

Sex and Ethnic Differences in the Association of *ASPN*, *CALM1*, *COL2A1*, *COMP*, and *FRZB* With Genetic Susceptibility to Osteoarthritis of the Knee

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Objective. To assess whether the association of genetic polymorphisms with osteoarthritis (OA) in other populations could be replicated in a large, multi-center, mixed-sex, case-control study of clinical knee OA.

Methods. Genetic polymorphisms in OA candidate genes were genotyped in 298 men and 305 women, ages 50–86 years, all of whom had a diagnosis of knee OA as assessed clinically and radiographically, and in 300 male and 299 female control subjects matched for age and ethnicity. Allele and haplotype frequencies for 5 genes (*ASPN*, *CALM1*, *COL2A1*, *COMP*, and *FRZB*) previously tested for association with hip and/or knee OA in other populations were compared between patients and control subjects, analyzing men and women separately.

Results. The same *FRZB* 2-marker single-nucleotide polymorphism (SNP) haplotype associated with hip OA in other populations of Caucasian women was shown to increase the risk of knee OA among the women (but not the men) in the current study (odds ratio [OR] 2.87, $P < 0.04$). The *CALM1* SNP, which affects the risk of hip OA in Japanese individuals, was not shown to be associated with susceptibility to OA in men or women. *COL2A1* haplotypes were demonstrated to be associated

with a decreased risk of knee OA in men (OR 0.68, $P < 0.005$) but not in women. *COMP* haplotypes that were associated with susceptibility to knee OA were different in men and women ($P < 0.014$ and $P < 0.032$, respectively). A meta-analysis of these data and those from previously published reports indicated a strong association between the *FRZB* G324 allele ($P < 0.0003$) and suggested that an *ASPN* allele is protective against the risk of knee OA in Caucasians ($P < 0.02$).

Conclusion. Our results indicate that genetic polymorphisms affecting knee OA vary between populations (Japanese versus Caucasian) and sexes and indicate a role for *ASPN*, *COMP*, *FRZB*, and *COL2A1* in Caucasians.

Several factors play a role in the risk of osteoarthritis (OA), including age, sex, genetics, ethnicity, behavioral influences, obesity, and occupation (1). In addition, epidemiologic studies in women suggest that estrogen loss may be accompanied by an increase in the prevalence and incidence of knee and hip OA (2), which may help explain the sex differences in the prevalence of OA.

A genetic contribution to OA has been suggested in several epidemiologic studies (3). Twin studies, segregation analyses, linkage analyses, and candidate gene association studies have generated important information about inheritance patterns and the location in the genome of potentially causative mutations, although results across studies are, to date, inconsistent. Linkage and family studies have suggested that both sex-specific and anatomic site-specific genes are likely to influence an individual's risk of developing OA (3,4).

In recent years, there has been considerable success in identifying genes that are involved in susceptibility to primary OA. In the current study, we focused

Supported by the Arthritis Research Campaign.

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Submitted for publication April 2, 2006; accepted in revised form September 22, 2006.

on 5 genes: *ASPN*, *CALMI*, *COL2A1*, *COMP*, and *FRZB*. *FRZB* codes for secreted Frizzled-related protein 3, an antagonist of Wnt signaling. Wnt/ β -catenin signaling regulates chondrocyte phenotype, maturation, and function (5). Through its influence on Wnt signaling, *FRZB* is a powerful and direct modulator of chondrocyte maturation (6). Accelerated cartilage breakdown has been shown to develop in knockout mice deficient in this gene (7). The original study in which an association of *FRZB* with hip OA was reported involved a cohort of women (8); that study showed that the associated alleles at *FRZB* reduced the activity of the protein encoded. In particular, the haplotype composed of substitutions at 2 highly conserved aspartic acid residues in *FRZB* (R200W and R324G) was highlighted as a strong risk factor for primary hip OA. A role for the same alleles/haplotypes in generalized radiographic OA (9) and in hip OA (10) has been reported in other studies in Caucasian populations. Evidence for a differential association of the *FRZB* R200W single-nucleotide polymorphism (SNP) with hip OA and osteoporosis has been reported (11).

