Author's response to reviews

Title: ACE gene D/I polymorphism as a modulator of severity of cystic fibrosis

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Version: 2 Date: 9 May 2012

Author's response to reviews: see over
Letter

We appreciate the considerations made to the article. The suggestions made were important to improve the quality of the final manuscript and contributed significantly to the construction of text and data presentation, and facilitated the discussion of the results. The suggestions were accepted, and each question was answered directly to each reviewer.

Assessor

Editorial comments:

We recommend that you ask a native English speaking colleague to help you copyedit the paper. – the article was reviewed by: (1) translation service of the University, (2) specialist in the area of genetic research, and (3) a professional with fluency in American English.

Please include a 'Competing interests' section between the Conclusions and Authors' contributions. – We have included a section: “Competing interests: The authors declare that they have no competing interests”.

Please include an Authors' contributions section before the Acknowledgements and Reference list. – We have included a section: “Authors' contributions:

FALM: made substantial contributions to conception and design, acquisition of data, and analysis and interpretation of data; involved in drafting the manuscript and revising it for critically important intellectual content.

TDRH: participated in the design of the study and in the collection of clinical markers.

CSB: carried out the molecular genetic studies and drafted the manuscript.

AFR: has been involved in drafting the manuscript and revising it critically for important intellectual content.

LCB: performed genotyping for CFTR mutation.
JDR: has been involved in drafting the manuscript and revising it critically for important intellectual content and has given final approval for the publishing of this version.

Acknowledgments

Reviewer's report
Title: ACE gene D/I polymorphism as a modulator of severity of cystic fibrosis
Version: 1 Date: 9 March 2012
Reviewer: Frauke Stanke

Reviewer's report:

1. Stratification of patient subgroups according to CFTR mutation genotype must ensure that mild PS CFTR genotypes and severe PI CFTR genotypes are not pooled. In this respect, patient with one identified class I, II and III mutation cannot be pooled with patients who carry two identified class I, II and III mutations. The former might be severe/unidentified_mild_mutation while the latter are obligatory PI. The analysis must be repeated to circumvent this bias. At least one subset of analyses must be restricted to patients with known and reasonably homogeneous CFTR mutation genotype, of which those with two class I, II and III mutations will be the only sufficiently large subpopulation.

Cystic fibrosis is influenced by environmental factors, modifier genes and mutations in the CFTR gene. The severity of cystic fibrosis associated with the classes of mutation in the CFTR gene is well known and reported in the literature. In contrast, studies of modifier genes have gained importance in demonstrating the significance of gene modulation in the severity of cystic fibrosis. The study of gene modulation depends on sample size and there is difficulty in obtaining a sample of patients that enables the model of the proposed study. In this context, we decided to conduct four different analyses in the sample, comparing the severity and the genotype of the ACE gene: (1) all
patients at the same time – without taking CFTR gene genotype into account, (2) patients diagnosed by alterations in sodium and chlorine, but without identified mutations in the CFTR gene identified, (3) patients diagnosed with alterations in sodium and chlorine and with a mutation belonging to class I, II or III in the CFTR gene identified, and finally (4) patients with two mutations belonging to class I, II and/or III in the CFTR gene – the group of major importance for the validation of these associations, because of "homogeneity" for mutations in the CFTR gene. In the final manuscript we included the following paragraph: "We performed four different analyses in the sample in order to detail the effect of the genotype of the CFTR gene on clinical severity. The analyses were performed in the cohorts: (1) all patients with CF (180 patients); (2) patients with no identified mutation in the CFTR gene (44 patients); (3) patients with a mutant allele identified in the CFTR gene (51 patients); and (4) patients with two mutations identified in the CFTR gene (85 patients) - main cohort used to analyze the influence of modifier genes associated with clinical variation in CF."

2. The authors’ conclusions that ACE is a modifier of CF rely to a large amount on the parameter BS, which is not explained in sufficient detail within the manuscript. The following points must be clarified before the work can be appreciated: What is BS and how does it differ from other, more commonly used assessments of CF disease severity? Can the authors explain why BS detects ACE as a modifier while lung function values, BMI and various other clinical parameters do not? – The Bhalla score is rarely used in cystic fibrosis compared with other scores because it is based on computed tomography. In order to better explain the association of this score, the following paragraph was added to the discussion: "The BS is a computed tomography score, which measures pulmonary involvement, therapeutic effects and selection of patients for transplantation, which detects anatomical changes of the lung parenchyma [15, 25]. The BS has low variation between examiners, good reproducibility, high sensitivity and specificity, and high correlation with pulmonary function test [15]. The values obtained in the score can predict severity associated with deterioration of the structure of the lung parenchyma,
which later in clinical evolution can be observed by other variables such as BMI and lung function."

Perhaps because of its sensitivity, accuracy and importance in the verification of early pulmonary changes, it has been associated with the genotype of the ACE gene and other markers of severity, also important, as mentioned (Spirometry and BMI), have not been associated. An important factor in this context is that the score was associated with severity in patients with two mutations in the CFTR gene identified.

