

## Genetic Variation in the *SMAD3* Gene Is Associated With Hip and Knee Osteoarthritis

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**Objective.** *Smad3* (or, *MADH3*) is a key intracellular messenger in the transforming growth factor  $\beta$  signaling pathway. In mice, *Smad3* deficiency accelerates growth plate chondrocyte maturation and leads to an osteoarthritis (OA)-like disease. We undertook this study to investigate the role of genetic variation in *SMAD3* in the risk of large-joint OA in humans.

**Methods.** Ten tag single-nucleotide polymorphisms (SNPs) in the *SMAD3* gene region were tested in a discovery set: 313 patients who had undergone total knee replacement, 214 patients who had undergone total hip replacement, and 520 controls from the UK. The SNP associated with both hip and knee OA was subse-

quently genotyped in 1,221 controls and 1,074 cases from 2 cohorts of patients with hip OA and 2,537 controls and 1,575 cases from 4 cohorts of patients with knee OA.

**Results.** A SNP (rs12901499) mapping to intron 1 of *SMAD3* was associated with both knee and hip OA ( $P < 0.0022$  and  $P < 0.021$ , respectively) in the discovery set. In all study cohorts, the major allele (G) was increased among OA patients relative to controls. A meta-analysis for knee OA yielded an odds ratio (OR) of 1.22 (95% confidence interval [95% CI] 1.12–1.34),  $P < 7.5 \times 10^{-6}$ . For hip OA, the OR was 1.22 (95% CI 1.09–1.36),  $P < 4.0 \times 10^{-4}$ . No evidence for heterogeneity was found ( $I^2 = 0\%$ ).

**Conclusion.** Our data indicate that genetic variation in the *SMAD3* gene is involved in the risk of both hip OA and knee OA in European populations, confirming the results from animal models on the potential importance of this molecule in the pathogenesis of OA.

Transforming growth factor  $\beta$  (TGF $\beta$ ) is a pleiotropic cytokine/growth factor with important anabolic effects on chondrocytes. It stimulates proteoglycan and type II collagen synthesis, can down-regulate cartilage-degrading enzymes, and is able to counteract interleukin-1-induced suppression of proteoglycan synthesis (1). Increasing evidence suggests that TGF $\beta$  plays an important role in the pathogenesis and progression of osteoarthritis (OA). This role in OA is likely to derive from its contribution to the maintenance of the stable phenotype in articular chondrocytes (for review, see ref. 2). TGF $\beta$  signals mainly through the TGF $\beta$  type I and type II transmembrane serine/threonine protein kinase receptors and the Smad signaling cascade. The pathway is initiated by C-terminal phosphorylation of the intracellular mediators Smad2 and/or Smad3 (also known as MADH3) by activated TGF $\beta$  receptors. Upon activa-

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tion, Smad2 and Smad3 oligomerize with Smad4 and translocate to the nucleus, where they interact with DNA, transcription factors, coactivators, and/or corepressors to modulate the transcription of target genes (3).

TGF $\beta$ /Smad3 signaling has been shown to be essential for maintaining articular cartilage, and mutant mice homozygous for a targeted disruption of *Smad3* exon 8 develop degenerative joint disease resembling human OA, as characterized by chondrocyte hypertrophy in the articular cartilage, with the presence of type X collagen-positive cells, progressive loss of the joint surface, formation of large osteophytes, and decreased production of proteoglycans in synovial joints (4). No studies have yet investigated whether genetic variation in

this molecule also contributes to the risk of OA in humans, although a mutation in the *SMAD3* gene seen in a single OA patient has been characterized (5). The investigators in that study screened 32 patients with knee OA and 50 controls from China and found that 1 of the OA patients carried an Asp197Ile mutation that was accompanied by higher expression of matrix metalloproteinases 2 and 9 (5).