Asporin is a member of the leucine-rich repeat (LRR) proteins, a series of noncollagenous glycoproteins that contribute to the regulation of tissue assembly and properties (12). Like similar LRRs such as decorin and biglycan, asporin binds to transforming growth factor (TGF). In particular, asporin suppresses TGF β -mediated expression of the genes encoding 2 cartilage structural component genes, aggrecan and type II collagen, and reduces proteoglycan accumulation in an in vitro model of chondrogenesis. Kizawa and coworkers (13) reported significant association between a polymorphism in the aspartic acid (D) repeat in the asporin-encoding gene (*ASPN*) and knee and hip OA, in 2 independent populations of Japanese individuals. The effect on TGF β activity is allele specific, with the D14 allele resulting in greater inhibition than that associated with other alleles. An association of the same polymorphism with OA was later reported among patients of Greek origin with knee OA (14), but no such association was observed among female patients with hip or knee OA in a Caucasian UK population (although a weaker association was observed among male patients) (15), and no association was observed among Spanish patients of either sex (16).

Calmodulin is an intracellular protein that interacts with several proteins involved in signal transduction. Mechanical compression of articular chondrocytes is known to trigger changes in aggrecan expression, and such changes are dependent on calmodulin signaling

(17). A group of Japanese investigators (18) reported a significant association between hip OA and an SNP (IVS3 -293CT) located in intron 3 of the calmodulin 1 gene (*CALMI*). *CALMI* was expressed in cultured chondrocytes and articular cartilage, and its expression was increased in OA. Subsequent linkage disequilibrium mapping identified 5 SNPs showing significant association equivalent to that of IVS3 -293C>T. Functional analyses indicate that the alleles in the promoter in linkage disequilibrium with this variant decreased *CALMI* transcription in vitro and in vivo. More recently, however, Loughlin and colleagues (19) were unable to replicate those results in a large and well-powered study of UK Caucasian women with hip OA (the Oxford study).

The cartilage oligomeric matrix protein gene (*COMP*) is a member of the thrombospondin gene family, which is known to be expressed more abundantly in OA cartilage than in normal cartilage (20). Prospective studies have shown that elevated serum levels of *COMP* are observed early in patients in whom chronic knee pain without radiographic OA progresses to radiographic disease (21). An association of serum concentrations of *COMP* with prevalent OA has also been reported, for *COMP* alone and in combination with other serum markers (21), and elevated serum levels of *COMP* may be a marker of rapid radiographic progression (22). Several *COMP* mutations produce osteochondral dysplasias (Online Mendelian Inheritance in Man, Johns Hopkins University, Baltimore, MD; online at <http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=600310>). Such disorders produce severe, early-onset OA and are models for common idiopathic OA.

Based on such data, Mabuchi et al (23) hypothesized that OA, as a common disorder, may be at the mild end of the phenotypic gradation produced by *COMP* mutations. Using 6 polymorphisms spanning the entire gene, those investigators examined the association of *COMP* in Japanese patients with OA of the knee and hip joints and observed no statistically significant evidence for association. Given that some genes appear to be associated in Japanese but not Caucasian individuals, we explored the possibility that there is a genetic association between *COMP* variants and knee OA in Caucasians.

Type II collagen is the major collagen in cartilage. Mutations in the type II collagen gene, *COL2A1*, have been observed in various types of chondrodysplasias. Studies performed in the early 1990s could not provide any evidence for an influence of *COL2A1* variants in OA susceptibility (24). Analyses of *COL2A1*

performed a few years later, however, suggested an association of *COL2A1* polymorphisms with hip and knee OA in subjects from the Rotterdam study (25) and in independent Japanese cohorts (26). *COL2A1* is in close physical proximity to the vitamin D receptor gene (*VDR*); however, a group of Dutch investigators showed that both *VDR* and *COL2A1* influence the risk of knee OA in a manner that is not attributable to linkage disequilibrium between the 2 genes (27). Other investigators failed to identify any such association in populations in the US (28) and Finland (29).

The strongest evidence for a real connection between a gene and a disease or trait should come from a systematic replication of a statistically significant association, in which any source of bias or inconsistency has been eliminated (30). After several independent groups of investigators replicate a finding, it seems reasonable to conclude with sufficient certainty that a link between a gene and a disease has been demonstrated (31). Three possible reasons accounting for the inconsistency of results are the different ethnicities of the subjects investigated, the sites of OA that were chosen, and the sex of the patients and controls. We recently reported that genetic associations with knee OA are strongly influenced by sex (32). In the current study, we assessed genetic variants in the above-mentioned 5 genes in an ethnically homogeneous cohort of patients with knee OA, analyzing men and women separately.

