Author's response to reviews

Title: Genetic susceptibility to Chagas disease cardiomyopathy: involvement of several genes of the innate immunity and chemokine-dependent migration pathways.

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Author's response to reviews: see over
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To BMC infectious diseases

Dear Editor,
First of all we thank the academic editor and the reviewers for their helpful and constructive comments and suggestions which markedly improved the manuscript.
Everybody were so enthusiastic about our manuscript
Please find enclosed a revised version of our manuscript entitled “Genetic susceptibility to Chagas disease cardiomyopathy: involvement of several genes of the innate immunity and chemokine-dependent migration pathways”.

We also provide a point by point reply to all the comments.
Thank you for your consideration.
Sincerely,

Chevillard Christophe
Dr. Werner Apt’s report
1. This is a good and important investigation in Chagas disease. Authors: Thanks to the reviewer for his constructive comment.

2. In the discussion the authors could mention other relevant works related to HLA antigens in Chagas cardiomiopathy Vg: Llope E, Rothhammer F, Acuña M, Apt W. 1988. HLA antigens in cardiomyopathic chilean chagasics. Am J Hum Genet 43:770-773 and from the same authors in 1991 in Rev Med Chile 119:663-636. These are pioneering works in relation to the susceptibility of human hosts to T.cruzi, considering HLA antigens and some genes. The above should considered as a discretionary revision. Authors: Thanks to the reviewer for his constructive comment. A list of genes that were previously associated to an increase risk of cardiomyopathy was added in the introduction with all the references. “Familial aggregation of CCC has been described, suggesting that there might be a genetic component to disease susceptibility [24]. Several genes were associated to an increased risk to develop cardiomyopathy (HLA, MHC, TNF, IL1A, IL1B, IL1RN, IL10, IL12B, TIRAP, CCL2, BAT1, LTA, IKBL, CCR5, MIF, IFNG, CXCL9, CXCL10) [25-50]. So far, up to 30 case control studies were done so far (see for review [51-53]). These studies often led to inconclusive results that may be explained in different ways: a) the use of seronegative subjects as controls which are inadequate controls, since it is unknown whether they were exposed to the pathogen; b) the relatively small size of the study groups which affected the power (the probability) to detect an association; c) the number of tested SNPs; d) the highly heterogeneous genetic background of the study population due to admixture; e) the sex ratio known to exist has not been taken in consideration [54].”

3. Minor correction: the last sentence of study population for polymorphism analysis, of 315 patients with CCC, 106..........................could be eliminated because it is a repetition. (The same is mentioned in the previous paragraph).
Authors: This sentence was removed.

Dr. Ines Zulantay’s report
1. This is a good and important investigation in Chagas disease. This investigation provides relevant original information on the role of the genetic in susceptibility to CCC development. With urgency, biological markers or tools of CCC with prognostic value are need. Thus, the study contributes to understand the Chagas disease in humans and project the application of these results. The design of study, methods and analysis of results are clear and understandable, considering that I am no expert to genetics. Authors: Thanks to the reviewer for his constructive comment.