3. The data presentation is very detailed. While a comprehensive presentation of all statistical analyses is suitable for an online supplement, the reader will profit from a carefully explained selection within the core manuscript. – The data analysis presented was intended to demonstrate the analysis performed. We agree that the possibility of supplements online can provide a gain in the final presentation of the article. Perhaps the changes made to the text have made it clearer and more readable, facilitating the understanding of the article.

4. The manuscript is hard to read. This is partially due to various mistakes in grammar and wording (see below), but also due to very technical sentences with numbers and abbreviations that have little or no explanatory bylines. Examples are listed below (e.g. Major point #9, #13). – Some changes were made in nomenclatures and presentation of the sentences. The grammar was reviewed. If there are more changes to be made, we will provide further corrections in accordance with the requests and suggestions.

Major Compulsory Revisions in point-by-point format:
1. Background, page 3: “Pro-inflammatory activity is activated by the TGF-ß1 enzyme and perhaps, for this property is related to the development of severe lung damage. “Please clarify the implied relationship of TGFB1 and ACE and its relevance for the author’s findings (as TGFB1 was not investigated). – We decided to remove this excerpt from the article. The TGFB1 gene was not analyzed in the research and perhaps could be a factor of contradiction and doubt if it had been kept in the final text.
2. Material and methods: heading should be changed to Patients and methods.  
   – **The alteration was made.**

3. Patients and methods: “Some mutations in adult patients with CF was obtained by …” Please specify which mutations were detected. Also check grammar: (mutations **WERE** obtained).  
   – **The grammar was changed and the following paragraph was added:**  
   Determination of mutations in the **CFTR** gene was performed by polymerase chain reaction (F508del) and restriction fragment length polymorphism method (G542X, R1162X, R553X, G551D and N1303K). Some mutations in patients with CF were obtained by sequencing or MLPA (Multiplex Ligation-dependent Probe Amplification) analysis: S4X, 2183A>G, 1717-G>A and I618T. For sequencing and MLPA, we also used MegaBace1000® (GE Healthcare Biosciences, United States of America, Pittsburgh) [13]. The **CFTR** genotype was used as a correction factor for statistical analyses. All mutations identified were included in classes one, two or three of the **CFTR** gene. Other identified mutations, such as class IV (P205S e R334W) were not included in the statistical analysis.”

4. Patients and methods: “Clinical scores of Kanga, Shwachman-Kulczycki and BS were performed …” Please specify BS. Explicitely, as BS is not a standard phenotype and the authors conclusion rely largely on BS, this parameter must be explained in more detail.  
   – **We included a paragraph in the results and discussion section to explain the importance of the score:**  
   “BS is a computed tomography score, which measures pulmonary involvement, therapeutic effects and selection of patients for transplantation, by detecting anatomical changes of the lung parenchyma [15, 25]. The BS has low variation between examiners, good reproducibility, high sensitivity and specificity, and high correlation with the pulmonary function test [15]. The values obtained in the score can predict severity associated with deterioration of the structure of the lung parenchyma, which later in clinical evolution can be observed by other variables, such as, BMI and lung function.”

5. Same paragraph: BMI, although a commonly used abbreviation, needs to be spelled out when first mentioned.  
   – **The change was made to the manuscript.**
6. Statistical analysis: “Variables described for the onset of illness (age at diagnosis and onset symptom pulmonary, digestive and first isolated P. aeruginosa) were categorized into two groups according to the median of the data.” Please reword to make it easier to understand that two subgroups of equal size were analyzed in all quantitative parameters. – We reworded the paragraph: “Variables described for the onset of illness (age at diagnosis, onset of pulmonary and digestive symptoms and first isolation of P. aeruginosa) were categorized into two groups according to the median of the data, due to the non-normal distribution of data. Data categorized by the median are divided into two cohorts with similar sample size.”

7. Statistical analysis: “the level of significance $\alpha$, was adjusted using Bonferroni correction”. Please specify how many independent tests were considered by the authors for correction. – Four tests were used for the correction. We reworded the paragraph: “In order to avoid spurious data due to the problem of multiple testing [21], the level of significance $\alpha$, was adjusted using Bonferroni correction on four tests.”

8. Statistical analysis: “All mutations observed in our study were included in classes I to III, thus, patients with two identified mutations, presented no influence of CFTR gene in analysis.” Please reword – the authors state in their abstract that conclusions were obtained “in the different groups of mutations in the CFTR gene.” How were the patients grouped to exclude an influence of the CFTR mutation genotype on the outcome? To explain how the analyses were carried out in a clear and direct method in the section "statistical analysis" we added the paragraph: “All mutations analyzed in our study were included in classes I, II or III. We performed four different analyses in the sample in order to detail the effect of the genotype of the CFTR gene in clinical severity. The analyses were performed in the cohorts: (1) all patients with CF (180 patients); (2) patients with no identified mutation in the CFTR gene (44 patients); (3) patients with a mutant allele identified in the CFTR gene (51 patients); and (4) patients with two mutations identified in the CFTR gene (85 patients) - main cohort to analyze the influence of modifier genes associated with clinical
variation in CF.” In the section “Results and discussion” this paragraph was added: “Genotypes of the identified mutations in \textit{CFTR} gene: 44 patients (24.44\%) without identified mutation, 51 (28.33\%) with one identified mutation (25\% F508del/-, 2.78\% G542X/-, 0.56\% R1162X/-) and 85 (47.22\%) patients with two identified mutation (31.67\% F508del/F508del, 6.67\% F508del/G542X, 2.78\% F508del/R1162X, 2.22\% F508del/N1303K, 0.56\% F508del/R553X, 0.56\% F508del/S4X, 0.56\% F508del/1717-1G>A, 0.56\% G542X/R1162X, 0.56\% G542X/I618T, 0.56\% G542X/2183AA>G and 0.56\% R1162X/R1162X).”

