (Queiroz et al. Pediatr Res. 2005;58:892-6); International Journal of Cancer (Rocha et al. Int J Cancer. 2005 Jul 10;115:678-8).

In respect to the results of the table 2, the results refer to the logistic regression model, as we have been using in many of our previous studies. Please, see in references cited above.

2. For the association between rs744166 polymorphism and in vitro expression of STAT3, the PBMCs were only extracted from subjects who carried rs744166 GG and AA genotype. However, the G allele should include both GG and GA.

A- In respect to association between rs744166 polymorphism and in vitro expression of STAT3, In order to evaluate the effect of the STAT3 rs744166 different genotypes on the
mRNA expression, we used PBMCs from nine healthy subjects (three AA, three AG and three GG) of the laboratory team who were \textit{H. pylori} negative, as determined by $^{13}$C-urea breath test. Fresh PBMCs were obtained from each healthy subject after 8-hour fast in five different days and were independently assayed. All assays were performed in triplicate, including the qRT-PCR. We clarified this issue in the manuscript. As you can observe, we added the results of the GA genotype to Figure 1 as you suggested.

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests:

I declare that I have no competing interests.
Parecer nº: ETIC 018/00

DECISÃO:


Prof. Dr. Dirceu Bartolomeu Greco
Presidente do COEP

Av. Alfredo Balena, 110 – 1º andar
Bairro Santa Efigênia – Cep 30.130-100 – Belo Horizonte-MG
Telefone: (031) 248 9364
FAX: (031) 248-9380 – Telex: (031) 2544
Título do Projeto de Pesquisa: Resposta imune à infecção pelo *Helicobacter pylori* em crianças: um estudo comparativo com adultos.

**SUJEITOS DA PESQUISA**

2. Número de sujeitos
   - No Centro: 300
   - Total: 700

3. Grupos Especiais:
   - Menor de 18 anos (x)
   - Portador de deficiência mental (x)
   - Embrião/feito (x)
   - Relação de dependência (militares, presidiários...) (x)
   - Outros (x)
   - Não se aplica (x)

**PESQUISADOR RESPONSÁVEL**

4. Nome: Dulcine Maria de Magalhães Queiroz

5. Instituição a que pertence: Faculdade de Medicina, UFMG

**INSTITUIÇÃO (ÓE) ONDE SERÁ REALIZADO**

6. Nome: Faculdade de Medicina e Hospital das Clínicas da UFMG

7. Unidade/Órgão:

8. Participação Estrangeira: Sim (x)
   - Não (x)

9. Projeto Multiúncenario: Sim (x)
   - Não (x)
   - Nacional (x)
   - Internacional (x)

**PATROCINADOR**

10. Nome:  

11. Data de Entrada: 21/10/02  
   12. Registro no CEP: 096  
   13. Data de análise: 06/12/02

14. Objetivos: Estudar o papel do hospedeiro na gênese e progressão das diferentes doenças associadas à infecção pelo *Helicobacter pylori*, através do estudo comparativo da resposta imune celular e humoral de adultos e crianças positivos e negativos para *H. pylori*, com e sem úlcera duodenal, e da investigação do polimorfismo e/ou microsatélite na região promotora dos genes que codificam as interleucinas IL-1β, IL-2, IL-4, IL-10, TNF-α e IFN-γ em pacientes com úlcera duodenal, carcinoma gástrico e controles.

15. Sumário do Projeto:

Os sujeitos envolvidos na pesquisa são de 3 diferentes grupos:
- 115 crianças com queixas dispêpticas atendidas no Setor de Endoscopia Digestiva do Hospital das Clínicas (UFMG), sendo 50 *H. pylori*-negativas, 50 *H. pylori*-positivas sem úlcera péptica e, pelo menos 15 com úlcera péptica.
- 300 adultos com queixas dispêpticas atendidas no mesmo setor, sendo 100 *H. pylori*-negativos e 200 *H. pylori*-positivos com gastrite, úlcera péptica e carcinoma gástrico.
- 300 doadores de sangue da Fundação Hemominas, como grupo controle.

Dos primeiros 2 grupos, será feita a colheita de fragmentos de mucosa gástrica para cultura, teste da urease, estudo histopatológico e dosagem de citocinas. Também serão colhidos, incluindo do grupo controle, 5 ml de sangue para pesquisa de anticorpos anti-*H. pylori* e análise de polimorfismo das regiões promotoras das interleucinas em estudo.

16. Comentário dos Relatores:

Sugerimos que em caso de resultado positivo para *H. pylori* no grupo controle, o sujeito seja convocado por carta e que os esclarecimentos devidos lhe sejam prestados pessoalmente. Salientamos a necessidade de um local que garanta a privacidade do sujeito durante a entrevista. Estas propostas foram discutidas e aceitas pela pesquisadora responsável.

O projeto encontra-se adequado às normas da Resolução 196/96, e foi aprovado por este Comitê.

17. Parecer:

   Aprovado (x)  
   Pendência ( )  
   Não Aprovado ( )

Data: 05/05/03  

18. Encaminho a CONEP:

   Os dados acima para registro ( )
   O projeto para apreciação ( )

Data:  

19. Coordenador

   [Assinatura]
   [Nome]
   Coordenadora Comitê de Ética em Pesquisa
   Fundação Hemominas