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# Genetic Variation in Inflammatory and Bone Turnover Pathways and Risk of Osteolytic Responses to Prosthetic Materials

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# Genetic Variation in Inflammatory and Bone Turnover Pathways and Risk of Osteolytic Responses to Prosthetic Materials

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**Running title:** Genetic variation and osteolysis susceptibility

Genetic variation and osteolysis susceptibility

**ABSTRACT**

Wear particle-induced inflammatory bone loss (osteolysis) is the leading cause of total hip arthroplasty (THA) failure. Individual susceptibility to osteolysis is modulated by genetic variation. In this 2-stage case-control association study we examined whether variation within candidate genes in inflammatory and bone turnover signaling pathways associates with susceptibility to osteolysis and time to prosthesis failure. We examined two cohorts, comprising 758 (347 male) Caucasian subjects who had undergone THA with a metal on polyethylene bearing couple; 315 of whom had developed osteolysis. Key genes within inflammatory, bone resorption, and bone formation pathways were screened for common variants by pairwise-SNP tagging using a 2-stage association analysis approach. In the discovery cohort 4 SNPs within RANK, and 1 each within *KREMEN2*, *OPG*, *SFRP1*, and *TIRAP* ($p<0.05$) were associated with osteolysis susceptibility. Two SNPs within *LRP6*, and 1 each within *LRP5*, *NOD2, SOST, SQSTM1, TIRAP* and *TRAM* associated with time to implant failure ($p<0.05$). Meta-analysis of the 2 cohorts identified 4 SNPs within *RANK*, and one each within *KREMEN2*, OPG, *SFRP1*, and *TIRAP* associated with osteolysis susceptibility ($p<0.05$). Genetic variation within inflammatory signaling and bone turnover pathways may play a role in susceptibility to osteolysis.

**Key words:** hip; arthroplasty, osteolysis; genetics, innate immunity, inflammation

Genetic variation and osteolysis susceptibility

## INTRODUCTION

A recent meta-analysis of worldwide total joint register data has shown that 12% of first (primary) THRs fail within 10 years of implantation, requiring revision surgery[1]. Adverse local tissue reactions to prosthesis materials characterized by periprosthetic osteolysis and resulting in prosthesis loosening accounts for 60% of these failures [2].

Osteolysis arises as a cell-mediated adverse inflammatory immune response to the wear debris materials shed from the implant surfaces[3; 4]. Several investigators have shown that particulate debris from prosthetic materials initiate inflammatory signaling through pattern recognition receptors (PRR) including toll-like receptors (TLRs)[5-9]. Furthermore, TLRs are expressed in osteolytic membrane taken from patients with failing prostheses[10; 11]. Bone turnover is closely regulated by the equilibrium between osteoblasts and osteoclasts that is regulated by the interplay between the Wnt and RANK signaling pathways[12; 13]. P2 purinergic receptors are important regulators of both inflammation and bone remodeling[14].

Patients vary in their osteolytic response to particulate wear debris[15]. Macrophage responses to a particulate challenge in-vitro varies between individuals[16; 17], and monocytes taken from patients with a susceptibility to osteolysis exhibit greater inducible cytokine responses to titanium particles compared to patients without this susceptibility[18]. Candidate gene studies also show that single nucleotide polymorphisms (SNP) within several pro-inflammatory cytokines associate with osteolysis susceptibility[19-24]. Similarly, variations within several genes involved in the regulation of bone turnover associate with osteolysis susceptibility[24-27]. To date these studies have focused on specific candidate variants within genes that are known to regulate inflammatory responses or bone turnover, and thus have not explored for other potential variants within or adjacent to candidate genes.

Genetic variation and osteolysis susceptibility

Here we used a 2-stage genetic case-control association study design to identify susceptibility loci for osteolysis and quantitative trait loci (QTL) for time to prosthesis failure within pivotal genes that modulate inflammatory signaling and bone turnover. We applied a SNP-tagging approach to systematically and economically screen for common variants both within in the flanking regulatory regions of the genes studied.