9. Results and discussion, second paragraph: “Bacteria in secretion: 76 (42.2\%) with \textit{P. aeruginosa} mucoid and 101 (56.1\%) non-mucoid, 141 (78.3\%) \textit{S. aureus}, 25 (13.9\%) \textit{B. cepacia} and 18 (10\%) \textit{A. xylosoxidans}. Comorbidities were: 143 (79.4\%) with pancreatic insufficiency, 33 (18.3\%), nasal polyps, 33 (18.3\%) diabetes mellitus, 29 (16.1\%) osteoporosis and 27 (15\%) meconium ileus. For variables with numerical distribution; see data listed in Table 1.”

Firstly, the sentence is incomplete (no verb). – We changed the sentence to: “The spectrum of isolated Bacteria in secretion was: 76 (42.2\%) with mucoid and 101 (56.1\%) non-mucoid \textit{P. aeruginosa}; 141 (78.3\%), \textit{S. aureus}; 25 (13.9\%), \textit{B. cepacia}; and 18 (10\%), \textit{A. xylosoxidans}. Comorbidities associated with CF severity were: 143 (79.4\%) with pancreatic insufficiency; 33 (18.3\%), nasal polyps; 33 (18.3\%), diabetes mellitus; 29 (16.1\%), osteoporosis; and 27 (15\%), meconium ileus. For variables with numerical distribution, see data listed in Table 1.”

Secondly, a repetition of the data that is presented in tabular format is unnecessary unacceptable. – In Table 1 we present the numerical distribution data; in Table 2 the associations between clinical variables with mutations in the \textit{CFTR} gene and \textit{ACE} gene polymorphism; and, in Tables 3 and 4 the analyses for the categorical variables that showed statistically significant value were included.

Thirdly, no conclusion is drawn from the accumulated number set– is the data as expected for the CF population? Are some comorbidities more frequent than expected? – We find data presentation to be extremely important. Some variables showed variation in relation to other population studies of patients with cystic fibrosis. However, due to the attempt to direct the
association of the ACE gene with the clinical variables, we decided not to argue with the emphasis on the distribution of dependent data in comparison with other studies. In this context, we try to be direct and demonstrate "just" the association with the CFTR gene and ACE gene polymorphism in association with clinical variables. Perhaps, with the volume of data collected, it is interesting to carry out another study that characterizes the clinical and laboratory characteristics of patients in our referral center compared with published studies that report these characteristics. Important surveys such as those held by the Cystic Fibrosis Foundation can provide important data for this association, since in Brazil this model of study is seldom performed in comparison to other countries.

10. Results and discussion, third paragraph: “ACE gene D/I polymorphism was associated with severity of CF.” This sentence need to be backed up with data – the main conclusion should not precede the findings. – We decided to change the paragraph and put the information at the end of the article.

11. Results and discussion: “…. when only one mutation in the CFTR gene was identified.” Are these carriers confirmed CF patients by other techniques? See also major point #8 above, the population structure should be better explained in the methods section. – After changes were made to the article, the section "diagnosis of patients" was clear and direct. The paragraph now reads: “Diagnosis of CF was confirmed in patients with two doses of sodium and chloride from the sweat with values greater than 60 mEq/L. In a cohort of patients we identified two mutations in the CFTR gene. No patient had received a neonatal screening test performed for CF.”. All patients had the examination of sodium and chlorine in sweat altered. Other changes were referred to in item # 8.

12. Results and discussion: “The D allele in the ACE gene is associated with a higher gene expression and, consequently, promotes a greater inflammatory response in the body, leading to early symptoms. The earliest onset of signs and symptoms are accompanied by early onset of inflammation and
deterioration of lung and pancreatic function. These symptoms characterize severe patients.” This paragraph is better suited for an introduction. This is not a result by the authors and unlinked to the surrounding information. – The information in that paragraph is contained in the introduction. After changes in the “results and discussion”, we believe that the paragraph enables an emphasis on demonstrating that the polymorphism can be considered a risk factor for increased severity of cystic fibrosis.

13. Results and discussion: “Association of the infection/colonization by B. cepacia with ACE gene D/I polymorphism was identified only for patients without distribution according to the CFTR gene mutation, OR: 3.309 (1.476 to 6.256) to D/D genotype (Table 4).” Please reword: what was observed in an elevated frequency in which subgroup? Which covariates were considered to mask/unmask the association? – Changes in Table 4 were performed to leave the information clearer. The analysis showed a significant association in patient cohorts: (1) Without taking CFTR mutation into account and (2) taking into account one CFTR mutation identified in class I, II or III, as show in the table 4. The genotype for mutations in the CFTR gene was used as a correction factor by dividing the patients into different groups.

