Author's response to reviews

Title: Measurement of absolute copy number variation reveals association with essential hypertension

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Author's response to reviews: see over
Dear Dr Broët

Thank you for your positive letter and the Reviewers’ comments.

We have now addressed all of the comments. The changes are detailed on the following pages. As a result we provide a revised version that we believe should be acceptable for publication in *BMC Medical Genomics*.

We look forward to learning of your decision subsequent to evaluation of the emendations required.

Very best wishes

Yours sincerely

Fadi Charchar
RESPONSE TO COMMENTS BY EDITOR

The Editor’s comments are quoted first (in italics), followed by our response.

*This work is interesting but some essential revisions are required by the reviewers. Additional work should be done before the final decision.*

We have now addressed the comments by the reviewers and we believe it should be in a format to be accepted for publication.

*Please update your ethics statement to include the name of the ethics committees that approved your study.*

We have now added in page 4: “All subjects gave informed consent and the study was approved by the human research ethics committee at the Alfred Hospital, Melbourne, Australia.” and in page 5: “All subjects gave informed consent and the study was approved by the human research ethics committee at the Medical University of Silesia, Poland.”
RESPONSE TO COMMENTS BY REVIEWERS

The Reviewers’ comments are quoted first (in italics), followed by our response.

Referee #1 Alexandre Stewart

*The manuscript by Marques et al., “Measurement of absolute copy number variation reveals association with essential hypertension”, is of interest to the readership of BMC Molecular Genomics. In this manuscript the authors used a technique of droplet digital PCR to quantify copy number variants in the vicinity of genes previously identified by GWAS to associate with blood pressure using a sample of phenotypic extreme cases and controls.*

The question posted by the authors is well defined and the methods are appropriate and well described. The data seem straightforward and relatively sound.

R: We thank the reviewer for his positive comments.

**Major concerns**

In addition to providing blood pressures after “adjustment” using some factor to account for antihypertensive medication in their extreme cases of hypertension, the authors should provide actual blood pressure measurements before and after initiation of medication (where possible) or actual blood pressure measurements even on antihypertensive meds.

R: The blood pressure of those on antihypertensive therapy previous to treatment was not known. It is common practice to adjust the blood pressure by adding 10 mm Hg to systolic blood pressure and 5 mm Hg to diastolic blood pressure in those taking antihypertensive medication. It was previously described by Cui *et al.* (2003, *Hypertension*) that “the use of measured pressures from treated subjects is not ideal; they represent a biased distortion in a quantitative analysis because treatment lowers blood pressure and is usually applied to those with the highest values. (...) A potential solution is to adjust measured pressures in treated subjects so that they better reflect the inherent untreated levels.” The adjustment is based on the average treatment effects described by Neaton *et al.*, (1993, *JAMA*). We have added a short explanation (page 4): “Briefly, 10 mm Hg was added to systolic blood pressure and 5 mm Hg to diastolic blood pressure, as previously described.”

Our main result is that a deletion in the CNVs esv27061 and esv2757747 is significantly more prevalent in those with extreme high blood pressure. Since this is a case-control study, the results do not change if adjusted or unadjusted blood pressure is used.

Also, the nomenclature for copy number variants seems outdated. The authors must provide genomic landmark (start and end of CNV and chromosome number) and preferably should use the esv or essv identifiers so that readers can readily refer to their sequences in the genome browser.

R: The genomic landmark is described in both Table 1 and Table S1. We have updated the nomenclature of the CNVs (and thus the genomic landmark) as requested by the reviewer.

**Minor revisions: none**
Otherwise the manuscript adheres to the relevant standards for reporting and data deposition. The discussion is fine and the limitations are clearly stated. The authors acknowledge work upon which their study builds and the title and abstract appropriately reflect the content.

R: We thank the reviewer for his positive comments.
Referee #2 Hoh Boon Peng

This paper attempts to report an association study of essential hypertension with CNVs located in the regions of GWAS signals, using a new technology namely droplet digital PCR (ddPCR). As far as I concern, association studies between CNV and essential hypertension have been relatively lacking, hence I feel that this manuscript valid for publication. Although using a relatively small number of samples, the selection of case-control using the extreme ends approach has greatly increased the power of study, thus the statistical analysis is believed to be reliable.

R: We thank the reviewer for his positive comments.

**Major concern:**
My major concern lies on the quality of the copy number calls. The authors acknowledge the challenges in accurately calling the CNV, and that spurious CN calls could lead to false association. It is therefore important to convince the readers by presenting the robustness and the quality of the ddPCR CN calls, at least as an Additional File. This can be done by plotting:
i) a histogram by clustering the raw CN calls into bins of 0.1
ii) scatter plot graph with the raw CNV calls with replicates
iii) scatter plot graph of FAM vs VIC intensity

 Ideally, a distinct cluster around an integer should be seen.

R: We have now added figures showing independent replicates, scatter plot graphs with calls, and FAM and VIC intensity to the online supplemental file (now Figure S2), to reassure the quality of our copy number calls.

