Author's response to reviews

Title: Polymorphisms of the insertion / deletion ACE and M235T AGT genes and essential hypertension: surprising new findings and meta-analysis of data

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Author's response to reviews: see over
Response to reviewers:

General remarks:

The authors thank both reviewers for their polite and thorough assessment of the manuscript. The revised text has profited greatly form this.

Please note that reference numbers given here refer to the reference list given in this document, and NOT to the reference list in the manuscript.

Please note that, since A.L. Zhu has left Bioinformatics Institute to study for a higher degree with National University of Singapore, most of the revision work was done by two other team members, to whom we attribute full author status in the revised manuscript.

With regards to the partially missing values in the figures: these were included in the originally sent files. We have noted, however, that the display of the figures is not consistent in some viewing programs. They can all and fully be seen on Macintosh in all three figures, but not on PC. If this JPEG version (as opposed to the previous high resolution TIFF images) poses the same problem to the reviewer, we will need to seek technical guidance from the BMC editors.

Response to Dr. Bostrom:
Major compulsory revisions:
1. “Abstract and Background: The aim of the study is to investigate how the ACE and AGT genes account for the prevalence of hypertension… But the subjects are not a random sample from the population but instead selected from blood donors that presumably are very healthy and patients from a clinic treating kidney diseases. The excess of males in the hypertensive sample also points towards selection bias. Usually hypertension is as common or even more common in women at that age (60 years). The genotypes of the AGT polymorphism were not in Hardy-Weinberg equilibrium which might also implicate selection bias.”

We apologize for the un-scientific use of the word “prevalence”, which here was meant only to state the presence of hypertension. We have changed the two sentences in abstract and background
accordingly: “This study examines how polymorphisms of the insertion/deletion (I/D) ACE and M235T AGT genes account for presence and severity of hypertension, and embeds the data in a meta-analysis of relevant studies.” The correct use of the word “prevalence”, as assumed by the reviewer, implies a cross-sectional analysis of the general population with the aim of investigating both the quantitative presence of hypertension, and the specific genotypes, and then to find an association. This is NOT the aim of our study. We do follow the case-control design of most of the previous studies done in this field by looking at distinctively hypertensive subjects (here defined by that sub-population of the clinic’s patients who are on anti-hypertensive medication) and compare them to a distinctively non-hypertensive population, the blood donors. As the inclusion criteria for the hypertensive patients were as simple as stated, the excess males rather point to a selection bias on the side of primary care providers, who might refer men rather than women, or to a lower health consciousness in the women who would be more reclusive in going for medical examination. However, these potential reasons are speculative as no reliable data is available. It should be noted, however, that the current health care system in Germany does not obviously reward specialist referral for conditions such as hypertension (Mondry et. al. 2004, BMC Nephrology, revised manuscript under review), so that only “hard to treat cases” may have been present in this study.

2. “Is the use of antihypertensive treatment validated? Some medications might be used for other purposes such as heart failure, angina pectoris or renal failure.” All medications were validated as being prescribed to hypertensive patients by M.H. before including the respective patients in the study. Of course, a number of them suffer from the conditions named, too, so that the actual “composition” of the prescription is customized.

3. “Results: There is no description of the subjects’ medical history or clinical data such as presence of obesity, diabetes, dyslipidemia and cardiovascular and kidney diseases. These conditions might influence the interpretation of the results.” We have added a more detailed description of the cases in the methods section. Apart from basic demography as detailed, no detailed information was available
for the controls (blood donors). Blood samples were obtained from donors following informed consent. This informed consent, however, did not include blood analysis beyond the gene polymorphism studies as described in materials and methods, nor any other data (body weight, medical history with regards to prevalence of other diseases). Blood donations in Germany follow strict rules (the full text at http://www.bundesaerztekammer.de/30/Richtlinien/Richtidx/Blutprodukte/). Post myocardial infarct patients are excluded, and so are any who suffer from chronic disease that requires regular medication. These rules, however, are checked only by asking the questions required according to the regulations before donation; therefore, it cannot be excluded that some donors did suffer from diabetes, kidney disease or pronounced cardiovascular disease at time of donation.

4. We thank the reviewer for pointing out this inconsistency. In fact, using and reporting a p-value derived from chi-square test is inappropriate here. The chi-square test does not examine the differences between T and M allele frequencies, but only whether there is a difference in genotype (be it M/M, M/T or T/T) distribution between the two groups: hypertensives and controls. As such, in the revised manuscript, we report only the frequencies of the genotypes. Looking at the odds ratio, which is the only relevant test used here, one sees a p-value of 0.01 for women, which is significant in answering the question, is the M allele associated with hypertension. The still significant p-value of 0.034 describes this association for both genders combined. For men, we find the insignificant p-value of 0.66. We have changed the text in the manuscript accordingly.