2. Discretionary revisions I suggest define, for better understanding of manuscript the concepts: -Protection CCC -Susceptibility incremented I think it would be interesting added to introduction or discussion: - What others markers no genetic have been evaluated in the CCC development?
Authors: A new section on immunological markers in CCC development was added at the Introduction. “As a result of persistent infection, both CCC and ASY chronic Chagas disease patients show a skewed Th1-type immune response [11, 12], but those who develop Chagas cardiomyopathy display a particularly strong Th1-type immune response with increased numbers of IFN-γ-producing T cells in peripheral blood mononuclear cells (PBMC) [13] as well as plasma TNF-α in comparison with uninfected or ASY patients [14]. PBMC of CCC patients also display increased levels of IFN-γ- or TNF-α producing CCR5/CXCR3+ CD4+ T
cells [15, 16]. In addition, CCC patients display a reduced number of CD4^+CD25^{high}IL-10^+ and CD4^+CD25^{high}FoxP3^+ regulatory T cells in their peripheral blood as compared to patients in the ASY form of Chagas disease, suggesting such cells may play a role in the control of the intensity of inflammation in chronic Chagas disease [15, 17]. Furthermore, PBMC from CCC patients displayed increased numbers of CD4^+CD25^{high}FoxP3^{CTLA-4^+} T cells, and decreased numbers of as compared to ASY patients. These reports suggest that a smaller CD4^+FoxP3^+/CD25^{high}Treg compartment with deficient suppressive activity exists in CCC patients, leading to uncontrolled production of Th1 cytokines [18]. Circulating CD4^+IL-17^+ T cells appear in low frequency in PBMC from CCC patients as compared with ASY patients and non-infected individuals [18, 19]. On the whole, these results suggest that proinflammatory cells and cytokines are markers associated with progression to CCC, whereas the production of IL-10, IL-17 and increased numbers of regulatory T cells are markers of protection from CCC development, indicating that failure to regulate Th1 responses may be the underlying immune defect of patients who progress to CCC.”

3. Level of interest: An article of outstanding merit and interest in its field.

Authors: Thanks to the reviewer for his constructive comment.

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Dr. Daniel Hoft’s report

1. This is an interesting manuscript presenting data in support of the hypothesis that there is a genetic component to Chagas disease progression. The authors focus on three immune genes (CCR5, CCL2, TIRAP) and find an association between polymorphisms in these genes and disease susceptibility or protection. While this study focuses on previously identified genes, the strengths of this report include: 1) it is the largest study done to date, 2) the authors use asymptomatic seropositive individuals instead of seronegative ones as controls, and 3) the Tag SNP approach is used to expand the areas of the genes studied. Through these techniques, they were able to identify new polymorphisms in the CCR5, CCL2, and TIRAP genes. This could potentially have prognostic or therapeutic value in the future and therefore should be published.

Authors: Thanks to the reviewer for his constructive comment. These SNPs may be used in the future as biomarker for prognostic studies in association with others biomarkers.

2. However, there are limitations to this study that are not clearly mentioned in the manuscript. First, this is a cross-sectional study which compares T. cruzi-infected individuals with no disease symptoms (ASY) to those with either moderate or severe chronic Chagas cardiomyopathy (CCC). With no knowledge of when these individuals were infected, we do not know whether or not the ASY patients will develop disease manifestations in the future or whether the CCC group has simply been infected for a longer period of time. A longitudinal study would be more ideal; however this would be very difficult, especially with this large and mostly rural population.

Authors: Thanks to the reviewer for this critical comment. A sentence was added in the study population section. “Regarding progression of the ASY cases to CCC, the yearly progression rate –regardless of age group- is ca. 1-2%/year. The average age of Subjects with asymptomatic form was above 55 years. Taking into account that they were all born in endemic areas before vector transmission was interrupted, it is likely that in most if not all cases vector-borne infection occurred in early childhood. The odds that a significant number of such mature patients convert to CCC, and that this thwarts our statistical calculation is...
rather low; however, this is a pitfall of all cross sectional studies on diseases that display progression.”

3. Second, this report involves a “test” population, and the results require validation in additional populations.

Authors: Thanks to the reviewer for this critical comment. In this study we confirmed the involvement of three genes that were already reported to be associated to susceptibility to chronic Chagas disease. It is true that the SNPs were different than those previously identified, but we believe this study stands as a “candidate gene confirmation study. These associated markers are in linkage disequilibrium with the previous associated markers. These SNPs may be considered as biomarkers. However, so far several additional studies will be necessary to be sure to have identified the functional variants.

4. Third, the study identified polymorphisms but does not correlate these polymorphisms with levels of gene expression. Without knowing how the SNP affects expression or function, it is hard to deduce the importance of each SNP.