The CNV 3306 is too long, 3.3Mb, comprising 37 genes. Although it is shown to be significantly associated with hypertension, the size confers considerable challenge to identify gene(s) responsible for blood pressure. I suspect the GWA signal rs2932538 could help narrowing down the gene searching.

R: We thank the reviewer for his suggestion, but finding which gene was driving the association was outside the scope of this manuscript. As stated in page 4 “the hypothesis of our study is that changes in genomic copy number may be associated with essential hypertension and/or BP variation but may have been missed in previous studies.” Association studies using SNPs only show that the region is associated with blood pressure and/or hypertension, and the SNP (or gene where the SNP is located) associated with the condition might not be the one driving it. One example is the recent findings by Smemo et al., (2014, Nature), showing that SNPs located in the intron of the FTO gene, and found associated with the risk for obesity and type 2 diabetes, had a functional role in the expression of the gene IRX3, located several megabases from the SNPs.

**Minor correction:**
Background (pg 3): “In contrast to SNPs,… varying from 10 kilobases to 5 megabases…” I would prefer to define the CNV with size range from 1 KB to several MBs, as proposed by Redon et al (2006), although I acknowledge the potential of false positive calls with CNV less than 10 KB.
R: We have now changed this sentence to (page 3): “In contrast to SNPs, copy number variations (CNVs) are large polymorphisms such as insertions, deletions, translocations and inversions of genomic material varying from 1 kilobase to several megabases.”

**Methods (pg 6):** “Denaturation and … between positive and negative droplets (Table 1)” I presume it meant “(Table S1)”?

R: We thank the reviewer for spotting this mistake. We meant Table S1. This has now been fixed.

**Results (pg 8):** “… but no significant difference in SBP… (Figures 1E and 2F)”

*** I could not locate Figure 2F. Is that a typo?

R: We thank the reviewer for spotting this typo. We meant Figure 1F. This has now been fixed.

**In Legend to Figure:** “… while (E) there was a significant decrease in DBP in the extreme high BP group (* indicates P=0.024), and (F) no change in SBP…”

*** The DBP figure should be (F); and the SBP figure should be (E).

R: We have now changed the text according to the suggestion by the reviewer (page 8): “Subjects from extreme high BP group with 3 copies of CNV dgv1306e1 had no significant difference in SBP (–4.5 mmHg, P=0.308) than those with 2 copies, but they had significantly lower DBP (–5.5 mmHg, P=0.024) (Figures 1E and 1F).”
Reviewer #3 Xiangyin Kong

The authors used a new approach named ddPCR to validate the CNVs overlapped with significant genomic regions, which associated with blood pressure in previous GWA studies in two groups of extreme phenotype patients. They identified a deletion of the CNVs 3306 and 64617 were significantly more prevalent in extreme high BP subjects. The results presented here are reasonable and will be a reference for CNVs quantitation.

R: We thank the reviewer for his positive comments.

However, here I have two suggestions:
1. The author selected the CNVs, which overlapped with the reported significant genomic regions using DGV database. However, the boundaries of CNVs may be quite different from the real variant for the method of CNV detecting, which means the overlap between a CNV and a gene may not be accurate. So here I suggest the author consider the CNVs nearby the candidate genomic regions. Moreover, the author mentioned that some genes do not map within the candidate CNV (i.e. CNV 4094) in the newest human genome browser (GRCh37/hg19). Therefore the authors need to use hg19 to annotate the CNVs and genome regions in the selection process.

R: We have updated the nomenclature of the CNVs and the genome regions described in the manuscript.

2. It needs to use some other method to quantify the relative quantity of CNVs as control.

R: There is no gold standard for the measurement of CNVs, therefore making it difficult to use another (better) method to quantify CNVs as a control. Most studies in the literature have used real-time quantitative PCR (qPCR). Recent findings from us (Pinheiro et al., 2012 Anal Chem) and others (Strain et al., 2013 PLoS One; Morisset et al., 2013 PLoS One; Lock et al., 2014 Hum Gene Ther Methods; Bharuthram et al., 2014 Infect Genet Evol), however, have shown that droplet digital PCR (ddPCR), the technique used in this manuscript, is more reliable and accurate than qPCR for the quantification of DNA and CNVs. For example, the difference between having 2 or 3 copies is only 0.5 cycle threshold (Ct) in a qPCR, which is usually only consider a pipetting error. Bharuthram et al., (2014, Infect Genet Evol) concluded in their study that “droplet digital PCR was shown to be a far superior method to qPCR for assessment of CCL4 gene copy number variation, the accuracy of which is essential for studies of the contribution of variable gene copy number to phenotypic outcomes of host infection and disease course.” The only more robust method for measurement of CNVs would be whole-genome sequencing, which is not possible due to the high cost for a large cohort and low sample availability. Using a less reliable technique to quantify CNVs as a control would not add to the study and quality of the data presented here.