Author's response to reviews

Title: A comprehensive investigation of variants in genes encoding adiponectin (ADIPOQ) and its receptors (ADIPOR1/R2), and their association with serum adiponectin, type 2 diabetes, insulin resistance and the metabolic syndrome.

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Author's response to reviews: see over
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Author's response to reviews: see over
A comprehensive investigation of variants in genes encoding adiponectin (ADIPOQ) and its receptors (ADIPOR1/R2), and their association with serum adiponectin, type 2 diabetes, insulin resistance and the metabolic syndrome.

Response to reviewer(s)' and Editor's Comments to Author:

All changes to the manuscript are marked in colored, underlined type (track changes).

We would like to thank the reviewers for their comprehensive review of this paper. We believe it is greatly improved through their input.

Reviewer: 1

General Comments:
In this report, KE Peters and colleagues assessed the association of tagged SNPs in the ADIPOQ, ADIPOR1 and ADIPOR2 genes with serum adiponectin level and metabolic traits in two population-based studies and one T2D case sample of European-Australian individuals. They found not surprisingly an association between SNPs in ADIPOQ (but not in ADIPOR1 and ADIPOR2) and adiponectin level and no association of the SNPs in the three genes with metabolic traits.

Major Comments:
1. Gene selection: I do not see the interest to study the association of SNPs in ADIPOR1 and ADIPOR2 genes with adiponectin level. Indeed multiple studies already tested this hypothesis and did not find any significant association. Choosing SNPs in the 11 loci (in addition to ADIPOQ) identified by the five GWAS for serum adiponectin level would have been a far more informative strategy.

Authors Response:
The ADIPOR 1 and ADIPOR 2 tagged SNPs for this project were chosen using HapMap and selecting SNPs that were spaced across the genes. The authors agree that there is no evidence associating these genes with adiponectin levels but there is evidence in the literature linking SNPs in these genes to insulin resistance (Obesity Reviews 2007;8:419–423, Hum Genet. 2009;125: 21–28). Previous studies have been conducted in small populations while our study was performed in a large cohort that consisted of a type 2 diabetic and a non-diabetic subjects and in our opinion further investigation was warranted in this large population.

2. The authors may test which of the nine SNPs is an independent contributor to adiponectin variation using conditional regression models. Several of the nine SNPs are likely to be in modest linkage disequilibrium with each other and may represent redundant association signals.

Authors Response:
The following was added to the results section:
“To determine which of the 9 SNPs identified in the meta-analysis independently contribute to adiponectin variation, we conducted a conditional regression analysis in all three cohorts; this analysis showed that 3 of the top 9 SNPs were independently associated with adiponectin levels in the BHS, including rs12637534, rs16861209 and rs17366568. Similar
results were found in the other two studies, where both rs16861209 and rs17366568 remained significant in FDS, while rs16861209, rs17366568 and rs1648707 were significant in CUDAS."

3. Were the T2D case cohort and the two population-based cohorts recruited in different geographical area of Australia? If yes, did the authors adjust the tests for population stratification? Population stratification, if not properly taken into account, can lead to false negative and false positive results.

Authors Response:
All three studies recruited individuals from the Western Australian population. For the Busselton Health Study, we have genome-wide data which was used to generate principle components for population stratification in the EIGENSTRAT software (Ref: Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, et al. (2006) Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet 38: 904-909). These principle components reveal no obvious population stratification, with low lambda values for other phenotypes tested. Given the area in which the other studies were recruited, we have no reason to believe that there is population stratification that will affect the conclusions of this manuscript.

4. Methods: ‘A random (10%) sample was analyzed in duplicate, with reproducibility found to be 98%’. As the authors double-genotyped 64 SNPs, we expect them to describe a range of concordance rate (e.g. SNP individual concordance rates were comprised between 97.1 and 100%) rather than a single value. Is the 98% concordance rate a mean value? A general rule in the field is to discard SNPs that did not display a concordance rate > 99%. Please be more specific.

Authors Response:
The following was added to the methods section:
….reproducibility found to be 100% for the Busselton population and 99.9% for both CUDAS and FDS populations. Individual SNP concordance rates were between 99.3 and 100%.

5. Methods: I do not think that adjusting adiponectin genetic association tests for BMI make any sense. Previous studies have shown an association between SNPs in ADIPOQ and obesity-related traits (Bouatia-Naji et al Diabetes 2006, Siitonene et al BMC Med Genet 2011, Yu et al Obesity 2012), so BMI cannot be considered as a confounding factor in this specific context.

Authors Response:
In this specific analysis, we wanted to investigate whether the SNPs were associated with adiponectin level; by adjusting for BMI we are ensuring that the association we see is not due to an underlying effect between the SNPs and obesity, rather a genetic effect directly with adiponectin levels themselves.