## METHODS

Local ethics committees approved the studies and all subjects provided informed written consent prior to participation. All subjects were of Caucasian North European background and had received a cemented primary THA with a metal on conventional polyethylene bearing couple for idiopathic osteoarthritis. Subjects who had undergone THA for inflammatory arthropathy, neck of femur fracture, secondary osteoarthritis or suspicion of infection were excluded.

### Discovery Cohort

The 'discovery' cohort included patients recruited from Northern England between April 2000 and April 2006. The definitions of cases and controls, inclusion and exclusion criteria have been described previously[21]. Briefly, cases comprised patients undergoing revision surgery for osteolysis or aseptic loosening, and controls were patients who had undergone THA greater than 7 years previously and had no current radiographic evidence of osteolysis or prosthesis loosening. Polyethylene wear in both patient groups was quantitated as previously described[15; 28], and used as an analysis covariate.

### Replication Cohort

The 'replication' cohort was recruited from the North West of England between 2002 and 2004. The definitions of cases and controls, inclusion and exclusion criteria are described

elsewhere[24]. Briefly, cases comprised patients undergoing revision surgery for osteolysis or aseptic loosening within 6 years of implantation, and controls were patients who had undergone THA at least 10 years previously and were currently asymptomatic, and had no radiographic evidence of osteolysis or aseptic loosening.

**Genotyping**

DNA was extracted from peripheral whole blood using standard methods and genotyped by competitive allele specific PCR (KASP), (LGC Genomics, Hoddesdon, UK). Tagging SNP selection was performed using Hapmap Gene Browser (release #24, phase 1 and 2 – full dataset, www.hapmap.ncbi.nlm.nih.gov) and Haploview software (v4.2, www.broadinstitute.org/haploview/haploview) using a pairwise tagging approach ($r^2$=0.8). Common variants (MAF≥0.05) within the gene of interest, and extending 5Kb upstream and 2Kb downstream to include variants within the adjacent regulatory flanking sequences, were tagged using this approach. The following genes were selected for genotyping: *MD2, MSK1, MSK2, MyD88, NOD1, NOD2, P2Y1, P2Y6, P2X7, SQSTM1, TLR1, TLR2, TLR4, TLR5, TLR6, TLR9, TRAM, TIRAP, TRIF* (inflammatory signaling); *DKK1, KREMEN2, LRP5, LRP6, SFRP1, SOST, Wnt3A* (bone formation); *TNFRSF11A* (encoding RANK), *TNFRSF11B* (encoding OPG), and *TNFSF11* (encoding RANK) (bone resorption). A total of 318 SNPs were directly genotyped using this approach (full tagging SNP details given in Supplementary Table 1).

**Association analysis**

Quality control (QC) and association analyses were carried out using an open source whole genome association analysis toolset (PLINK version 1.07, www.pngu.mgh.harvard.edu/~purcell/plink). Subjects with missing phenotype data and variants that were incompletely typed across the population were excluded from analysis, as

were SNPs that failed Hardy-Weinberg equilibrium at $p>0.05$. Two genotype-phenotype association analyses were performed: A case-control association analysis for susceptibility loci for osteolysis, and a quantitative trait analysis (QTL) for time to prosthesis failure (cases only and defined as the time from insertion to osteolysis diagnosis) using linear regression. Signals reaching a statistical significance threshold of $p<0.05$ in the discovery cohort after adjustment for age, sex, time since surgery, and annual prosthesis wear rate were taken forward for analysis in the replication cohort. Finally, case-control and time-to-failure meta-analyses of signals genotyped in both cohorts were performed using Genome Wide Association Meta-Analysis (GWAMA) software (version 1.4, Wellcome Trust Sanger Institute, Cambridge, UK).