PATIENTS AND METHODS

Subjects. Six hundred three Caucasian patients with knee OA (298 men and 305 women) were recruited from families with a history of OA and from clinic populations in Nottingham. OA was assessed both clinically and radiographically. For each patient, standardized anteroposterior radiographs of the knees were obtained with the patient standing and bearing weight. Among patients with knee OA, 44% of women and 25% of men were affected by nodal OA, which was defined as at least 2 rays on each hand affected with Heberden's and/or Bouchard's nodes unrelated to overt trauma, and 11% of women and 7% of men had undergone or were waiting to undergo hip replacement surgery. In addition, 596 age-matched Caucasian control subjects (ages 50–80 years) without signs or symptoms of OA were recruited from 2 centers: Nottingham (111 women and 50 men) and Oxford (185 women and 250 men). Radiographs were not obtained for most control subjects, who were characterized according to clinical criteria. The mean \pm SD age of female patients was 73.5 ± 7.16 years, and that of female controls was 72.1 ± 8.5 years. The mean \pm SD ages of male patients and controls were 72.1 ± 6.9 years and 71.0 ± 7.8 years, respectively. Allele frequencies at each SNP were compared between the Oxford and Nottingham

control groups and between male and female control subjects. No significant differences between either set of controls were observed.

Genotyping. Multiplex polymerase chain reaction (PCR) and SNP analyses were performed using the GenomeLab SNPstream Genotyping System (Beckman Coulter, Fullerton, CA) and the accompanying automated SNPstream software suite. Primers for the multiplex PCR and single-base extension reactions were optimally designed using Web-based software (online at www.autoprimer.com). Following a multiplex PCR, the PCR-amplified fragments were treated with a mix of exonuclease I and shrimp alkaline phosphatase to degrade unincorporated PCR primers and dNTPs. The tagged extension primers were extended using single-labeled TAMRA-fluorescein or Bodipy-fluorescein nucleotide-terminator reactions and spatially resolved by hybridization to the complementary oligonucleotides arrayed on the 384-well SNPware Tag Array microplates (Beckman Coulter).

The Tag Array microplates were imaged using the 2-laser, 2-color CCD-based GenomeLab SNPstream Array Imager. The individual SNPs within each multiplex were identified according to the position of the arrayed oligonucleotides within each well. Genotype data for individual samples were generated on the basis of the relative fluorescence intensities for each spot and were processed for graphic review using the automated SNPstream software suite. The genotyping success rate was 97.4% (range 93.8–100%). Internal genotyping controls were included on each plate, with a concordance rate of 100%. Genotype frequencies for all SNPs were in Hardy-Weinberg equilibrium among controls ($P > 0.10$).

ASPN SNP selection. In order to identify a set of *ASPN*-tagging SNPs that would cover linkage disequilibrium within the gene Caucasian *ASPN* SNP genotype, data available online (www.hapmap.org) were analyzed using Haploview software (online at <http://www.broad.mit.edu/mpg/haploview/index.php>). Five of the haplotype tag SNPs that were identified as marking the haplotype block using the confidence interval method were chosen to analyze genetic variation within the gene, which should capture the majority of the variation within *ASPN*.

Three hundred eighty-six of the control subjects in this study had also participated in a genetics association study by Mustafa and coworkers (15); the genotype for the aspartic acid repeat was available for this subset, thus enabling us to determine that the specific 5-SNP haplotypes were in positive linkage disequilibrium with particular aspartic acid repeat alleles (Figure 1). The product-moment correlation pairwise measure of linkage disequilibrium between 5-SNP haplotypes and microsatellite alleles was computed by isolating a specific allele or SNP haplotype and clumping all other alleles together.

Statistical analysis. *Individual polymorphism genetic associations.* The association between individual SNP genotypes and OA was tested by comparing SNP allele frequencies among patients and controls using Pearson's chi-square test. Odds ratios (ORs) for this model with the corresponding 95% confidence intervals (95% CIs) were also computed.

Haplotype frequency estimation and haplotype genetic associations. Two methods were used to estimate haplotype frequencies among female and male patients and controls.