6. I do not think relevant to include the T2D case study in the meta-analysis of quantitative traits. As the authors acknowledge themselves, important discrepancies are found between the T2D sample and the two population-based studies (higher BMI, lower adiponectin…). The disease status and anti-diabetic treatments have a major impact on QT values. I recommend using this cohort only in the T2D case control study.

Authors Response:
We included the T2D case study (FDS) in the meta-analysis of quantitative traits as, although we saw differences in the mean values of the traits, the genetic variants displayed similar effect size changes in the traits. In addition, the heterogeneity between the studies from the meta-analysis appeared to be low, however we conducted a random-effects meta-analysis to account for possible differences between the studies. For completeness, we conducted the meta-analysis without the FDS, and the same conclusions were made.

The following was added to the results section:

“As FDS displayed different mean levels of adiponectin and other quantitative traits, a second meta-analysis was conducted without the FDS study and similar results were found (data not shown)”

7. 17% of the SNPs genotyped using the Illumina Golden Gate technology do not pass the quality control criteria. How the authors explain this abnormally high rate of SNP genotyping failure?

Authors Response:
The sentence in the results section has been changed as follows: “Eleven of the sixty-four genotyped tSNPs, five failed and six were monomorphic quality control checks, leaving fifty-three.”.

8. Discussion: ‘Despite putative effects of the adiponectin receptors on tissue adiponectin activity, we found no evidence that tSNPs in ADIPOQ, or ADIPOR1/R2 were associated with insulin resistance, MetS or T2D. This supports the findings of three large studies [4, 32, 41]. I do not agree with this comment. Dastani et al (PLOS Genet 2012) (ref 32) evidenced that a multi-SNP genotypic risk score for adiponectin-decreasing alleles (including a SNP in ADIPOQ) was associated with BMI, WHR, fasting insulin, HOMA-IR, 2-hour post OGTT glucose, T2D, triglycerides and HDL-cholesterol. In order to gain statistical power in their analysis, the authors may assess the association of these metabolic traits using a multiple SNP genotype risk score that includes SNPs independently associated with adiponectin level. If an association is found between the gene score and a specific metabolic trait, a post-hoc analysis further adjusting for serum adiponectin may help to understand if the association between SNPs in ADIPOQ and the metabolic trait is mediated by circulating adiponectin (mendelian randomization).

Authors Response:
We looked at an allele score of the top 9 SNPs in our study and the 3 independent SNP identified in the conditional analysis; neither of these allele scores were significantly associated with HOMA-IR or MetS. We have decided not to incorporate this analysis into the manuscript, but have removed the reference 32 as supporting our finding. We did find a significant association between the allele score of the top 9 and 3 SNP independent SNPs and diabetes in the case-control study.

The following was added to the results section:

“There was a significant association between the allele score of the top nine SNPs and T2D in the case-control study without adjusting for adiponectin levels (OR=0.94, 95%CI=(0.91,0.98), P=0.0015) and this association strengthened after adjusting for adiponectin levels (OR=0.92, 95%CI=(0.89, 0.96), P=2.43x10^-5). The allele score of the 3 independent SNPs was significantly associated with T2D only after adjusting for adiponectin levels (OR=0.86, 95%CI=(0.75, 0.99), P= 0.0314).”

The following was added to the results section of the abstract:
A multi-SNP genotypic risk score for ADIPOQ alleles revealed that after adjusting for adiponectin levels an association with 3 independent SNPs; rs12637534, rs16861209, rs17366568 and type 2 diabetes (OR=0.86, 95%CI=(0.75, 0.99), P=0.0134).

The following was added to the conclusion of the abstract:
.."genetic variation in ADIPOQ and its receptors does not appear to contribute to the risk of insulin resistance or metabolic syndrome but did for type 2 diabetes in a European-Australian population.

The following changes have been made to the discussion section:
Despite putative effects of the adiponectin receptors on tissue adiponectin activity, we found no evidence that tSNPs in ADIPOQ, or ADIPOR1/R2 were associated with either insulin resistance, or MetS. This supports the findings of two other studies [4, 41]. However, we did find a significant association between the allele score of the top nine SNPs and T2D in the case-control study without adjusting for adiponectin levels. Multi-SNP genotypic risk score for adiponectin-decreasing alleles has been associated with BMI, WHR, fasting insulin, HOMA-IR, 2-hour post OGTT glucose, T2D, triglycerides and HDL-cholesterol [31]. In the current study a post-hoc analysis revealed that adjusting for adiponectin levels strengthened the association with 3 independent SNPs; rs12637534, rs16861209, rs17366568 and T2D. The strengthening of this association provides strong evidence that the genetic determinants of adiponectin levels are shared with T2D using a Mendelian randomisation approach[13].