## RESULTS

Seven hundred and fifty eight subjects were included in the analyses, of whom 315 formed the osteolysis group. The discovery cohort comprised 631 patients (275 osteolysis cases). The replication cohort comprised 127 patients (40 osteolysis cases). Subjects in the osteolysis group were younger, a greater proportion were male and they had higher annual polyethylene wear rate versus the control subjects (Table 1, $p<0.05$). These findings are consistent with known risk factors for osteolysis and were included as covariates in the association analyses.

### Osteolysis susceptibility

In the discovery case-control association analysis 4 SNPs within *TNFRSF11A*, including rs4524033, rs9960450, rs7226991, and rs4485469, and 1 each within *KREMEN2* (rs4786361), *SFRP1* (rs921142), *TIRAP* (rs8177375), and *TNFRSF11B* (rs11573847), met the significance threshold of $p<0.05$ for carriage forward into the replication analysis (Table 2). At replication, 4 of these SNPs showed an association in the same direction as for the

discovery cohort (rs4524033, rs7226991, and rs4485469, all in *TNFRSF11A*; and rs921142 in *SFRP1*), but none reached statistical significance. Meta-analysis of the 2 cohorts identified 8 SNPs which associated with susceptibility to osteolysis (Figure 1, $p<0.05$). Four of these signals lay within *TNFRSF11A*, and 1 each within *TNFRSF11B, SFRP1, KREMEN2*, and *TIRAP*.

**Time to Failure**

Two SNPs within *LRP6* (rs10743980 and rs2417086), and 1 each within *LRP5* (rs606989) *NOD2* (rs5743289), *SOST* (rs851056), *SQSTM1* (rs155788), *TIRAP* (rs1786704), and *TRAM* (rs10079000) associated with time to implant failure in cases at $p<0.05$, and were carried forward (Table 3). At replication 1 SNP in *TIRAP* (rs1786704) showed the same direction of association with the discovery cohort, but none reached statistical significance, and no SNPs were associated with time to failure following meta-analysis of the 2 cohorts (Figure 2, $p>0.05$).

## DISCUSSION

We conducted a 2-stage case-controlled association study to identify common genetic variants that associate with susceptibility to osteolysis, and with time to diagnosis in cases. At meta-analysis of the 2 stages we identified 8 SNPs that weakly associated with osteolysis susceptibility, 7 of these lay within bone resorption (6 signals) or bone formation (2 signals) pathways, and 1 within genes that regulate inflammatory signaling (*TIRAP*).

Variants within *TNFRSF11A* (encoding RANK) and *TNFRSF11B* (encoding OPG) showed the strongest association with susceptibility to osteolysis, and are consistent with the function of these genes in regulating osteoclast differentiation and activation. The functional

role of these variants is uncertain, as all lie in non-coding regions of the gene. However, they may act as markers for non-synonymous SNPs or regulate transcription and translation through effects on mRNA stability, splicing, or binding of miRNAs.

Variation within bone formation pathways associated with osteolysis, and previously showed variation within *SFRP3* associated with both osteolysis and heterotopic ossification susceptibility.[25] *SFRP1* is also a Wnt antagonist. *SFRP1*-/- mice have high trabecular bone mass,[29] and inhibition of *SFRP1* activity associates with increased bone formation.[30] The rs921142 variant within *SFRP1* lies in the 5'UTR region and may thus affect stability of the RNA transcript Kremen2 is a transmembrane protein that blocks the LRP6 receptor inhibiting Wnt signalling, and Kremen2 deficient mice have increased bone formation.[31] Our finding of an association between osteolysis and variation within Kremen-2 is previously unreported.

We found fewer than anticipated associations within the inflammatory signaling pathways, given their reported importance to osteolysis. The only consistent association found was between the *TIRAP* rs8177375 variant and susceptibility to implant failure. This variant also showed a trend toward association with time to prosthesis failure in the osteolysis cases (β—1.12, p=0.08), but failed to meet the threshold for genotyping in the replication cohort. TIRAP is an adaptor protein that modulates many inflammatory pathways, including inflammatory signaling through TLR-4, and also transduces signals from TLR2.[9] This *TIRAP* variant is located in the 3' UTR and associates with risk of sepsis-induced lung injury [32].