In the present study, we investigated whether common variations in the *SMAD3* gene are associated with large-joint OA in individuals of European descent. We tested 10 tag single-nucleotide polymorphisms (SNPs) mapping to the *SMAD3* gene region and, having identified a polymorphism associated with both hip and knee OA in a discovery set, we assessed whether this

**Table 1.** Descriptive characteristics of study subjects by cohort, with allele counts and major (risk) allele frequencies of the *SMAD3* intron 1 polymorphism rs12901499\*

	Discovery set, Nottingham (OA patients)/TwinsUK (controls)	Replication sets			
		Nottingham	Hertfordshire	Estonia	Chingford
Type of study	Case-control	Case-control	Population-based	Population-based	Population-based
No. of OA patients/no. of controls <sup>†</sup>	477/520	2,014/733	167/867	68/449	317/488
Origin	UK	UK	UK	Estonia	UK
Age, mean $\pm$ SD years	60.4 $\pm$ 8.2	67.2 $\pm$ 8.9	65.5 $\pm$ 2.7	47.1 $\pm$ 6.4	64.1 $\pm$ 6.0
BMI, mean $\pm$ SD kg/m <sup>2</sup>	26.4 $\pm$ 4.6	28.0 $\pm$ 4.9	27.1 $\pm$ 4.3	28.1 $\pm$ 5.4	26.7 $\pm$ 4.7
Women, %	83	58	49	69	100
Controls					
No. of subjects	520	733	867	449	488
Definition	TF K/L grade <2, hip K/L grade <2	No radiographic OA of hip or knee	TF K/L grade <2	TF K/L grade <2	TF K/L grade <2 and no definite hip JSN
SNP rs12901499					
No. with A/G allele	489/551	698/768	779/955	481/417	477/499
% M/F with G allele	NA/53	52/53	54/56	46/46	NA/51
Knee OA					
No. of patients	313	1,083	167	68	257
Definition	TKR	TF K/L grade $\geq$ 2 (73% TKR)	TF K/L grade $\geq$ 2	TF K/L grade $\geq$ 2	TF K/L grade $\geq$ 2
SNP rs12901499					
No. with A/G allele	246/380	938/1,228	134/200	67/79	234/280
% M/F with G allele	58/62	57/57	63/58	55/49	NA/54
OR (95% CI)	1.30 (1.08–1.57) <sup>‡</sup>	1.18 (1.02–1.36) <sup>‡</sup>	1.27 (0.98–1.65) <sup>‡</sup>	1.10 (0.73–1.66) <sup>‡</sup>	1.24 (0.99–1.57) <sup>§</sup>
Hip OA					
No. of patients	214	979	NA	NA	95
Definition	THR	Croft grade $\geq$ 3 (91% THR)	NA	NA	Definite JSN
SNP rs12901499					
No. with A/G allele	173/255	850/1,108	NA	NA	82/108
% M/F with G allele	60/60	57/56	NA	NA	NA/57
OR (95% CI)	1.34 (0.97–1.86) <sup>‡</sup>	1.19 (1.03–1.36) <sup>‡</sup>	NA	NA	1.23 (0.90–1.69) <sup>§</sup>

\* OA = osteoarthritis; TF = tibiofemoral; K/L = Kellgren/Lawrence; JSN = joint space narrowing; SNP = single-nucleotide polymorphism; NA = not applicable; TKR = total knee replacement; OR = odds ratio; 95% CI = 95% confidence interval; THR = total hip replacement.

<sup>†</sup> Number of OA cases, including individuals with knee OA or hip OA or with both hip and knee OA. The number of individuals with OA of both the knee and the hip is the number listed in this row minus the sum of the number of individuals with exclusively knee OA and the number of individuals with exclusively hip OA listed in the rows below.

<sup>‡</sup> Adjusted for age, sex, and body mass index (BMI).