Authors: Thanks to the reviewer for this critical comment. Indeed, we identified some genetic markers associated to chronic disease development. These associated markers are in linkage disequilibrium with the previous associated markers. These SNPs may be considered as biomarkers. However, so far several additional studies will be necessary to be sure to have identified the functional variants. A sentence was added in the conclusion section. “Our data show beyond reasonable doubt that polymorphisms affecting key molecules involved in several immune parameters (innate immunity signal transduction and T cell/monocyte migration to inflammatory regions) play a role in genetic susceptibility to CCC development. However, the functional impact of these markers remains unknown. This also points out to the multigenic character of CCC, each polymorphism imparting a small contribution. The confirmation of more genetic markers of susceptibility to CCC will provide information for pathogenesis as well as therapeutic targets. The identification of these marker sets may also have a combined prognostic value for disease progression at the individual patient level, allowing close follow up and early treatment of those carrying high-risk genetic signatures”

1. Overall, this study yields exciting results by analyzing polymorphisms from a large number of CCC patients, but it is important for the authors to recognize and clearly state the limitations. In the background section, a few statements are overstated. For example, the authors say that Chagas disease occurs exclusively in the Americas, but I think they meant to say that it is endemic to Latin America and vector-based transmission happens exclusively in the Americas.

Authors: Thanks to the reviewer for this critical comment. A sentence was added in the introduction. “The disease remains endemic in Latin America where the vector-based transmission is still active in some countries.”

Another example is stating that CCC is “by far the most important clinical consequence” since this is more of an opinion than a fact. Perhaps revise this to the most common consequence or one of the most important consequences?

Authors: Thanks to the reviewer for this critical comment. Sentence has been changed. “Decades after acute infection, approximately 30% of infected individuals develop Chronic Chagas cardiomyopathy (CCC), one of the most important consequence of T. cruzi infection.”
2. Also in the background section, the authors mention previous data with CXCL9 and then jump to a statement that this is consistent with an accumulation of CCR5+ Th1 T cells in the CCC heart tissue. Perhaps they could have another sentence directly linking CXCL9 to their decision to study CCR5?

Authors: The text in the manuscript indicates that CCR5 chemokine ligands (CCL3, CCL4, CCL5) are also upregulated; we now added the sentence “Importantly, median expression of CCL5, a CCR5 ligand, was the highest among all chemokines tested (166-fold increase over control).” This is data from the cited reference and is the underlying reason for mentioning CCR5+ as well as CXCR3+ T cell migration to the heart.

3. In the methods section, they mention that data for LVEF were missing for 10 patients with CCC, however fail to mention whether or not they used data from these 10 individuals for any of the analysis completed. If so, the authors should state their group assignments (moderate to severe CCC).

Authors: Thanks to the reviewer for this critical comment. A sentence was added in the study population section. “Data for left ventricular ejection fraction were missing for 10 patients with CCC. So, when we compared moderate patients to severe patients, these 10 individuals were excluded from the analysis.”

4. In the methods section, it would be nice if the authors could clarify the SNP selection process in more detail. Were the 15 tag SNPs in Table 1 the only 15 for those 3 genes with a minor allele frequency over 20%? And what is the rationale for using the 20% MAF cutoff?

Authors: Thanks to the reviewer for this comment. The SNP selection was not clear enough. Section was improved as follow: “Tag single nucleotide polymorphisms (SNPs) were selected on the basis of HapMap Data for the Caucasian and Yoruba reference populations. Tag SNPs were selected within a region extending 5 kb on either side of the candidate gene. The minor allele frequency (MAF) cut off value was arbitrarily set at 20% (so the markers characterized by a MAF<20% were excluded from the analysis by lack of power). In each reference population, the markers with MAF>20% are included in different blocks of correlation (based on the r² values). One marker in each block was selected and considered as a Tag SNPs. Indeed, markers located in the same block of correlation gave the same genetic information in association studies. Tag SNPs characterised by a MAF over 20% on at least one reference population were selected. So for this cut off value (20%) there is only 15 Tag SNPs. These Tag SNPs were defined to catch all the genetic information from the candidate gene. We selected three tag SNPs for CCR5, six tag SNPs for CCL2 and six tag SNPs for MAL/TIRAP genes. Taking into account a disease with a prevalence of 30%, a cutoff for significant association of 0.05, for a genotype relative risk of 1.3, the probability to detect a real association reaches 63% with 315 chronic cases and 118 ASY controls. We decided to use a cut off of 20% instead of 10% or 15%. For lower cut off, the number of Tag SNPs will increase and it will request a seriously large your study population to have a good statistical power.