A preliminary bioinformatics analysis suggests that the rs8177375 G allele encodes for a miRNA binding site (has-miR-1236) that is likely to increase mRNA degradation and/or regulate translation. In line with this, a miRNA1236 binding site, sharing 100% homology with the *TIRAP* sequence has recently been shown to regulate the expression of VEGFR-3 in endothelial cells.[33] miRNA1236 expression is upregulated in these cells in response to IL1,

suggesting that this miRNA may be involved in the regulation of inflammation. In this context, miRNA1236 was a regulator of translation, rather than mRNA degradation *per se*.

Similar to the strategy we have used recently to the verifying functional relevance of a SNP in *TNF* [34], we have generated two reporter constructs, encoding for Renilla luciferase under the control of the constitutive EF1 promoter, and incorporating the A or G allele of the *TIRAP* 3'UTR. We have transfected these constructs into Raw 264.7 cells and measured the Renilla activity. Data from three independent experiments suggest that the luciferase signal for the 'G' allele containing construct is approximately 40% reduced (Figure 3), in line with the prediction of increased mRNA degradation and/or inhibition of translation. This observation may offer a mechanistic model for the association between *TIRAP* variation and osteolysis susceptibility through an effect on TLR4 or TLR2 signaling.

We used knowledge of the haplotype architecture of candidate genes to conduct a screen for common variants across the whole of each gene, including its regulatory flanking sequences. We used a 2 stage design to reduce the likelihood of false positive associations, carrying forward to replication only those signals reaching a significance threshold of $p<0.05$ in the discovery cohort. This approach also has several limitations. Although the presented sample size combines the 2 largest cohort studies of the genetics of osteolysis reported to date[21; 24], it remains relatively small in genetic association study terms. We aimed to address the issue of multiple testing and false positive associations through a 2-stage analysis process, followed by meta-analysis of the datasets only for signals undergoing replication analysis. Our study had only power to detect common variants with relatively large effects sizes and with modest p-values. For example this design had 80% power (at $p<0.0005$, joint analysis, multiplicative model, CaTS),[35] to detect a variant with an OR of 1.5 with a MAF of 0.35, but only 7% power to detect a variant with similar effect size and a MAF of 0.05. Although our

design aimed to reduce the number of candidate SNPs genotyped without loss of sensitivity for identifying disease loci by using Hapmap data on their haplotype structure, candidate pathways and genes were selected based on a-priori knowledge of their importance to the pathogenesis of osteolysis.

Current genotyping technology allows genome-wide scanning for disease-susceptibility loci, and with advanced imputation methodologies derived from haplotype datasets such as the UK10K initiative (http://www.uk10k.org/), high-resolution screens for rare variants with modest effect sizes are now feasible, but require substantial increases in osteolysis cohort size if such approaches are to be applied to the field of ALTR to prosthetic materials. Only through such approaches can the true genetic architecture of these responses be determined.

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Genetic variation and osteolysis susceptibility

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Genetic variation and osteolysis susceptibility

| Subject characteristics | Control Group | Osteolysis Group | P value |
|---|---|---|---|
| *Discovery cohort* | *n=356* | *n=275* | |
| Age at THA (years)[a] | 65±8 | 59±9 | <0.001 |
| Sex (male/female)[b] | 147/209 | 148/127 | 0.002 |
| Osteolysis free survival (years)[c] | 12±4 | 10±5 | <0.001 |
| Polyethylene wear (mm) | 0.77(0.49 to 1.23) | 1.18 (0.75 to 1.91) | <0.001 |
| *Replication cohort* | *n=87* | *n=40* | |
| Age at THA (years)[a] | 72±8 | 69±7 | 0.03 |
| Sex (male/female)[b] | 29/58 | 23/17 | <0.001 |
| Osteolysis free survival (years)[c] | 18±6 | 4±1 | <0.001 |