14. Results and discussion: “There was no difference between BS and the age of patients after categorization.” BS not a standard in CF phenotyping - is this an age-independent description of LF in a progressive disease? Does BS employ CF-specific centiles? – The score is associated with computerized tomography. CT may be associated with age, with older patients generally being more affected. In this context, we associated the age with the score and no association was found. This favors our results of association between the
score and *ACE* gene polymorphism. After alterations, the paragraph reads:

“There was no difference between BS and the age of patients after categorization. Younger patients (≤ 154 months) had the same distribution of BS as older patients (> 154 months) (p = 0.761). Age is not a variable that contributes to the association between the *ACE* gene D/I polymorphism and BS. The analysis of association between the BS and age of patients with CF was performed in order to show that age had no influence on the score value analysis. We can conclude that the *ACE* gene D/I polymorphism acts in genetic modulation by association with BS.”

15. Results and discussion: “Evolution of CF is secondary to the type of mutation and environment. Few studies have correlated mutations, polymorphisms and clinical variables in CF.” This is not correct. there are numerous publications on genotype-phenotype relations in CF. Please review the literature. – We agree with the information, and we reworded the paragraph: “Evolution of CF is secondary to mutation class in the *CFTR* gene and environment factors. Many studies have correlated mutations, polymorphisms and clinical variables in CF [5, 26]. Association studies usually face the problem of obtaining a sufficient sample size for the number of mutations in the *CFTR* gene to achieve a homogeneous population and characterize the follow up of chronic and persistent lung disease [27].”

16. Results and discussion: “The main environmental factor in the clinical variability of CF is the access of patients to treatment. At our center, treatment is guaranteed by the public health system, which allows equal access for all patients included in the study and it is not an additional factor in the analysis of data.” In the US, there is a considerable disadvantage for patients treated under the public health system (Schechter MS. Non-genetic influences on CF lung disease: the role of sociodemographic characteristics, environmental exposures and healthcare interventions. *Pediatr Pulmonol Suppl.* 2004;26: 82-85.). Can the authors rule out a similar effect for Brazil? – In Brazil, the treatment is carried out mainly in public health service, which is the service reference in the treatment of cystic fibrosis and without payment. In Brazil, the public service for the treatment of cystic fibrosis is distributed in referral centers, which
facilitates the access of patients to a multidisciplinary team, however, this is not a reality across the country. Patients treated in the private system also have excellent accompaniment, and perhaps have an advantage in private treatment in relation to the public in some regions of Brazil where access to health services is restricted. The paragraph was reworded as: “The main environmental factor in the clinical variability of CF is the patients’ access to treatment [28]. At our center, treatment is guaranteed by the public health system, which allows equal access for all patients included in the study, and it is not an additional factor in the analysis of data, which is not true in all CF centers in Brazil. Unlike the U.S. where the private system ensures better treatment in CF [29], in Brazil, the public health system is the reference.”

17. Results and discussion: “On the best of our knowledge, only the study by Arkwright et al. (2003) [8] had characterized the ACE gene as a potential factor in the clinical CF severity.” ACE was also investigated by Drumm et al (Reference 25) and others. – We reworded the paragraph: “To the best of our knowledge, few studies have characterized the ACE gene as a potential factor in the clinical CF severity [8, 30, 31].

18. page 12, table 1: CFTR mutation spectrum is missing. – we decided to put the description of mutations in the CFTR gene in the “results and discussion”: “Genotypes to mutation identified in CFTR gene: 44 patients (24.44%) without identified mutation, 51 (28.33%) with one identified mutation (25% F508del/-, 2.78% G542X/-, 0.56% R1162X/-) and, 85 (47.22%) patients with two identified mutation (31.67% F508del/F508del, 6.67% F508del/G542X, 2.78% F508del/R1162X, 2.22% F508del/N1303K, 0.56% F508del/R553X, 0.56% F508del/S4X, 0.56% F508del/1717-1G>A, 0.56% G542X/R1162X, 0.56% G542X/I618T, 0.56% G542X/2183AA>G and 0.56% R1162X/R1162X).”

19. page 14, table 3: “Association of ACE gene D/I polymorphism, in patients with one identified mutation (class I, II or III) in the CFTR gene, with onset of clinical symptoms of patients in months.” Please also give data for the other
patient subgroup. Stratification should ensure that mild PS CFTR genotypes and severe PI CFTR genotypes are not pooled. – **We changed the table (title and data): “Table 3. Association of ACE gene D/I polymorphism with onset of clinical symptoms of patients in months considering the cohorts to CFTR mutation.”** To details, see table 3.