5. In the results section, for the CCR5 rs11575815 A/T polymorphism, the authors combine individuals expressing the more frequent homozygous genotype (AA) and those with the heterozygous genotype (AT) for comparison with the rare homozygous genotype (TT). However, in all the other examples, they compare the more frequent homozygous expressing group to combinations of the heterozygous and rare homozygous genotypes. Is there a reason
why the analysis was done differently for the CCR5 rs1157815 SNP? If so, the rationale should be explained?

Authors: Thanks to the reviewer for this comment. The way, we performed association study, was not clear enough.

Section was improved as follow: “SPSS Statistics software v. 17.0 (IBM, Armonk, USA) was used for statistical analyses. We performed stepwise binary logistic regression analysis on the whole population, to analyse the relationship between the probability of an individual to develop chronic Chagas cardiomyopathy and the main covariates (sex and polymorphisms). Sex was considered as a binary covariate. In our stepwise binary logistic regression analysis, genotypes were considered as binary covariates. Indeed, for each polymorphism we had two alleles (A frequent one; a rare one). So, we obtained three genotypes (AA, Aa and aa). In our stepwise binary logistic regression analysis, genotypes were considered as binary covariates. So, we performed three different analyses (Analysis 1: AA vs Aa+aa (we supposed that the a allele is dominant); Analysis 2: AA+aa vs Aa (we supposed that the heterozygote carriers are different from the homozygote ones); Analysis 3: AA+Aa vs aa (we supposed that the A allele is dominant)). The best results are indicated in Tables 4, 5 and 8.

In multivariate analyses, several polymorphisms and gender were included as covariates. All the covariates are analyzed in the same time. In a stepwise approach, the worse associated covariate (non significant) is removed and the analysis is run again up to keep only significant associated covariates.”

For example, for the rs11575815 polymorphism, we compare ASY subjects to CCC patients. If we merged subjects carried TT genotype to subjects carried AA or AT genotypes the statistical test will give p=0.030; OR=1.41; 95%CI: 1.03-1.92. If we merged subjects carried AA genotype to subjects carried AT or TT genotypes the statistical test will give p=0.265; OR=1.13; 95%CI: 0.91-1.41. If we compared the heterozygous subjects to the homozygous subjects the statistical test will give p=0.748; OR=1.038; 95%CI: 0.83-1.30. So the best result was obtained in the first test suggesting that the Allele may be dominant.

6. In the results section text, the authors present results of the multivariate analyses for all the studied genes. However, all tables included with the manuscript present only univariate analyses. Inclusion of a table with an overview of the multivariate analyses would be helpful.

Authors: Thanks to the reviewer for this comment. An additional table that summarizes the Multivariate stepwise binary logistic regression analyses was added to the revised draft (new table 6).

7. In the discussion section, there should be more information on how the disease associated SNPs might affect gene expression and then how the altered gene expression patterns could influence disease progression. There is some mention of previous studies that looked at gene expression with different SNPs in these three genes, but it is not clear what the authors’ predictions are for these particular SNPs.