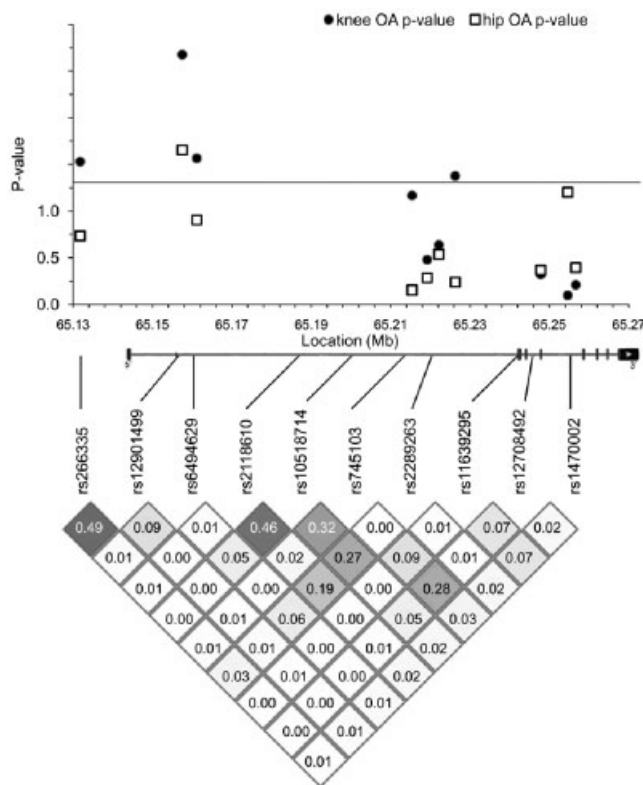
<sup>§</sup> Adjusted for age and BMI.

effect was reproducible in 4 independent cohorts of patients with knee OA and 2 independent cohorts of patients with hip OA.