20. Page 15, table 4: “….. without CFTR genotype distribution …..” please specify (without talking CFTR mutation genotype into account? – **We used the suggest term and we put some new data in table 4. For more information see table 4.**

Minor Essential Revisions:

1. Abstract: “….and Bhalla score (SB) (p= 0.015) ….” Abbreviation BS ? – **The abbreviation was changed and used in all of the article.**

2. Patients and Methods: “…..delF508, G542X, R1162X, N1303K, G551D and N1303K. “N1303K listed twice. – **The alteration was made.**

3. Patients and Methods: “Comorbidities (nasal polyposis, osteoporosis, diabetes mellitus, pancreatic insufficiency and meconium ileus) were analysis.” Check grammar (were analysed). – **The alteration was made.**

4. Sometimes, the font changes, e.g. page 5 “and the R program version 2.12 (Comprehensive R Archive Network, 2011).” and page 6: “derived and 15 (7,4 %) were African-derived individuals.” – **The alteration was made, and we put all article in the same formatting.**

5. Page 15, table 4: “Ausence” typo: absence – **The alteration was made.**

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Needs some language corrections before being published

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

1. I declare that I have no competing interests

Reviewer's report

Title: ACE gene D/I polymorphism as a modulator of severity of cystic fibrosis

Version: 1 Date: 13 March 2012

Reviewer: Rossella Tomaiuolo

Reviewer's report:

The scientific quality of the manuscript is good and the presentation is clear. However the study could be reinforced with the data about the prevalence of D allele in the ACE gene polymorphism in general population from the same geographical area. – We used a study of the same referral center, and we reworded the paragraph: “The ACE gene D/I polymorphism showed a higher frequency for ACE*D (228 patients) compared with ACE*I (132 patients). The genotype frequencies were: 72 (40.0%) with D/D; 84 (46.67%) with D/I; and 24 (13.3%) with I/I. The sample was in Hardy-Weinberg equilibrium (p>0.05). Analysis of 70 healthy control subjects in UNICAMP demonstrated the genotype frequency: 20 (29%) with D/D, 37 (53%) of D/I, and 13 (18%) I/I [23]. There was no difference in frequency of genotypes in relation to our study (p= 0.210). The analyses of the ACE gene D/I polymorphism with the clinical variables are denoted in Table 2, where it can be observed every association possible between the clinical trial, CFTR mutation identified and ACE gene D/I polymorphism.”

Minor revision

• Background, line 5 and result and discussion, line 45: to replace “expresses” with “codify”. – The alteration was made

• Materials and methods: The Authors could give supplemental information on the prevalence of the ESR2 AluI gene polymorphism in healthy population from the same geographical area (Sicily, Italy). – We reworded the article following the information in the first topic: We used a study of the same referral center, and we reword the paragraph: “The ACE gene D/I polymorphism showed a
higher frequency for ACE*D (228 patients) compared with ACE*I (132 patients). The genotype frequencies were: 72 (40.0%) with D/D; 84 (46.67%) with D/I; and 24 (13.3%) with I/I. The sample was in Hardy-Weinberg equilibrium (p>0.05). Analysis of 70 healthy control subjects in UNICAMP demonstrated the genotype frequency: 20 (29%) with D/D, 37 (53%) of D/I, and 13 (18%) I/I [23]. There was no difference in frequency of genotypes in relation to our study (p= 0.210). The analyses of the ACE gene D/I polymorphism with the clinical variables are denoted in Table 2, where it can be observed every association possible between the clinical trial, CFTR mutation identified and ACE gene D/I polymorphism.”

• Table 2: Probably the content of Table 2 1 can become “Supplemental data” to the article. – We agree that the possibility of supplements online can provide a gain in the final presentation of the article. Perhaps, given the changes made the text has become clearer and more readable, facilitating understanding.

• to replace “delF508” with “F508del”. – we reword the article, using the term “F508del”.

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

1. Dr Rossella Tomaiuolo, MD, PhD

3. Reviewer’s report
Title: ACE gene D/I polymorphism as a modulator of severity of cystic fibrosis
Version: 1 Date: 26 March 2012
Reviewer: SALMO RASKIN
Reviewer’s report:
MATERIALS AND METHODS

Authors say that “Diagnosis of CF was confirmed in patients with two doses of sodium and chloride in the sweat with values of greater than 60 mEq/L and/or by the finding of two mutations in the CFTR gene.” But there is no information in the entire text about patients CFTR genotypes, which is VERY important. – We rewrote the paragraph in “Determination of mutations in the CFTR gene” section to: “Determination of mutations in the CFTR gene were performed by polymerase chain reaction (F508del) and restriction fragment length polymorphism method (G542X, R1162X, R553X, G551D and N1303K). Some mutations in patients with CF were obtained by sequencing or MLPA (Multiplex Ligation-dependent Probe Amplification) analysis: S4X, 2183A>G, 1717-G>A and I618T. For sequencing and MLPA, we also used MegaBace1000® (GE Healthcare Biosciences, United States of America, Pittsburgh) [13]. The CFTR genotype was used as a correction factor for statistical analysis. All mutations identified were included in the class one, two or three of the CFTR gene. Other identified mutations, class IV (P205S e R334W) were not include in statistical analysis.”