Table 1. Association of individual SNPs with knee OA in study subjects, according to sex*

Gene	Position	SNP alias	Reference SNP	SNP description	DNA change	HWE†	Minor allele frequency, %											
							Women						Men					
							OA (n = 305)	Controls (n = 299)	OR	95% CI	P	OA (n = 298)	Controls (n = 300)	OR	95% CI	P		
<i>ASP</i> (chr 9)	92295895	aspn_1	rs7022562	5'-UTR	T>C	<1.0	30.0	31.5	0.93	0.73-1.19	<0.573	33.0	29.2	1.19	0.92-1.54	<0.182		
	92303535	aspn_2	rs7033979	Intron	T>C	<0.79	25.8	28.3	0.88	0.68-1.14	<0.322	28.4	25.3	1.17	0.90-1.53	<0.247		
	92308602	aspn_3	rs13301537	Intron	T>C	<0.57	25.3	27.3	0.90	0.69-1.17	<0.434	28.2	25.1	1.17	0.90-1.54	<0.246		
	92316777	aspn_4	rs3739606	3'-UTR	C>A	<0.80	30.1	31.5	0.94	0.73-1.20	<0.601	33.6	29.3	1.22	0.94-1.58	<0.129		
	92323104	aspn_5	rs331377	3'-UTR	T>C	<1.0	50.0	50.0	1.00	0.80-1.25	<1.000	46.2	49.8	0.86	0.68-1.10	<0.230		
<i>CALM1</i> (chr 14)	89939666	ivs3-293	rs3213718	Intron 1	C>T	<0.89	35.8	39.8	1.19	0.94-1.50	<0.151	36.7	39.0	1.10	0.86-1.40	<0.439		
	46654096	col2_int	rs1635560	Intron 11	C>T	<0.76	24.8	24.5	1.01	0.77-1.32	<0.930	19.6	26.5	0.68	0.51-0.89	<0.005		
<i>COL2A1</i> (chr 12)	46654243	col2_1405	rs2070739	S1405G	C>T	<0.82	9.6	9.7	0.99	0.68-1.45	<0.958	10.9	9.7	1.13	0.77-1.67	<0.526		
	18758455	comp_386	Valdes et al (20)	N386D	A>G	<0.85	9.2	5.4	1.77	1.12-2.77	<0.013	5.4	5.5	0.97	0.59-1.60	<0.899		
<i>FRZB</i> (chr 2)	18764495	comp_5p	Mabuchi et al (23)	5'-UTR -1417	C>G	<0.59	8.8	10.1	0.86	0.58-1.27	<0.446	13.6	9.6	1.48	1.03-2.13	<0.032		
	183528842	frzb_200	rs288326	R200W	C>T	<0.77	13.0	9.5	1.43	0.99-2.05	<0.053	12.7	13.0	0.98	0.69-1.39	<0.894		
	183525090	frzb_324	rs7775	R324G	C>G	<0.66	9.3	6.6	1.45	0.95-2.23	<0.087	8.1	8.7	0.92	0.59-1.44	<0.728		

* SNPs = single-nucleotide polymorphisms; OA = osteoarthritis; OR = odds ratio; 95% CI = 95% confidence interval; 5'-UTR = 5'-untranslated region; chr = chromosome.

† P value for the test of Hardy-Weinberg equilibrium (HWE).