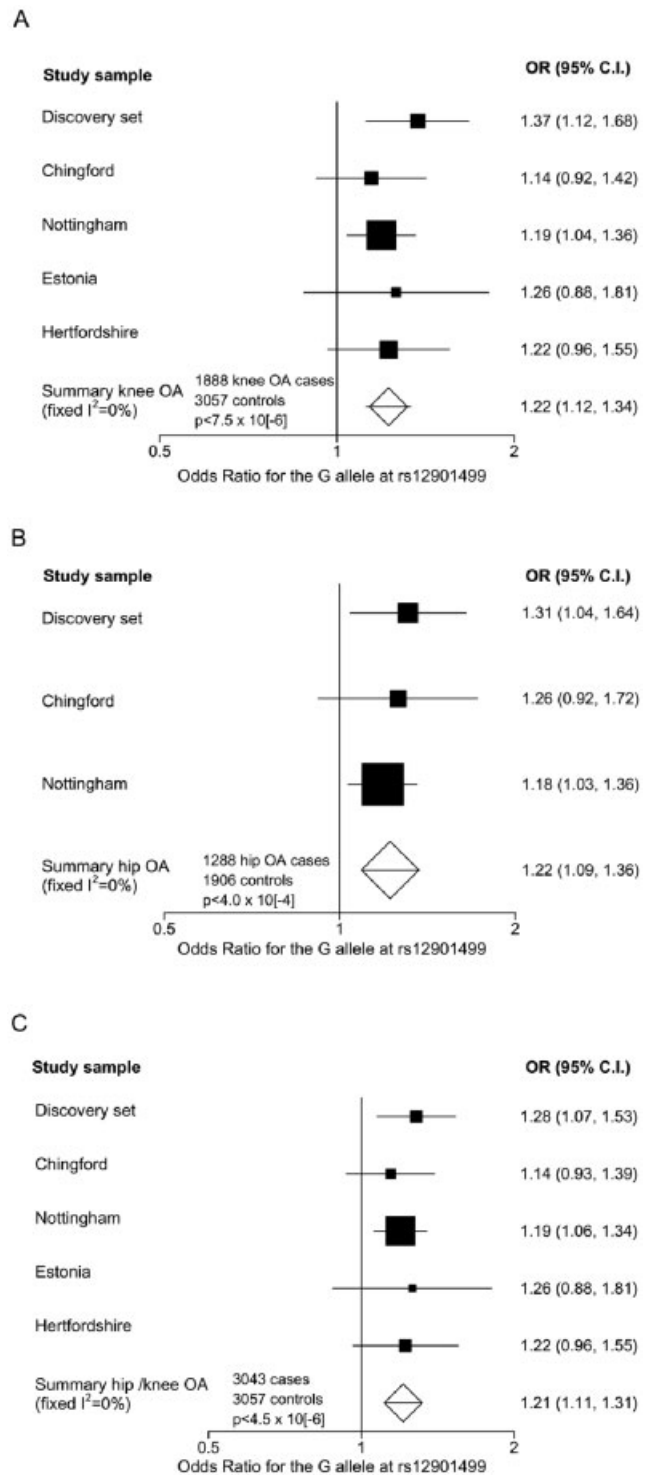
**PATIENTS AND METHODS**

**Study subjects.** *Discovery set.* Five hundred twenty unrelated women from the TwinsUK cohort (further information is available at <http://www.treatoa.eu/publicpdfs/SupplementaryMaterialAr-09-1758.pdf>) who had no radiographic knee or hip OA served as a control discovery sample. In addition, 313 patients who had undergone total knee replacement (TKR) and 214 patients who had undergone total hip replacement (THR) from the Nottingham case-control study were used to identify polymorphisms within the *SMAD3* gene associated with large joint OA.

*Replication sets.* A detailed description of each study cohort, recruitment, and radiographic and clinical assessments is available at <http://www.treatoa.eu/publicpdfs/SupplementaryMaterialAr-09-1758.pdf>. Study subjects from the UK came



**Figure 1.** Single-nucleotide polymorphisms (SNPs) mapping to the *SMAD3* gene region on chromosome 15, along with linkage disequilibrium ( $r^2$ ) in the discovery set controls. The  $r^2$  value is shown inside each square, with darker squares having higher  $r^2$  values. The gene structure corresponds to GenBank accession numbers NM\_005902.2/NP\_005893.1. The  $-\log_{10}$  of the P values is shown for genetic association of each of the 10 SNPs with hip and knee osteoarthritis (OA). The horizontal line indicates the P value of 0.05.



**Figure 2.** Forest plot of study-specific unadjusted estimates and fixed-effects summary odds ratios (ORs) and 95% confidence intervals (95% CIs) for the association between the G (major) allele at *SMAD3* polymorphism rs12901499 and knee osteoarthritis (OA) (A), hip OA (B), and either knee or hip OA (C).

from a case-control study in Nottingham, and population-based cohorts were from Chingford (North London) and Hertfordshire. Patients with knee and hip OA from Nottingham who were not part of the initial discovery experiment were compared with 733 controls from the same catchment area. A population-based cohort from Estonia was also included. All 4 studies were approved by the relevant Ethics Committee, and informed consent was obtained from all study participants.

A total of 2 hip OA replication sets and 4 knee OA replication sets were tested, totaling 1,221 controls and 1,074 cases for the hip OA study and 2,537 controls and 1,575 cases for the knee OA study. The descriptive characteristics of each cohort, including the diagnosis criteria, are presented in Table 1.

**Genotyping.** The control samples from the discovery set underwent genome-wide genotyping using the Illumina 300 and Illumina 600 Duo assays (6). For the Nottingham, Hertfordshire, Chingford, and Estonian study participants, genomic DNA was extracted from their blood using standard protocols. Genotyping was carried out by KBioscience. SNPs were genotyped using the KASPar chemistry, which is a competitive allele-specific polymerase chain reaction SNP genotyping system using fluorescence resonance energy transfer quencher cassette oligos. Genotyping accuracy was 99.6%, as determined from the genotype concordance between 250 duplicate samples. The polymorphisms studied were in Hardy-Weinberg equilibrium in all case and control sets tested separately ( $P > 0.40$ ), as assessed using Haploview version 4.1 software (<http://www.broad.mit.edu/mpg/haploview/>).

**SNP selection.** The *SMAD3* gene in humans maps to chromosome 15 from position 65,145,249 to position 65,274,586. We used genome-wide genotyping data available in the TwinsUK cohort in 520 women who had no radiographic evidence of knee or hip OA (further information is available at <http://www.treatoa.eu/publicpdfs/SupplementaryMaterial/Ar-09-1758.pdf>). Genotyping data in these control samples mapping to the *SMAD3* gene region were used to select SNPs to tag the genetic variation within this gene. From this subgroup, 10 tag SNPs with a minor allele frequency  $\geq 30\%$  were selected to tag blocks with a squared correlation coefficient ( $r^2$ )  $> 0.65$ . The subset of 10 SNPs was then assessed in HapMap ([www.hapmap.org](http://www.hapmap.org)) data from subjects of European descent using the Haploview pairwise tagger (version 4.1) and the HapMap National Center for Biotechnology Information B36 Rel24/phase II Nov08 genotype data from 90 samples of European descent. There are 88 markers mapping to the *SMAD3* gene in HapMap with a minor allele frequency  $\geq 30\%$ . The 10 SNPs genotyped in our study tag 50 of those 88 alleles (56.8%), with  $r^2 \geq 0.65$  and mean maximum  $r^2 = 0.824$ . The linkage disequilibrium between SNPs in our control samples as well as the positions along chromosome 15 are shown in Figure 1.