The only clue I could find was in Table 2, as authors show that there were 50 patients with only one identified mutation (class I, II or III) in the CFTR gene. I could not find how many from the 180 patients of the cohort had neither of the two CTR mutations identified. In Statistical Analysis, authors say that “All mutations observed in our study were included in classes I to III, thus, patients with two identified mutations, presented no influence of CFTR gene in analysis.” But how many from the 180 patients in the cohort had indeed two identified Class I-III CFTR mutations? It is understandable that due to the rarity of CF patients with the same two mutations genotypes, studies of genes influencing the main locus (CFTR) are very difficult to be done Therefore it is also understandable that many authors are including all patients with Class I, II and II CFTR mutations “as a single group”. – We reword the “Statistical analyses” topic to: “Data was compared with the linear and logistic regression analysis. For comparison between genotypes and the variables with numerical
distribution, T-student test was used. Genotyped data for the CFTR gene was used to establish association between the CFTR gene, ACE gene and clinical variables. All mutations analyzed in our study were included in classes I, II or III. We performed four different analyses in the sample in order to detail the effect of the genotype of the CFTR gene in severity of clinical. The analyses were performed in the cohorts: (1) all patients with CF (180 patients); (2) patients with no identified mutation in the CFTR gene (44 patients); (3) patients with a mutant allele identified in the CFTR gene (51 patients); and (4) patients with two mutations identified in the CFTR gene (85 patients) - main cohort to analyze the influence of modifier genes associated with clinical variation in CF.

and we added to the “results and discussion” section the paragraph: “Genotypes of mutations identified in CFTR gene: 44 patients (24.44%) without an identified mutation, 51 (28.33%) with one identified mutation (25% F508del/-, 2.78% G542X/-, 0.56% R1162X/-) and, 85 (47.22%) patients with two identified mutation (31.67% F508del/F508del, 6.67% F508del/G542X, 2.78% F508del/R1162X, 2.22% F508del/N1303K, 0.56% F508del/R553X, 0.56% F508del/S4X, 0.56% F508del/1717-1G>A, 0.56% G542X/R1162X, 0.56% G542X/I618T, 0.56% G542X/2183AA>G and 0.56% R1162X/R1162X).”

Authors of this work say that “All mutations observed in our study were included in classes I to III, thus, patients with two identified mutations, presented no influence of CFTR gene in analysis.”. I would be more careful to make this generalization, but more important, in this work, seems to me that at least 50 patients did not have at least one CFTR mutation identified, and I believe that this patients, as well as patients with no CFTR mutations identified, should not be included in the cohort, because they could have one or two “mild” (Class IV, V or VI) CFTR mutations, and this would introduce a very strong bias to the study. So, in my opinion, authors should inform all patients CFTR genotype in a Table, and exclude from the final cohort the ones with only one CFTR mutation identified as well as patients with no CFTR mutations identified. Analysis should be done only in the patients that are homozygous or compound heterozygous for two CFTR identified Class I-III mutations. I understand that this criteria may reduce the sample size of the cohort up to a point of turning the
work unviable, but I think the only way to try to escape of the huge obstacle of the rarity of CF patients with the same two CFTR mutations, is through working with a Consortium of Centers, to get a larger sample of patients with both CFTR mutations identified. – The genotypes for mutations in the CFTR gene were included in the study and had been previously mentioned in the text. Cystic fibrosis is influenced by environmental factors, modifier genes and mutations in the CFTR gene. The severity of cystic fibrosis associated with the classes of mutation in the CFTR gene is well known and reported in the literature. In contrast, studies of modifier genes have gained importance in demonstrating the significance of gene modulation in the severity of cystic fibrosis. The study of gene modulation depends on sample size and there is difficulty in obtaining a sample of patients that enables the model of the proposed study. In this context, we decided to conduct four different analyses in the sample, comparing the severity and the genotype of the ACE gene: (1) all patients at the same time – without taking CFTR gene genotype into account (2) patients diagnosed by altered sodium and chlorine levels, but without identified mutations in the CFTR gene identified, (3) patients diagnosed with the sodium and chlorine altered, but with a mutation belonging to class I, II or III in the CFTR gene identified, and finally (4) patients with two mutations belonging to class I, II and/or III in the CFTR gene - the group of major importance for the validation of these associations, because of "homogeneity" for mutations in the CFTR gene. In the final manuscript we included the following paragraph: " We performed four different analyses in the sample in order to detail the effect of the genotype of the CFTR gene in severity of clinical. The analyses were performed in the cohorts: (1) all patients with CF (180 patients); (2) patients with no identified mutation in the CFTR gene (44 patients); (3) patients with a mutant allele identified in the CFTR gene (51 patients); and (4) patients with two mutations identified in the CFTR gene (85 patients) - main cohort to analyze the influence of modifier genes associated with clinical variation in CF."

When we mentioned that patients with two mutations identified in the CFTR gene do not "suffer" influences in the analysis by CFTR gene, we were reporting that there was homogeneity in genotype in the CFTR gene, and that the association with the genotype for the polymorphism did not 'suffer'
influence of unknown mutation. However, the way in which this was described was difficult to understand. In this case, we reworded the paragraph to: “Data was compared with the linear and logistic regression analysis. For comparison between genotypes and the variables with numerical distribution, T-student test was used. Genotyped data for the CFTR gene was used to establish association between the CFTR gene, ACE gene and clinical variables. All mutations analyzed in our study were included in classes I, II or III. We performed four different analyzes in the sample in order to detail the effect of the genotype of the CFTR gene in severity of clinical. The analyses were performed in the cohorts: (1) all patients with CF (180 patients); (2) patients with no identified mutation in the CFTR gene (44 patients); (3) patients with a mutant allele identified in the CFTR gene (51 patients); and (4) patients with two mutations identified in the CFTR gene (85 patients) - main cohort to analyze the influence of modifier genes associated with clinical variation in CF.”