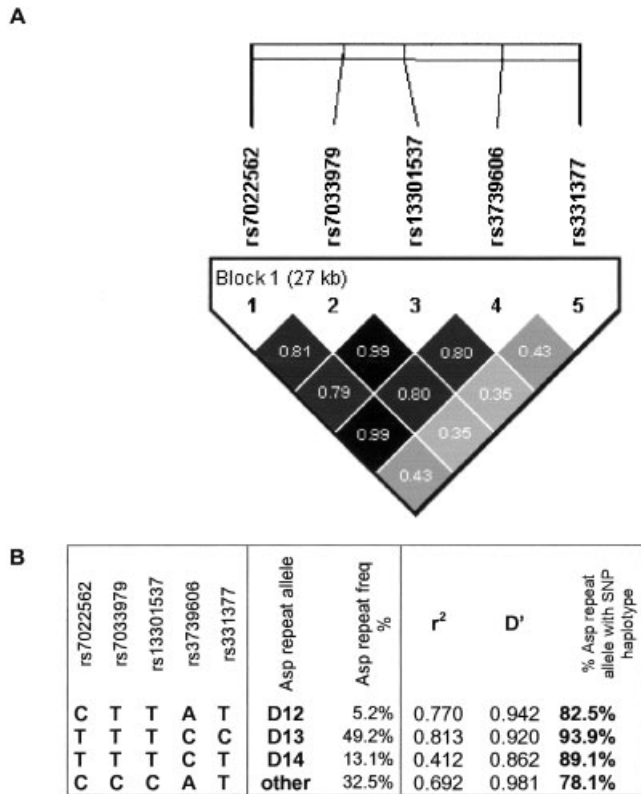


Figure 1. Pairwise linkage disequilibrium expressed as the squared correlation coefficient (r^2) between the *ASPN* single-nucleotide polymorphisms (SNPs) genotyped in this study (A) and between 5-SNP haplotypes and the most common Asp repeat alleles in a subset of 386 unaffected individuals (B). The normalized gametic disequilibrium coefficient (D') between all SNP pairs was >0.99 and was statistically significant for all comparisons.

Maximum-likelihood haplotype frequencies were computed using an expectation-maximization algorithm as implemented using Haploview software. In addition, the program PHASE version 2.02, which implements a Bayesian statistical method for reconstructing haplotypes from population genotype data (online at <http://www.stat.washington.edu/stephens/software.html>), was used to confirm haplotype frequency estimates in each of the 4 groups (male patients, female patients, male control subjects, and female control subjects). Haplotype frequencies estimated by both methods were very similar and were always within the standard error of the estimate. Contingency tables were generated by multiplying the number of chromosomes in patients and control subjects of each sex by the haplotype frequency estimate. Haplotype frequencies between patients with knee OA and control subjects were then compared using Pearson's chi-square test.

Meta-analyses. We conducted a Mantel-Haenszel meta-analysis of data from these studies, in order to assess the evidence of association between alleles (*ASPN*, *FRZB*, *COMP*) or genotypes (*CALMI*) at polymorphisms in these genes and OA. The Mantel-Haenszel chi-square test and the Mantel-

Haenszel estimate of the OR (for review, see ref. 33) were used. Data for *COL2A1* could not be incorporated, because different research groups tested different polymorphisms or different OA-related traits, or the data were not from a case-control study. Wherever possible, we attempted to use data that corresponded to the definition of the population (men, women, or both) used in the original study. Therefore, data for *FRZB* were analyzed separately for men and women, as in the original study, whereas data for both sexes were pooled for the analyses of *ASPN*, *CALMI*, and *COMP*. In the case of genes for which knee OA data were available from 1 or more independent studies, we included only data for knee OA and disregarded data for hip OA.

RESULTS

At the level of individual SNP allele frequency (Table 1), only the comp_386 SNP was statistically significantly ($P < 0.05$) associated with OA in women, and the comp_5p and the col2_int SNPs were associated in men. However, an almost significant association ($P < 0.053$) was observed with the *FRZB* R200W SNP among women. When we compared haplotype frequencies between patients with knee OA and control subjects, we observed that the same *FRZB* haplotype (W200/G324) reported to be associated with hip OA in other studies was associated with increased risk of knee OA (OR 2.87, $P < 0.04$) (Table 2), although the strongest association was the protective effect of the wild-type haplotype (OR 0.70, $P < 0.012$). These associations were observed only among women.

We also observed that haplotypes in *COMP* were associated with knee OA, but that the specific haplotypes involved were different in men and women. The *COL2A1* haplotype was also significantly associated with knee OA but only in men, with no association in women. We did not find evidence that the *CALMI* polymorphism affects the risk of OA, although the frequency of the minor allele was modestly increased in women with OA compared with controls.

None of the individual SNPs typed for *ASPN* was, by itself, associated with knee OA. However, the frequency of a 5-SNP haplotype (CTTAT) was modestly increased in both male patients and female patients compared with controls, but the difference was not statistically significant in patients of either sex. When the data for both sexes were combined, the haplotype frequency in patients (5.2%) was significantly higher than that in controls (3.5%) (OR 1.53, 95% CI 1.02–2.30). An analysis of pairwise linkage disequilibrium between *ASPN* 5-SNP haplotypes and the *ASPN* aspartic acid repeat alleles revealed that specific SNP haplotypes were commonly associated with the 3 most common

Table 2. Estimated haplotype frequencies of *ASPN*, *COL2A1*, *COMP*, and *FRZB* among patients with knee OA and controls, and their association with knee OA*