Authors: Thanks to the reviewer for this comment. For CCR5 gene, the main polymorphism rs3176763C/A is located in the promoter region of the gene and may affect the binding of transcription factors. The CCL2rs2530797A/G and TIRAPrs8177376A/C polymorphisms are located into the 3’ region of the gene and may affect stability of the transcript or the binding of regulatory elements. Sentences were added to the revised manuscript “Among these susceptibility studies, putative implication of genes crucially involved in the innate immunity—such as the Toll like receptors (TLR) and some of its most relevant signalling molecules like TIRAP was searched for. Two studies on the TLR and TIRAP failed to identify disease associations with TLR 1,2, 5, 6 and 9; in one of the reports an association was found with a
TLR4 SNP among Chilean chagasic patients [55], while in the second study – which enrolled nearly double the number of Brazilian Chagasic individuals - no association was found with TLR4, but instead with TIRAP S180L heterozygosity [41]. Chemokines are key players in controlling migration of specific cell types bearing their receptors to sites of tissue inflammation, and associations between CCR5 –involved in T cell and macrophage migration and CCL2 –involved in monocyte migration - with CCC were reported [42, 47, 48]. Both processes, TLR signaling and chemokine-mediated cell migration are of paramount importance in Chagas disease and are key to the pathogenesis of CCC."

8. In the discussion section, they mention a few previously identified SNPs in the CCR5, CCL2, and TIRAP genes. Could the authors speculate on why they did not identify these same SNPs? Were these polymorphisms present but at minor allele frequencies lower than 20% and thus excluded?

**Authors:** Thanks to the reviewer for this comment. The previous associated markers were not tested because they were not defined as Tag SNPs. Moreover in the previous studies they didn’t test the same polymorphisms than us. However, as we mentioned, each time the associated markers are in linkage disequilibrium with the previous marker. So, we can speculate that these two markers are carrying the same genetic information but we do not know yet which one is the functional polymorphism.

9. In the conclusion, the authors comment on the multigenic character of CCC, with “each polymorphism imparting a small contribution.” Have they analyzed whether or not the expression of multiple SNPs associated with CCC patients correlates with increased disease severity?

**Authors:** Thanks to the reviewer for his constructive comment. Several genes have been associated to an increase risk to develop chronic cardiomyopathy. We may raise the hypothesis that some interactions between these genes (so between polymorphisms). In order to confirm this hypothesis we performed a multivariate analysis on ASY subjects and CCC patients. In this analysis, we input as covariates the gender, the main CCR5 polymorphism, the main CCL2 polymorphism and the two TIRAP polymorphisms. The gender and one polymorphism in CCR5 and TIRAP genes remain associated.

“In order to detect interaction between the candidate genes a multivariate stepwise binary logistic regression analysis was performed on ASY subjects and CCC patients (see table 9). In this analysis, we included the gender, rs11575815A/T, rs2530797A/G, rs8177376A/C and rs17866704T/C as covariates. Polymorphisms CCR5rs3176763C/A (p=0.007; OR=1.879; 95%CI: 1.19-1.89), TIRAP rs8177376A/C (p=0.007; OR=1.393; 95%CI: 1.09-1.77) and the gender (p=0.001; OR=2.226; 95%CI: 1.39-3.55) were still significantly associated to CCC (see table 9). However, if we want to add a significant number of genes and polymorphisms at the first step of the multivariate analysis, the study population (which is one of the largest described so far) is underpowered. So, we are working toward obtaining a cohort between 1,500 and 2,000 subjects that would enable us to assess whether possessing a given combination of alleles in several SNPs contribute more strongly for prognosis than the individual SNPs.”

10. In the conclusion, it also would be informative if the authors could speculate on exactly how to proceed with these data and what are the next steps for translating this information into prognostic or therapeutic value.

**Authors:** Thanks to the reviewer for his constructive comment. The conclusion section has been improved.
“Our data show beyond reasonable doubt that polymorphisms affecting key molecules involved in several immune parameters (innate immunity signal transduction and T cell/monocyte migration to inflammatory regions) play a role in genetic susceptibility to CCC development. However, the functional impact of these markers remains unknown. This also points out to the multigenic character of CCC, each polymorphism imparting a small contribution.