**Table 1. Characteristics of study subjects.** Analysis is cases versus controls within each cohort by [a]Student's t-test, [b]Chi-squared test, or [c]Mann-Whitney U test, as appropriate.

| Gene | Chromosome | SNP ID | Minor allele | OR (95% CI) | P value |
|---|---|---|---|---|---|
| *TNFRSF11A* | 18 | rs4524033 | A | 1.69 (1.24 to 2.31) | 0.0009 |
| *SFRP1* | 8 | rs921142 | G | 1.32 (1.04 to 1.69) | 0.025 |
| *TNFRSF11A* | 18 | rs9960450 | C | 0.50 (0.28 to 0.93) | 0.025 |
| *TNFRSF11B* | 8 | rs11573847 | G | 1.67 (1.06 to 2.62) | 0.027 |
| *KREMEN2* | 16 | rs4786361 | A | 0.75 (0.57 to 0.97) | 0.028 |
| *TNFRSF11A* | 18 | rs7226991 | A | 0.73 (0.55 to 0.97) | 0.031 |
| *TIRAP* | 11 | rs8177375 | G | 0.65 (0.43 to 0.98 | 0.040 |
| *TNFRSF11A* | 18 | rs4485469 | G | 0.77 (0.59 to 1.00) | 0.046 |

**Table 2. Discovery cohort osteolysis susceptibility loci meeting the replication threshold (P<0.05).** Analysis is cases (n=275) versus controls (n=356) using PLINK (version 1.07).

Genetic variation and osteolysis susceptibility

| Gene | Chromosome | SNP | Minor allele | BETA (95% CI) | P |
|---|---|---|---|---|---|
| *LRP6* | 12 | rs10743980 | T | -0.91 (-1.69 to -0.14) | 0.022 |
| *SOST* | 17 | rs851056 | G | -0.86 (-1.62 to -0.11) | 0.026 |
| *TRAM* | 5 | rs10079000 | A | -0.91 (-1.73 to -0.10) | 0.029 |
| *SQSTM1* | 5 | rs155788 | C | -0.91 (-1.72 to -0.10) | 0.029 |
| *NOD2* | 16 | rs5743289 | T | 1.03 (0.10 to 1.97) | 0.030 |
| *LRP6* | 12 | rs2417086 | G | -0.84 (-1.60 to -0.07) | 0.033 |
| *TIRAP* | 11 | rs1786704 | C | -0.95 (-1.85 to -0.05) | 0.039 |
| *LRP5* | 11 | rs606989 | T | 1.60 (0.07 to 3.13) | 0.041 |

**Table 3. Discovery cohort quantitative trait loci associated with time to prosthesis failure in cases (n=275) meeting replication threshold (P<0.05).** Analysis made by linear regression using PLINK (version 1.07).