Gene, SNP	Haplotype	Women					Men				
		Frequency		OR	95% CI	P	Frequency		OR	95% CI	P
		OA	Controls				OA	Controls			
<i>ASPN</i>											
aspn_1, spn_2,	TTTCC	49.7	50.0	0.99	0.79–1.24	<0.929	46.2	50.1	0.85	0.67–1.08	<0.190
aspn_3, spn_4,	CCCAT	24.6	28.3	0.83	0.64–1.07	<0.151	27.9	25.0	1.16	0.89–1.52	<0.277
aspn_5	TTTCT	20.3	18.5	1.12	0.84–1.49	<0.451	19.7	20.8	0.93	0.70–1.25	<0.653
	CTTAT	4.9	3.2	1.56	0.87–2.82	<0.134	5.6	3.8	1.49	0.85–2.63	<0.163
<i>COL2A1</i>											
col2_int, col2_1405	CC	66.0	66.1	1.00	0.79–1.26	<0.974	69.6	63.9	1.30	1.02–1.65	<0.036
	TC	24.5	24.3	1.01	0.78–1.31	<0.945	19.6	26.4	0.68	0.52–0.89	<0.005
	CT	9.3	9.5	0.98	0.67–1.44	<0.909	10.8	9.7	1.12	0.77–1.64	<0.544
	TT	0.3	0.2	1.48	0.16–13.47	<0.709	0.0	0.0			
<i>COMP</i>											
comp_386, comp_5p	AC	82.1	84.5	0.84	0.62–1.14	<0.258	81.1	84.9	0.76	0.56–1.03	<0.080
	AG	8.8	10.0	0.86	0.59–1.27	<0.450	13.6	9.6	1.48	1.03–2.12	<0.032
	GC	9.1	5.4	1.75	1.12–2.74	<0.014	5.4	5.5	0.97	0.59–1.60	<0.900
<i>FRZB</i>											
frzb_200, frzb_324	CC	79.4	84.6	0.70	0.52–0.95	<0.012	83.3	83.7	0.98	0.71–1.35	<0.953
	CG	7.2	5.9	1.24	0.78–1.97	<0.222	5.4	5.4	1.00	0.58–1.73	<0.997
	TC	11.4	8.8	1.33	0.91–1.95	<0.105	10.5	9.8	1.08	0.72–1.63	<0.728
	TG	2.0	0.7	2.87	0.92–8.95	<0.039	0.8	1.1	0.88	0.42–1.84	<0.743

* OA = osteoarthritis; SNP = single-nucleotide polymorphism; OR = odds ratio; 95% CI = 95% confidence interval.

Table 3. Previous associations, summary of current results, and meta-analysis results*

Gene	Ref.	Population	Trait	Replications (refs.)	Known/hypothesized function in OA	Observed result in current study	Meta-analysis results
<i>ASP</i>	13	Japanese	Knee, hip OA	Negative (15,16); positive on a different allele (14)	Cartilage extracellular protein that regulates the activity of TGFβ	Weak association of <i>ASP</i> haplotype in LD with D12 in both sexes	D14, Cauc + Jap; OR 1.31 (95% CI 1.10–1.56), $P < 0.002$ † D14, Cauc only; OR 1.14 (95% CI 0.94–1.40), P NS D13, Cauc only; OR 0.85 (95% CI 0.74–0.97), $P < 0.02$
<i>FRZB</i>	8	UK	Hip OA	Positive (9,10)	Modulator of chondrocyte maturation	Association only in women	W200, F only; OR 1.25 (95% CI 1.07–1.47), $P < 0.004$ ‡ W200, M only; OR 0.95 (95% CI 0.75–1.19), P NS W200, M + F; OR 1.04 (95% CI 0.91–1.19), P NS G324, F only; OR 1.38 (95% CI 1.16–1.65), $P < 0.0003$ G324, M only; OR 0.82 (95% CI 0.61–1.11), P NS G324, M + F; OR 1.21 (95% CI 1.02–1.42), $P < 0.022$ –1417 G; OR 1.05 (95% CI 0.86–1.29), P NS§
<i>COMP</i>	23	Japan	No association with hip/knee OA	None	Cartilage matrix macromolecule	Association in both sexes, but different haplotypes	
<i>COL2A1</i>	25	Netherlands	Knee, hip OA	Negative (24,27,36); positive (26)	Major cartilage collagen, structural cartilage component	Associated only in men	NA
<i>CALMI</i>	18	Japan	Hip OA	Negative (19)	Intracellular protein, interacts with proteins involved in signal transduction	No association observed	TT, Cauc + Jap; OR 1.15 (95% CI 0.99–1.33), $P < 0.055$ ¶ TT, Cauc only; OR 1.02 (95% CI 0.88–1.20), P NS

* OA = osteoarthritis; Cauc = Caucasian; Jap = Japanese; OR = odds ratio; 95% CI = 95% confidence interval; TGFβ = transforming growth factor β; LD = linkage disequilibrium; NS = not significant; NA = not applicable.