**Statistical analysis.** Allele odds ratios (ORs) were calculated by comparing the numbers among cases and controls, and  $P$  values were computed using Pearson's chi-square test. In the absence of interstudy heterogeneity within samples, we constructed a Mantel-Haenszel meta-analysis of data from the samples to assess the overall evidence of association. The Mantel-Haenszel chi-square test and estimate of the OR (7) were computed with or without the inclusion of covariates using the Meta-Analyst software (<http://tuftscaes.org/>

meta\_analyst/) (8). The assumption of heterogeneity for each analysis was tested using the DerSimonian-Laird method (7).

## RESULTS

The 10 selected tag SNPs were genotyped in the discovery set of 313 TKR cases and 214 THR cases. The  $P$  values for genetic association with hip OA and knee OA are shown in Figure 1. Four SNPs (rs266335, rs12901499, rs6494629, and rs2289263) were found to be nominally significantly associated with knee OA ( $P < 0.05$ ), but only 1 of them, rs12901499, was nominally associated also with hip OA ( $P < 0.021$ ) (Figure 1). This SNP was the only one associated with knee OA after adjusting for multiple testing ( $P = 0.0211$  with Bonferroni correction). Two of the SNPs typed in this study (rs266335 and rs12901499) map to the 14-SNP haplotype block (as defined by European descent data from HapMap) in which rs12901499 is located. One of them is located 5' of the *SMAD3* gene (rs266335), and rs12901499 maps to intron 1 ( $r^2 = 0.49$  between both) (Figure 1). We investigated whether any haplotype defined by these 2 markers would result in a stronger association that would suggest the need for finer mapping. We found that the strongest association was seen with the variation defined by rs12901499 rather than by any haplotype (results not shown). Therefore, we pursued only this marker for replication in 2 additional cohorts of patients with hip OA and 4 additional cohorts of patients with knee OA.

Table 1 shows the SNP allele counts in the cases and controls, with descriptive statistics for each cohort. In every instance, we observed that the major allele G was found at a higher frequency among OA patients than among controls. We then carried out a meta-analysis of this SNP with knee OA (Figure 2A), hip OA (Figure 2B), and either knee or hip OA (Figure 2C). For all 3 analyses, we found no evidence of heterogeneity of the genetic effect ( $I^2 = 0\%$ ,  $Q$  [4df] = 1.80,  $P < 0.77$  for knee OA;  $I^2 = 0\%$ ,  $Q$  [2df] = 0.58,  $P < 0.75$  for hip OA; and  $I^2 = 0\%$ ,  $Q$  [4df] = 0.86,  $P < 0.93$  for combined hip and/or knee OA). A fixed-effect meta-analysis resulted in Mantel-Haenszel ORs of 1.22 (95% confidence interval [95% CI] 1.12–1.34),  $P < 7.5 \times 10^{-6}$  for knee OA, 1.22 (95% CI 1.09–1.36),  $P < 4.0 \times 10^{-4}$  for hip OA, and 1.21 (95% CI 1.11–1.31),  $P < 4.5 \times 10^{-6}$  for combined hip and/or knee OA.