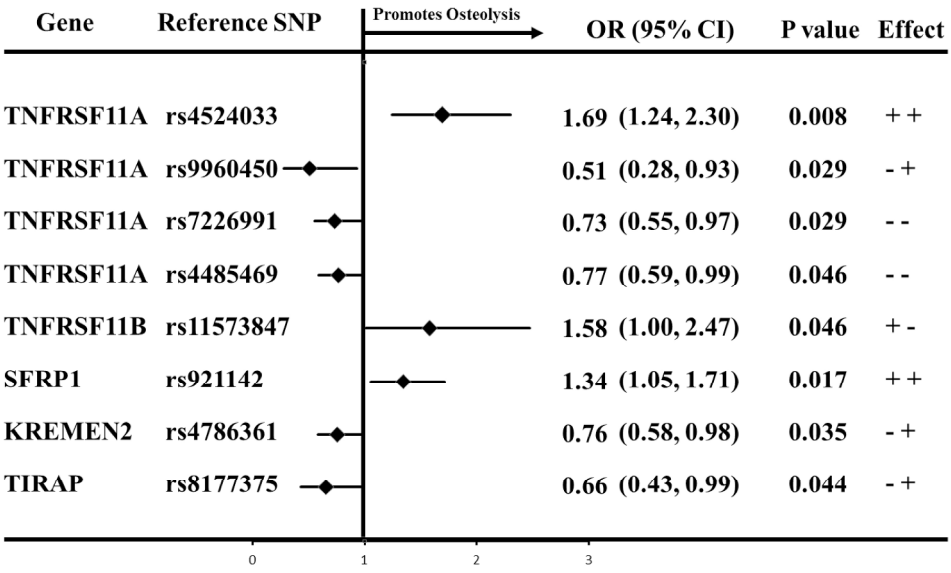
Genetic variation and osteolysis susceptibility

## LEGEND TO FIGURES

**Figure 1. Meta-analysis discovery and replication cohorts of susceptibility loci for development of osteolysis.** Analysis is cases (n=315) verses controls (443) across discovery and replication cohorts and made using GWAMA software (version 1.4). The effect column indicates the effect direction of the given allele in the stage 1 and stage 2 cohorts.

**Figure 2. Meta-analysis discovery and replication cohorts of quantitative trait loci associated with time to prosthesis failure.** Analysis is within cases only (n=315) across discovery and replication cohorts and made using GWAMA software (version 1.4). The effect column indicates the effect direction of the given allele in the stage 1 and stage 2 cohorts.

**Figure 3. Renilla activity of the 2 TIRAP 8177375 variants**. Raw 264.7 cells were transiently transfected with a CMV-driven firefly luciferase construct, in combination with an EF1 promoter driven Renilla luciferase reporter, that contained either the A or G allele of the 3'UTR of the TIRAP mRNA. Cells were lysed 24 hrs post-transfection and reporter activity was measured by a dual luciferase assay. Renilla activity was normalized to that of the firefly within the same sample and expressed as a relative value. Unpaired t-test was used for statistical analysis (* $p<0.05$)