† Includes pooled data for knee OA in men and women; data for Cauc + Jap were from refs. 13–16; data for Cauc only were from refs. 14–16 and the current study. When the 5–single-nucleotide polymorphism haplotype was used as surrogate, the OR for D14 (Cauc only) was 1.09 (95% CI 0.94–1.25 [$P < 0.27$]) and the OR for D13 was 0.88 (95% CI 0.79–0.98 [$P < 0.017$]).

‡ Includes data for knee OA (current study), hip OA (refs. 8–11), and generalized radiographic OA (ref. 9). Data for females (F) only were from the current study and refs. 8, 10, and 11; data for males (M) only were from the current study and ref. 8; data for M + F were from the current study and refs. 8 and 9.

§ Includes pooled data for knee OA in men and women from the current study and ref. 23.

¶ Includes pooled data for hip and knee OA in men and women from the current study and refs. 18 and 19. The current study and ref. 18 refer to rs3213718; ref. 19 refers to rs12885713, which is in complete LD with rs3213718.

aspartic acid repeat alleles (Figure 1). These data indicate that although not all of the information on allele D14 was captured by the 5-SNP haplotypes, sufficient information on alleles D12 and D13 was captured by the 5 SNPs ($r^2 > 0.77$).

We summarized the data from the current study, compared them with those from previous association studies, and carried out meta-analyses for all genes except *COL2A1* (Table 3). The strongest evidence from the meta-analysis was for the *FRZB* G324 allele in women ($P < 0.0003$), followed by the *FRZB* W200 allele in women ($P < 0.004$) (Table 3). The meta-analysis also indicated that neither of these 2 variants influences the risk of OA in men. Combining the data for *ASPN* genetic variants in Caucasians and Japanese populations resulted in a significant association for the D14 allele, although data sets for Caucasians alone provided no evidence for this (Table 3). However, the meta-analysis of 3 data sets for Caucasians indicated that D13—the protective allele in Japanese individuals—is statistically significantly associated with a decreased risk of knee OA in Caucasian individuals. Combining data for Caucasians and Japanese resulted in a nearly significant association between *CALMI* and the TT genotype, but no evidence for an association was seen in Caucasians only. Finally, combining the current data with published data for Japanese populations provided no evidence for an association between *COMP* and knee OA.

DISCUSSION

Our data confirm striking sex-related differences in certain genes, in particular *FRZB*, and are supportive of previously published results regarding hip OA in Caucasian populations, namely, that the *FRZB* T/G haplotype is involved in the pathogenesis of OA, but only in women. However, the present study is, to our knowledge, the first to show a genetic association with knee OA. We must note that some of the subjects used in the current study (the 185 female control subjects from Oxford) are shared between this study and the original study by Loughlin et al (8). For this reason, the 2 studies are not totally independent. However, the trends observed for the combined set of control subjects were the same as those observed when only the Nottingham control subjects were used. For example, when only the Nottingham control subjects were considered, the *FRZB* R200W polymorphism had an OR of 1.57; when the Oxford control subjects were included the OR was 1.31.

Because the biologic rationale for involvement of

ASPN in OA susceptibility is very strong and is based solely on the functional properties of asporin, Kizawa and coworkers (13) decided to test *ASPN* for association with OA. They not only observed a genetic association with an aspartic acid repeat but also demonstrated that it is abundantly expressed in OA articular cartilage and found that asporin inhibits the expression of the genes encoding aggrecan and type II collagen. Three studies in Caucasian populations were unable to demonstrate an association with allele D14, the risk allele in the Japanese study, and, according to our own data, the 5-SNP haplotype in linkage disequilibrium with this allele is not associated with knee OA, although our study did not capture sufficient genetic variation to fully mark this allele.