When all the genetic markers will be identified, we will be able to performed multivariate analyses using several genes (gene polymorphisms) as covariates. In order to perform this kind of analysis it is essential to enroll a study population including at least 1,500 and 2,000 cases and 1000 ASY controls. It will allow us to detect gene–gene interactions and additive or antagonist effects between the associated polymorphisms. A panel of markers will be defined to early detect individuals with a highest risk to develop chronic cardiomyopathy. It will provide information for pathogenesis as well as therapeutic targets. The identification of these marker sets may also have a combined prognostic value for disease progression at the individual patient level, allowing close follow up and early treatment of those carrying high-risk genetic signatures.”

11. Level of interest: An article of importance in its field.

Authors: Thanks to the reviewer for his constructive comment.

**Editor’s Comment**

1. In the background section, please provide a rationale for including TIRAP SNPs (and not the more primary TLRs) into the typing strategy, and quote the respective literature, i.e. there are at least two previous papers on TIRAP SNPs in Chagas disease.

Authors: Thanks to the editor for his constructive comment. A complete section was added to introduction. “Among these susceptibility studies, putative implication of genes crucially involved in the innate immunity—such as the Toll like receptors (TLR) and some of its most relevant signalling molecules like TIRAP was searched for. Two studies on the TLR and TIRAP failed to identify disease associations with TLR 1, 2, 5, 6 and 9; in one of the reports an association was found with a TLR4 SNP among Chilean chagasic patients [55], while in the second study – which enrolled nearly double the number of Brazilian Chagasic individuals - no association was found with TLR4, but instead with TIRAP S180L heterozygosity [41]. Chemokines are key players in controlling migration of specific cell types bearing their receptors to sites of tissue inflammation, and associations between CCR5 –involved in T cell and macrophage migration and CCL2 –involved in monocyte migration - with CCC were reported [42, 47, 48]. Both processes, TLR signaling and chemokine-mediated cell migration are of paramount importance in Chagas disease and are key to the pathogenesis of CCC. Here, we conducted a study focusing on TIRAP, CCL2 and CCL5. Thorough genetic analysis, testing multiple tag SNPs per gene and thus detecting any possible relevant genetic variants in a large Brazilian population and ASY subjects as controls we could have a sensitive assessment of the contribution of genetic variants in prognosis to CCC either confirming or finding additional associated SNPs in the mentioned genes. This can be considered a candidate gene replication study, performed with a larger cohort of Chagas patients and only comparing CCC to the asymptomatic seropositive (ASY) patient group. Significant associations were found for CCR5, CCL2, and TIRAP genes.”

2. Please provide a rationale for excluding patients with digestive manifestations and state their number.
Authors: Thanks to the editor for his constructive comment. Here, we were focusing on cardiac manifestations of the disease. Patients with the symptomatic digestive form have a different course of the disease. Mixing two clinical forms may introduce a bias in our study. So we decided to exclude patients with the digestive form.

3. I doubt that linear regression is the most suitable way of analysing the data. As a reader I would rather like to see the various genotype (!) proportions among ASY and CCC. By arbitrarily grouping genotypes into a binary variable, information is lost. Such an illustration of genotype proportions (in a table) should be supplemented by the crude odds ratio, and by the adjusted odds ratio from multivariate logistic regression including age and the other associated SNPs of all (!) examined genes (i.e., after stepwise removal). So far, I find the statistical analysis not convincing and its description (?univariate analysis including gender as covariate?) misleading and overly long.

Authors: Thanks to the Editor for this comment. Indeed, we made a mistake we talk about linear regression instead of binary regression. Sentence has been changed. Statistical section was improved as follow: “SPSS Statistics software v. 17.0 (IBM, Armonk, USA) was used for statistical analyses. We performed stepwise binary logistic regression analysis on the whole population, to analyse the relationship between the probability of an individual to develop chronic Chagas cardiomyopathy and the main covariates (sex and polymorphisms). Sex was considered as a binary covariate. In our stepwise binary logistic regression analysis, genotypes were considered as binary covariates. The various genotype proportions are clearly described in tables 2 and 7. The way, we conduct the association were better described now in the materials and methods section. Grouping genotypes into a binary variable has been applied in several studies. It is useful in order to avoid misinterpretations due to the fact that one genotype is always relatively rare.