Meta-analysis discovery and replication cohorts of susceptibility loci for development of osteolysis. Analysis is cases (n=315) verses controls (443) across discovery and replication cohorts and made using GWAMA software (version 1.4). The effect column indicates the effect direction of the given allele in the stage 1 and stage 2 cohorts.
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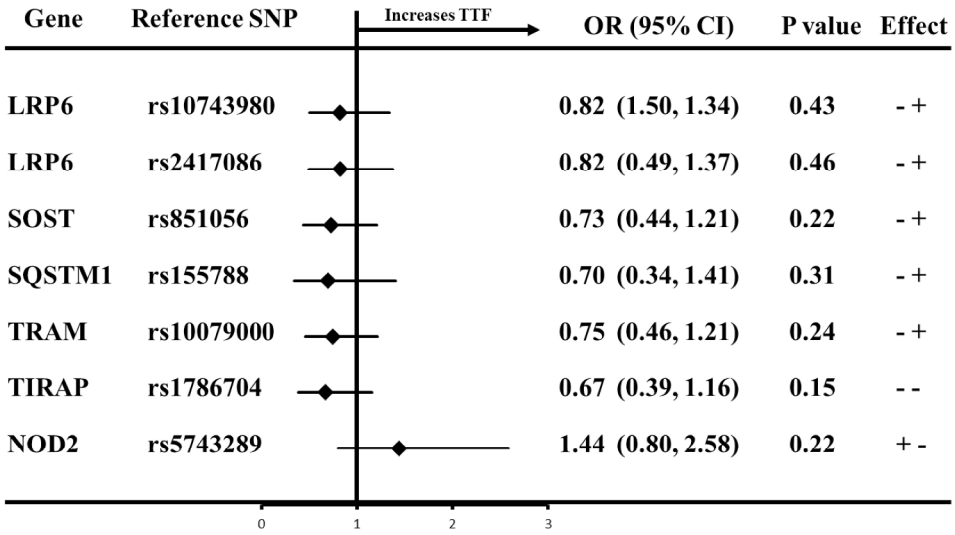
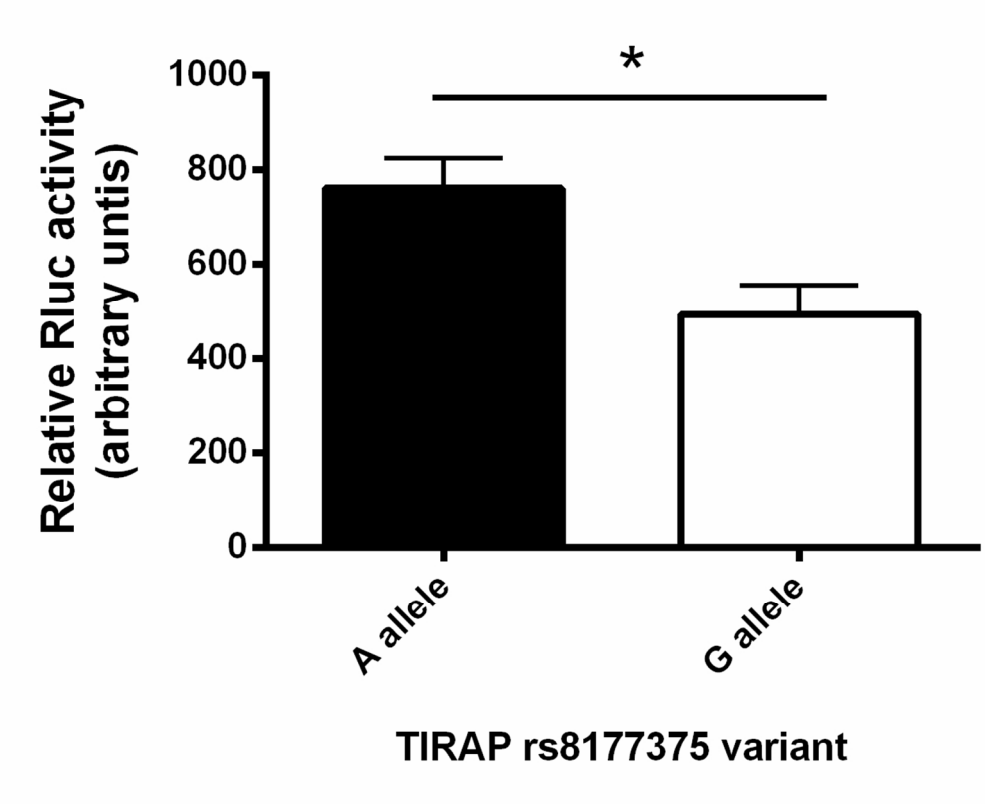



Meta-analysis discovery and replication cohorts of quantitative trait loci associated with time to prosthesis failure. Analysis is within cases only (n=315) across discovery and replication cohorts and made using GWAMA software (version 1.4). The effect column indicates the effect direction of the given allele in the stage 1 and stage 2 cohorts.
282x156mm (300 x 300 DPI)




Renilla activity of the 2 TIRAP 8177375 variants. Raw 264.7 cells were transiently transfected with a CMV-driven firefly luciferase construct, in combination with an EF1 promoter driven Renilla luciferase reporter, that contained either the A or G allele of the 3'UTR of the TIRAP mRNA. Cells were lysed 24 hrs post-transfection and reporter activity was measured by a dual luciferase assay. Renilla activity was normalized to that of the firefly within the same sample and expressed as a relative value. Unpaired t-test was used for statistical analysis (* $p<0.05$)
107x86mm (300 x 300 DPI)