The haplotype that is modestly increased in patients with knee OA relative to controls is in linkage disequilibrium with allele D12, an allele that was not implicated in OA risk in any of the previous studies. However, evidence from combined data for 3 independent Caucasian populations from Greece, Spain, and the UK (14–16) indicated that the D13 allele is indeed associated with a reduced risk of knee OA. According to our own data, the haplotype in linkage disequilibrium with the D13 allele (TTTCC) was less frequent among patients with knee OA than among controls (Table 2), although the difference was not statistically significant. Thus, although none of the studies of Caucasians has provided evidence of an association between the D14 allele and an increased risk of OA, the combined data for Europeans point toward a decreased risk of knee OA in carriers of the D13 allele and, taken together, would confirm a role for *ASPN* in susceptibility to knee OA not only in Japanese individuals but also in Caucasians.

Unlike the situation with *ASPN*, in which we covered a vast proportion of variation within the gene, we tested only one SNP for *CALMI*, and we were unable to detect any association with knee OA for this specific SNP. Because we tested no other polymorphisms within *CALMI*, we cannot exclude the possibility that other variants in weak or no linkage disequilibrium with the current SNP could influence the genetic risk of knee OA. However, another UK-based study (19) was unable to detect an association between hip OA and a *CALMI* SNP that is in complete linkage disequilibrium with the SNP we studied here. This could suggest that whatever role this variant within *CALMI* plays in genetic susceptibility to OA in Caucasians, it is likely to be a modest one.

Unlike a previous Japanese study, in the present study we observed that *COMP* contributed to the risk of

knee OA, albeit in a different manner in men compared with women. The association we observed was not particularly strong but was consistent with expression data comparing normal and OA-affected cartilage (20), the fact that serum levels of *COMP* are increased in OA (22), and the fact that *COMP* levels are heritable (34). A meta-analysis including both sets of data, however, showed no evidence of association between knee OA and *COMP*. Further research is needed to clarify the role of genetic variation at this gene in susceptibility to OA in Caucasians.

Finally, *COL2A1*, which has been shown to be associated with OA in some studies (e.g., Rotterdam) (25) but not in others (e.g., Framingham) (28), appeared to be associated with OA only in men. Because we investigated only 2 genetic variants at this gene, we cannot exclude the possibility that other polymorphisms might also be involved in women in this cohort. Interestingly, one of the earlier studies of the risk of knee OA and *COL2A1* indicated that different VNTR alleles were associated in men compared with women (27).

Apart from the facts that it was not feasible to cover all genetic variation in the genes analyzed (which would be possible only through resequencing) and that only common variants were studied, there are other limitations to the present study. Only limited data were available for control subjects, which did not allow us to test possible confounders such as obesity. However, previous modeling studies showed no genetic correlation of knee OA with obesity (35), making it unlikely that we had merely confirmed obesity genes. It is also possible that the use of different genotyping methodologies can result in lower call rates for some assays, and that this could be the source of inconsistencies across studies. Such error, however, is most likely to be unbiased, and its effect would be a small reduction in power (corresponding at most to a 6% reduction in sample size). Multiple testing was not a problem, because all of the tests were considered a priori, with the exception of the test for *ASPN*, in which we did not test the exact same variants as those tested in previous studies.

Another potential source of error is that haplotype comparisons derived from unphased data carry the possibility of obtaining a larger Type I error than the nominal one (36). However, because we analyzed our data with those from several published studies, for the most part as individual polymorphisms, this is unlikely to have biased our conclusions. A final confounding factor could be population stratification; however, all of the study subjects were ethnically matched (Caucasians from the UK), which makes this an unlikely possibility.

In conclusion, our results highlight the strong reproducibility of several important candidate gene associations with OA and the fact that many of these associations are strongly dependent on sex and ethnicity and are often probably site specific. OA is now one of the few common diseases in which a large number of candidate genes have been consistently and independently replicated. Additional research to exploit the combined effects of these and other candidate genes and to determine how environmental factors modify the genetic risk will greatly help in understanding the etiology of this complex disease and forming the basis of clinical susceptibility tests.

ACKNOWLEDGMENTS

We thank 2 anonymous referees for their very valuable comments and suggestions regarding an earlier version of this manuscript.

AUTHOR CONTRIBUTIONS

Dr. Spector 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 design. Drs. Valdes, Loughlin, Doherty, and Spector.

Acquisition of data. Mr. van Oene, Dr. Chapman, and Ms Surdulescu.

Analysis and interpretation of data. Drs. Valdes, Loughlin, and Spector.

Manuscript preparation. Drs. Valdes, Loughlin, and Spector.

Statistical analysis. Dr. Valdes.

Patient enrollment. Dr. Doherty.

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