RESULTS AND DISCUSSION
The relative frequencies of polymorphisms of ACE were not tested in local controls without CF and were not compared with published frequencies of these polymorphisms in normal Brazilian populations, to test if there were significant differences between the frequencies of these alleles and genotypes in this CF cohort when compared to control subjects. ACE polymorphism frequencies in 70 controls that live in the same city were the authors and CF patients live, were previously published in the literature and should be used (Yugar-Toledo JC et al. Gene variation in resistant hypertension: multilocus analysis of the angiotensin 1-converting enzyme, angiotensinogen, and endothelial nitric oxide synthase genes. DNA Cell Biol. 2011 Aug;30(8):555-64.) – After revision we decided to change the article and we reword the paragraph: “The ACE gene D/I polymorphism showed a higher frequency for ACE*D (228 patients) compared with ACE*I (132 patients). The genotype frequencies were: 72 (40.0%) with D/D; 84 (46.67%) with D/I; and 24 (13.3%) with I/I. The sample was in Hardy-Weinberg equilibrium (p>0.05). Analysis of 70 healthy control subjects in UNICAMP demonstrated the genotype frequency: 20 (29%) with D/D, 37 (53%)
of D/I, and 13 (18%) I/I [23]. There was no difference in frequency of genotypes in relation to our study (p= 0.210)."

Authors say that “The ACE gene D/I polymorphism showed a higher frequency for allele D (154 patients) compared with allele I (108 patients). But if there were 180 patients, then there were 360 alleles...What was the frequency of allele D and allele I among the 360 alleles typed? I understand that the genotype frequencies were: 84 (46.4%) (or 46.7%) D/I, 73 D/D (40.3%) (or 40.5%) and 24 (13.3%) I/I. “. But 84+73+24= 181 patients and not 180...Besides, allele and genotypes polymorphism frequencies were not tested to see if they conform to the Hardy–Weinberg equilibrium, to test any possible bias in sampling in this population, such as the presence of sub-structure, or problems with genotyping. – We calculated and added the equilibrium. The calculation was wrong, we had 181 patients genotyped for polymorphism, but got only 180 patients with clinical variables that were included in subsequent analyses. In this way, we reword the paragraph: “The ACE gene D/I polymorphism showed a higher frequency for ACE*D (228 patients) compared with ACE*I (132 patients). The genotype frequencies were: 72 (40.0%) with D/D; 84 (46.67%) with D/I; and 24 (13.3%) with I/I. The sample was in Hardy-Weinberg equilibrium (p>0.05).”

CONCLUSION
Authors conclude that “An association between the D allele in the ACE gene with the severity of CF was found in our study.” In Results, authors show that “There was association of the genotype D/D with early initiation of clinical manifestations (OR: 1.519, CI: 1.074 to 2.146), bacterium Burkholderia cepacia colonization (OR: 3.309, CI: 1.476 to 6.256), and Bhalla score (SB) (p= 0.015). Would this 3 associations, even if true, be enough to conclude that the ACE polymorphism is associated with severity of CF? How to explain that in more then 20 other clinical variables studied, no association was found, including the Kanga and Shwachman-Kulczycki score? - The association with clinical severity is dependent on several factors. The study seeks to understand how severity can be associated with different polymorphisms, and we believe there are three factors of influence, CFTR genotype, environment and other
In the study we found three associations with variables in different groups. The most significant variable was the score of Bhalla, and in that context we include a few quotes. The Bhalla score is rarely used in cystic fibrosis compared with other scores, because it uses computed tomography. In order to better explain the association of this score, we added the paragraph in the discussion: “The BS is a computed tomography score, which measures pulmonary involvement, therapeutic effects and selection of patients for transplantation, which detects anatomical changes of the lung parenchyma [15, 25]. The BS has low variation between examiners, good reproducibility, high sensitivity and specificity, and high correlation with pulmonary function test [15]. The values obtained in the score can predict severity associated with deterioration of the structure of the lung parenchyma, which later in clinical evolution will be observed by other variables, such as, BMI and lung function.” Perhaps because of its sensitivity, accuracy and importance in the verification of early pulmonary changes, it has been associated with the genotype of the \textit{CFTR} gene and other markers of severity, also important, as mentioned (Spirometry and BMI), have not been associated. An important factor in this context is that the score was associated with severity, also in patients with two mutations in the \textit{CFTR} gene. We emphasize that most studies associate little or only one variable with genotype polymorphism analysis, and in our study, we analyzed 24 variables, to understand how the disease with extremely variable expressivity among patients may suffer influence of modifier genes. Due to sample size and different factors that influence the severity, it was expected that few variables would have an association. Our blurring, to assess the conclusion, based primarily on study design, and the approach to the clinical characterization, which was conducted by staff of the university.