There have been numerous studies investigating the association of adiponectin gene and related polymorphisms with T2D. Most of these have used small sample sizes with results being inconclusive and conflicting. However, Two recent large meta-analyses showed that the G vs C allele of rs266729 might be a risk factor for T2D [42, 43], while the rs17300539 A allele was shown to be a risk factor only in European Caucasians [43]. The latter SNP is in complete LD with rs16861209 that we have identified in our Caucasian populations. Despite the fact that rs266729 was not successfully genotyped in the current study, LD with other tSNPs suggests that this SNP is not associated with T2D in our cohort. Likewise, rs17300539, which was tagged by rs16861209, was not found to be associated with T2D in the present study. Although this may be due to insufficient power in our cohort of people with diabetes, our findings could be useful when combined with other large cohorts in Mendelian randomisation studies [13], to investigate further the potential aetiological relationship of total circulating adiponectin level with cardiometabolic disease.

The following was added to the methods section:
“In addition to investigating the tSNPs independently, an allele score was created by summing the number of risk alleles an individual had in those tSNPs that were shown to be associated with adiponectin levels in the meta-analysis.”

Minor Comments:
1. Abstract: please detail the sample size of the three cohorts.

Authors Response:
Sample sizes have been added to the abstract.

2. Is HOMA-IR normally distributed? Logarithm transformations of HOMA-IR have been reported in literature (Cauchi et al Diabetes 2006).

Authors Response:
We used log transformed HOMA-IR (lnHOMA-IR) in the analyses, and have updated the manuscript to reflect this.
3. Do haplotypes explain more variation of serum adiponectin than single SNPs tagging the same LD blocks?

Authors Response:
We have added the amount of variation explained collectively by the haplotypes in each block and by the SNPs in each block for both the BHS and CUDAS studies.

The following sentences have been added to the results section:
“In all haplotype blocks, the single SNPs tagging the block accounted for the same, or slightly more of the variation in adiponectin levels than the haplotype block itself (difference of between 0-0.8% between the tSNPs and the haplotypes).”

4. The lack of replication of the association between adiponectin and several SNPs previously identified in GWAS is intriguing. As several rare population-specific coding variants have been reported to be strongly associated with adiponectin level (Vasseur et al. HMG 2002, Croteau-Chonka et al. HMG 2012, Warren et al. Diabetes 2012) one explanation for the observed discrepancies may be synthetic associations (the fact that population-specific rare variants are in partial linkage disequilibrium with common variants) (Dickson et al. PLOS Biol 2010). This may be mentioned in the discussion.

Authors Response:
This suggested comment has now been included in the discussion.

5. The in silico functional tests for SNPs have little value without confirmatory in vitro studies. Please discard the discussion about in silico experiments, as the discussion is too long.

Authors Response:
The in silico sections in the methods, results and discussion have been deleted.

6. Please do not cite and discuss the results of the reference 36. This study is clearly underpowered and has not scientific value.

Authors Response:
The following has been deleted from the discussion.
Broedl et al. [36] found a 3-SNP haplotype in ADIPOR2 (rs16928751-Ile290Ile-rs9805042) that was associated with increased serum adiponectin, although the sample size used in the study was small (n=20). SNPs rs16928751 and rs9805042 are both tagged by rs2058112 in our study which was not associated with serum adiponectin.

7. As the power of the present study is limited, the lack of association of SNPs in ADIPOQ with metabolic traits must be interpreted with caution in the discussion (negative result or lack of power to evidence a subtle genetic effect).

Authors Response:
This point is covered in the discussion in the following section.

“Likewise, rs17300539, which was tagged by rs16861209, was not found to be associated with T2D in the present study. Although this may be due to insufficient power in our cohort of people with diabetes, our findings could be useful when combined with other large cohorts in Mendelian randomisation studies [13], to investigate further the potential aetiological relationship of total circulating adiponectin level with cardiometabolic disease.”
Reviewer: 2

General Comments:
The authors have performed a tag SNP-based association analysis of three adiponectin-related genes (ADIPOQ, ADIPOR1, and ADIPOR2) with total adiponectin level and risk of type 2 diabetes and metabolic syndrome in three European-Australian populations. They find that associated common variants in ADIPOQ explain a modest proportion of variation in adiponectin level consistent with other studies, but do not find evidence of association of these SNPs with metabolic phenotypes.

Major Comments:
1. Figure 1 is missing from the submission.

Authors Response:
Figure 1 has now been included.

2. In the eighth paragraph of the discussion, the authors make a claim about "insufficient power" to detect associations with T2D. Formal power calculations for detecting adiponectin and T2D/MetS associations and for the study's replication capability should be included in the results section.

The following was added to the methods section:
"A power calculation, performed in the Quanto software (reference: Gauderman WJ (2002) Sample size requirements for association studies of gene–gene interaction. Am J Epidemiol 155:478–484), indicated that this study has at least 90% power at an alpha level of p=0.05 to detect an odds ratio for diabetes (population risk of 10%) of 1.45 for a minor allele frequency of 0.05 and an odds ratio of 1.2 for a minor allele frequency of 0.45, under an additive genetic model."