The individual contribution of each cohort was further explored, and a significant association with all traits investigated was seen, excluding 1 cohort at a time (further information is available at <http://www>.

treatoa.eu/publicpdfs/SupplementaryMaterialAr-09-1758.pdf). A significant association with each trait was observed in each sex independently. For knee OA, the association from meta-analysis in women alone was as follows: OR 1.20 (95% CI 1.08–1.34),  $P < 9.5 \times 10^{-4}$ ,  $I^2 = 0\%$ . In men alone, the association was as follows: OR 1.28 (95% CI 1.09–1.51),  $P < 0.0027$ ,  $I^2 = 0\%$ . For hip OA, the association in women alone was as follows: OR 1.21 (95% CI 1.06–1.38),  $P < 0.0044$ ,  $I^2 = 0\%$ . In men alone, the association was as follows: OR 1.24 (95% CI 1.01–1.52),  $P < 0.0417$ .

Summary genetic effect sizes were then computed, adjusting for age, sex, and body mass index (BMI), and the results were similar to the unadjusted estimates: for knee OA, OR 1.22 (95% CI 1.11–1.34),  $P < 2.6 \times 10^{-5}$ ; for hip OA, OR 1.21 (95% CI 1.08–1.36),  $P < 0.00144$ .

## DISCUSSION

In this study, we found convincing evidence that genetic variation in the *SMAD3* gene is involved in genetic susceptibility to large-joint OA. The relevance of this molecule in vivo (4) and in chondrocyte metabolism and function in vitro (9) is well documented, and our data lend support to the extensive body of existing knowledge, while also representing the first demonstration of the involvement of Smad3 in OA in human populations.

Current data suggest that signaling of TGF $\beta$  through its classic pathway involving phosphorylation of Smad2/3 molecules stimulates chondrocyte anabolism and helps maintain the typical phenotype of the articular chondrocyte. Loss of Smad3 appears to enhance bone morphogenetic protein signaling in the articular chondrocytes, leading to hypertrophy and OA-like changes (10). The key role of Smad3 is further supported by the observation that Smurf2 overexpression leads to dephosphorylation of Smad3 and is associated with a spontaneous OA phenotype in transgenic mice (10).

We note that there are some limitations to our study. We cannot claim to have fully tagged the *SMAD3* gene region, and other SNPs in *SMAD3* may show a stronger association that may be identified by finer mapping of this gene.

Moreover, our data apply only to subjects of European descent. Differences in the strength or even the presence of genetic associations have been reported between Asian and European patients (for example, see ref. 11). Therefore, it is possible that this variant may not be associated in other ethnic groups.

The SNP that we found to be associated with large-joint OA is an intronic SNP with no obvious function, and further research is needed to identify the functional role of this variant or to determine whether this variant is in linkage disequilibrium with other variants that affect the levels or function of the *SMAD3* gene product. Of specific interest, a recent study on the role of *SMAD3* in graft-versus-host disease suggested that interindividual differences in *SMAD3* expression levels could not be attributed to in-cis genetic interactions in a panel of 22 SNPs tested (12). It remains to be investigated whether this also applies to articular chondrocytes.

In addition, investigators in the graft-versus-host disease study reported that Smad3 levels are lower in women than in men, which is consistent with other data showing that estrogens inhibit *SMAD3* transcriptional activity (13). Nevertheless, we found that the genetic association of the *SMAD3* intronic SNP with OA was significant in both men and women and that effect sizes were extremely similar between sexes, confirming the robustness of the result.

Another potential limitation of our study is that of the heterogeneity between cohorts and the mixture of radiographic and clinical phenotypes. In terms of the genetic effects, we found no evidence of heterogeneity between cohorts, and the effect sizes were extremely similar if only clinical outcomes (total joint replacement) were used or if radiographic data were also included. Hence, we conclude that this did not bias the overall result. Unlike other genetic associations with OA previously reported (for example, see ref. 14), the effect that we found was almost identical for both hip and knee OA, and the effect was very consistent even after adjusting for age, sex, and BMI. In conclusion, our data support a role for genetic variation in the *SMAD3* gene in the risk of large-joint OA.

## AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Valdes had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study conception and design.** Valdes, Lories, Arden, Michael Doherty. **Acquisition of data.** Valdes, Spector, Agu Tamm, Kisand, Sally A. Doherty, Dennison, Ann Tamm, Kerna, Hart, Wheeler, Cooper, Arden, Michael Doherty.

**Analysis and interpretation of data.** Valdes, Mangino, Lories, Michael Doherty.

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