- Minor Essential Revisions

ABSTRACT

In Abstract/Results, authors define Bhalla Score as (SB), but latter use “BS” instead of “SB” in the entire text; - we reworded this in the article.
MATERIALS AND METHODS

Authors say that “Two hundred and fifteen patients were selected for the study. Twenty-five patients without clinical data for statistical analysis and those who did not sign the consent were excluded”. So I understood that 190 CF patients were included in the study. But latter in the text, only in RESULTS, authors say that “From a population of 215 patients attending our Center, 35 were excluded for lack of data and collection of material, resulting in 180 patients analyzed. I suggest that all this exclusions should be explained in Materials and Methods. –

Changes were made and in the patients and methods section we reworded the paragraph: “Two hundred and fifteen patients were selected for the study. Thirty five patients without clinical data for statistical analysis and those who did not sign the consent form were excluded. Patients’ DNA was obtained by phenol-chloroform extraction. The concentration of DNA used for analysis was 50 ng/mL, evaluated using GE NanoVue™ Spectrophotometer (GE Healthcare Biosciences, United States of America, Pittsburgh).”

Authors should include the full references of the methods used for CFTR genotyping; - We include the reference and we reword the text to:

Determination of mutations in the CFTR gene was performed in the Laboratory of Molecular Genetics, for principal mutations in the Brazilian population: delF508, G542X, R1162X, N1303K, G551D and N1303K. Some mutations in adult patients with CF were obtained by sequencing or MLPA (Multiplex Ligation-dependent Probe Amplification) analysis. For sequencing and MLPA, we used the same MegaBace1000® equipment. The CFTR genotype was used as a correction factor for statistical analysis. All mutations identified were included in the classes one, two or three of the CFTR gene.”

Authors say that “Each initial sample with D/D genotype passed to a second PCR reaction. Primers used: Hace 5a, 5’-TGG GAC CAC-AGC GC C CGC CAC
TAC-3′ and have 5c, 5′-TCG CCA GCC CTC CCA TGC CCA TAA-3′. What is the exact reference for this new set of primers and why they were not used as first option of the authors, if it seems that with this set of primers and a higher annealing temperature, there was no preferential amplification of allele “D”? It is not clear in the text how did the authors figure out that there was a preferential amplification of D allele in heterozygous individuals. Did they know this by literature or personal experience before they started to genotype the samples? Or they figure out in the initial genotyping by the different intensity of the two expected bands in heterozygous? In the same context, it is not clear what did authors mean by writing “A 335 bp sequence was amplified in the presence of at least one allele” Were authors trying to say that in homozygous “D/D” patients a single 335bp sequence was amplified? And if this is the case, what was the expected fragment size in heterozygous and homozygous I/I, with the new set of primers? – Upon completion of the first PCR, we observed preferential amplification of the deleted allele, and where was the genotype D/D, we performed a new PCR to confirm that the patient had this genotype, or was, D/I. We stand on Article: Ogus C, Ket S, Bilgen T, Keser I, Cilli A, Gocmen AY, Tosun O, Gumuslu S: Insertion/deletion polymorphism and serum activity of the angiotensin-converting enzyme in Turkish patients with obstructive sleep apnea syndrome. Biochem Genet 2010; 48:516-523.

RESULTS AND DISCUSSION
Authors say that from 180 subjects, Ninety (49.7%) were male,” but 90/180 is exactly half, 50% and not 49.7%; - The problem was explicated in the section about ACE gene genotype, and we did the alteration.
Authors say that from 180 subjects, 165 (91.7%) were European-Caucasian derived and 15 (7,4 %) were African-derived individuals”. But 15/180 equals 8,3% and not 7,4%. Were there subjects that are not European-Caucasian derived and also not African-derived? – We reworded the paragraph with the correct data: “From a sample of 180 patients analyzed; 90 (50%) were male, 165 (91.7%) were European-Caucasian derived and 15 (8.3 %) were African-derived individuals.”
Discretionary Revisions

RESULTS AND DISCUSSION

Authors say that “The main environmental factor in the clinical variability of CF is the access of patients to treatment”, and we agree. They also say that “treatment is guaranteed by the public health system, which allows equal access for all patients included in the study and it is not an additional factor in the analysis of data”, and then we disagree. As Table 1 shows, there is a 21 months mean delay between age at fist symptoms and age of diagnosis, showing the difficulties of diagnosis of CF in Brazil, and even if access to the Reference Center is free, it is obvious that patients arrive at the Center with different stages of the disease, which is an additional factor biasing the data.

– This generalization is really wrong, so we decided to change the paragraph to: “The main environmental factor in the clinical variability of CF is the patients’ access to treatment [28]. At our center, treatment is guaranteed by the public health system, which allows equal access for all patients included in the study. It is not an additional factor in the analysis of data, but this is not true in all CF centers in Brazil. Unlike the U.S. where the private system ensures better treatment in CF [29], in Brazil, the public health system is the reference.”

There is also the problem of patient compliance- this factor was not studied and is difficult to control in any study.

Authors say that “The ACE gene expressed the ACE protein, which acts on the inflammatory response [8, 10]. The expression of the ACE gene is influenced by the D/I polymorphism. ACE protein levels in plasma and tissues, including the lung, is determined in part by the D/I polymorphism [12]“. This information has been already informed in the “Background” section and therefore can be excluded from DISCUSSION. – We made the change and excluded the portion measured.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Needs some language corrections before being published
**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