3. The results of the haplotype analyses need more context in the discussion. How much more collectively informative are these results than merely the single-SNP associations alone, say in terms of percent phenotypic variation explained? How might the findings motivate further genetic or molecular characterizations of the ADIPOQ locus?

Authors Response:
This point has been covered under reviewer one, minor comment 3.

4. Much of the fourth paragraph of the discussion reads as results text and so should be moved to the last section of the results about in silico analyses, which itself should have more detail. In place of this discussion text, the authors might talk about how the specific transcription factors that bind sites overlapping the associated SNPs are related to adiponectin biology.

Authors Response:
In silico discussion has been removed from the manuscript as suggested by reviewer 1.

Minor Comments:
1. In the first sentence of the conclusions, the indefinite article is missing before "well phenotyped general population".

Authors Response:
This sentence has been changed to “comprising 3,322 well phenotyped subjects”.
2. Under the “Selection and genotyping of tSNPs” section in the results, it is not specified how the tag SNP selection was actually performed. Was it done manually or using Haploview?

Authors Response:
This sentence has been changed to “tSNPs were selected using Haploview to represent the common genetic variation of each gene, including an additional 10kb upstream and downstream, as well as variants previously reported in the literature to be associated with serum adiponectin levels”.

3. The exact number of control individuals used in the T2D case-control analysis isn’t specified. It would be helpful to list in Table 1 the number of non-diabetic subjects for the BHS/CUDAS subjects and list that total in the results section under “Analysis of association between tSNPs and T2D, HOMA-IR and MetS”.

Authors Response:
All BHS and CUDAS subjects were non-diabetic. We have added the following statement to reflect this in the Methods section:
“The BHS and CUDAS cohorts are representative of the general population and do not contain individuals with T2D, while FDS…..”

4. In the last paragraph of the results section called “Genotype distribution and linkage disequilibrium”, there should either be a comma added between “from each pair” and "i.e." or "i.e." should be replaced with a colon.

Authors Response:
The sentence has been changed to read ..” one tSNP from each pair: rs1648707…”.

5. In the second paragraph of the discussion, the last two sentences about association results in the additional SNPs tagged don’t really belong in the discussion as they aren’t mentioned in the results section. The authors also make a point in the results about reporting only the associations of SNPs that were not strongly correlated with each other.

Authors Response:
These two sentences have been deleted.

6. In the second-to-last sentence of the conclusions, the authors state that the "present study shows that circulating adiponectin is affected by gene-environment interactions" when no formal GxE analyses have been performed. This sentence should be re-phrased or dropped.

Authors Response:
This sentence has been deleted.

7. In the acknowledgements, "principle investigator" should be "principal investigator" and there should be a "the" in "version of manuscript".

Authors Response:
Both these points have been fixed.

Discretionary Revisions
1. In the first sentence of the third paragraph of the introduction, points i) and ii) appear redundant. They should be combined and the numbering dropped.
Authors Response:
The following changes to this sentence have been made: "..Adiponectin cellular signalling is mediated by two adiponectin receptors, 1 and 2, with down regulation or altered function of these proteins believed to affect the activity of adiponectin. The genes for these adiponectin receptors 1 and 2 (ADIPOR1 and ADIPOR2), although generally not..

2. Given the low observed heterogeneity in the meta-analysis results, wouldn’t a fixed-effects meta-analysis have had a little more statistical power? Perhaps a sentence stating that association results not shown were consistent using this approach.

Authors Response:
As in the response to reviewer 1, question 6, we were concerned the particularly the type 2 diabetes study (FDS) may be different to the two general population cohorts. For this reason, we conducted a random-effects meta-analysis. For completeness, we conducted a fixed effects meta-analysis for those SNPs where no heterogeneity was detected and the results remained the consistent (data not shown) and hence the conclusions stay the same.

3. In the results, it isn't clear to me whether the three tSNPs showing significant departure from HWE were dropped from later analysis. If so, perhaps it should be explicitly stated in the results.

Authors Response:
These 3 SNPs were not dropped from the analysis.

4. A citation for Wu et al. (2010) (PMID: 20876611) would be appropriate in the fifth paragraph of the discussion as it is actually the first adiponectin GWAS by a very slim margin to report the CDH13 signal as genome-wide significant (in this case, in an Asian population).

Authors Response:
This reference has been included in the text.

5. The authors state in the sixth paragraph of the discussion that more complete sequencing of the ADIPOQ region should be performed. A paper by Warren et al. (2012) (PMID: 22403302) describes just such an effort in ~14,000 subjects and would be worth discussing briefly.

Authors Response:
This reference has been included in the text.