5. The present study detects, based on odds ratio (TT vs MM), significant differences between hypertensives and controls for both overall and especially the female group. The p-values were 0.034 and 0.01 respectively, which are considered well below the cutoff point of P=0.05. Thus, the results presented here should be considered as sufficiently significant, especially for the female group. Hence, the results are unlikely to be due to chance. We agree that the number of TT homozygotes is quite low but this is not uncommon and it is likely that this could indeed be reflective of the actual genotype distribution in the population. We also concur that the results should be interpreted with care since the genotypes were not in Hardy-
Weinberg Equilibrium. Please see also our response to Dr. Bantis with regards to the power analysis.

6. “Results and Discussion of meta-analysis: The tests for heterogeneity were significant pointing at a considerable variation among the studies included. The reason for this heterogeneity should be discussed and the result of the meta-analysis should be interpreted with great caution.” We agree with the reviewer, and have, therefore, included the phrase: “Nevertheless, the quality of meta-analysis results depends on the quality of the individual studies included, and unusual sample sizes might bias the finding. For example, one single study [1] included in the previously largest meta-analysis [2] was exceptionally large, giving it enormous weight. The highly variable study quality implies that all interpretations must be made with great caution, as was explicitly pointed out by Kunz et al. [3]’” (New text in italics).

7. “Discussion: The authors should explain further why they propose a recessive effect of the T-allele on of hypertension. How could that be a conclusion from the fact that the allele frequency in the present study was below the CI of frequencies in an earlier meta-analysis (ref # 7)? Can recessive inheritance exist in a polygenic condition?” We thank the reviewer for pointing out this wrong statement. What we meant was that the T- allele’s inheritance may follow a recessive pattern. However, as hypertension is a polygenic and multicausal disease, this is an observation with regards to the genotype that may never become visible in the phenotype. The genotype can follow a recessive inheritance pattern. However, the polygeny may obscure the inheritance pattern of the phenotype. We have changed the sentence accordingly: “This may reveal the specific genetic background of this particular German population, suggest a recessive inheritance pattern of the T allele, and maybe provide an opportunity to test the recently proposed method to analyze for the origin of dominant and recessive traits[4]”

Minor essential revisions: The manuscript was adjusted accordingly. In particular, the reviewer noted the use of “Nagel” to denominate the present study in figure 4,
and asked whether the results have been published before. This is not really the case. We had submitted a partial analysis, with M.N. as first author because of his ASN membership, to the ASN 2003 conference, where it was accepted for “publication only” (PUB 435 in the abstract book). Maybe due to this, A-L. Z. denominated the present study in this way in Fig. 4. With regards to the comment on Frossard’s[5] study on the associations of AGT mutations with hypertension in Arabs, it is true that no significant association has been found. However, Frossard stresses the point several times that the T frequency decreases with age (last paragraph of discussion), to the extent of putting this finding in prominent position in the abstract (last sentence). As we have never stated that Frossard’s finding was statistically significant, we would prefer to leave the text unchanged as in the original manuscript. We use this reference only as basis for discussion of potential influences that might bias the findings of our own study.

**Discretionary revisions:**

The dissertation (ref. 3) is available online at http://diss.ub.uni-duesseldorf.de/ebib/diss/show?dissid=474

**Response to Dr. Bantis:** We believe much has been addressed already in the response to Dr. Bostrom, so we cover only the remaining questions.

1. Impact of ACE and AGT polymorphisms on phenotype: We have included the following sentence in the background section: “Subjects carrying the ACE D allele have unanimously been shown to have increased ACE serum activity[6, 7], while the T235 AGT variant has been associated with elevated angiotensinogen levels[8].”

2. The term isoform has been replaced by genotype throughout.

3. The numbering of tables has been corrected.

4. Definition of subgroups in Siani’s article is described in the results section. Briefly, he combines the M235T AGT with the I/D ACE variants, and A1166C of the AT2R gene and C344T of the CYP11B2 gene, and then proceeds to do multiple comparisons.

5. Power analysis:
   Consider the equation \( n = 10.51 \cdot p_A^2(1 - p_A)^2/D_A^2 \).
Where: \( n \) = number of individuals needed for 5% test with 90% power
\( p_A \): population gene frequency of allele A
\( D_A \): disequilibrium coefficient

When \( p_A = 0.5 \), the above equation is maximised, holding the other variables constant. We let \( D_A = 0.1 \), \( p_A = 0.5 \) and we will get \( n \approx 66 \). Thus, given our overall sample size being so much bigger, multiple comparison does not impact inappropriately on the statistical power.

**References** within responses. Please note that some, but not all of the articles are also referred to in the main manuscript. We chose this separate reference list to avoid confusion.

7. B Rigat, C Hubert, F Alhenc-Gelas, F Cambien, P Corvol, F Soubrier: An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene