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# Significance of Valine/Leucine$^{247}$ Polymorphism of $\beta_2$-Glycoprotein I in Antiphospholipid Syndrome

## Increased Reactivity of Anti–$\beta_2$-Glycoprotein I Autoantibodies to the Valine$^{247}$ $\beta_2$-Glycoprotein I Variant

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***Objective.*** **To clarify the consequences of the valine/leucine polymorphism at position 247 of the $\beta_2$-glycoprotein I ($\beta_2$GPI) gene in patients with antiphospholipid syndrome (APS), by investigating the correlation between genotypes and the presence of anti-$\beta_2$GPI antibody. The reactivity of anti-$\beta_2$GPI antibodies was characterized using recombinant Val$^{247}$ and Leu$^{247}$ $\beta_2$GPI.**

***Methods.*** **Sixty-five Japanese patients with APS and/or systemic lupus erythematosus who were positive for antiphospholipid antibodies and 61 controls were analyzed for the presence of the Val/Leu$^{247}$ polymorphism of $\beta_2$GPI. Polymorphism assignment was determined by polymerase chain reaction followed by restriction enzyme digestion. Recombinant Val$^{247}$ and Leu$^{247}$ $\beta_2$GPI were established to compare the reactivity of anti-$\beta_2$GPI antibodies to $\beta_2$GPI between these variants. The variants were prepared on polyoxygenated plates or cardiolipin-coated plates, and the reactivity of a series of anti-$\beta_2$GPI antibodies (immunized anti-human $\beta_2$GPI monoclonal antibodies [Cof-19–21] and autoimmune anti-$\beta_2$GPI monoclonal antibodies [EY1C8, EY2C9, and TM1G2]) and IgGs purified from patient sera was investigated.**

***Results.*** **A positive correlation between the Val$^{247}$ allele and the presence of anti-$\beta_2$GPI antibodies was observed in the patient group. Human monoclonal/polyclonal anti-$\beta_2$GPI autoantibodies showed higher binding to recombinant Val$^{247}$ $\beta_2$GPI than to Leu$^{247}$ $\beta_2$GPI, although no difference in the reactivity of the immunized anti-$\beta_2$GPI between these variants was observed. Conformational optimization showed that the replacement of Leu$^{247}$ by Val$^{247}$ led to a significant alteration in the tertiary structure of domain V and/or the domain IV–V interaction.**

***Conclusion.*** **The Val$^{247}$ $\beta_2$GPI allele was associated with both a high frequency of anti-$\beta_2$GPI antibodies and stronger reactivity with anti-$\beta_2$GPI antibodies compared with the Leu$^{247}$ $\beta_2$GPI allele, suggesting that the Val$^{247}$ $\beta_2$GPI allele may be one of the genetic risk factors for development of APS.**

The antiphospholipid syndrome (APS) is characterized by arterial/venous thrombosis and pregnancy morbidity in the presence of antiphospholipid antibodies (aPL) (1–3). Among the targets of aPL, $\beta_2$-glycoprotein I ($\beta_2$GPI), which bears epitopes for anticardiolipin antibodies (aCL), has been extensively studied (4–6). APS-related aCL do not recognize free $\beta_2$GPI, but do recognize $\beta_2$GPI when it is complexed with phospholipids or negatively charged surfaces, by exposure of cryptic epitopes (7) or increment of antigen density (8).

The significance of antigen polymorphism in the production of autoantibodies or the development of autoimmune diseases is now being widely discussed. It is speculated that amino acid substitution in antigens can lead to differences in antigenic epitopes of a given protein. In particular, $\beta_2$GPI undergoes conformational

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alteration upon interaction with phospholipids (9). $\beta_2$GPI polymorphism on or near the phospholipid binding site can affect the binding or production of aCL (anti-$\beta_2$GPI autoantibodies), the result being altered development of APS. Polymorphism near the antigenic site, or which leads to alteration of the tertiary structure of the whole molecule, may affect the binding of autoantibodies. Five different gene polymorphisms of $\beta_2$GPI attributable to a single-nucleotide mutation have been described: 4 are a single amino acid substitution at positions 88, 247, 306, and 316 (10), and the other is a frameshift mutation associated with $\beta_2$GPI deficiency found in the Japanese population (11). In particular, the Val/Leu$^{247}$ polymorphism locates in domain V of $\beta_2$GPI, between the phospholipid binding site in domain V and the potential epitopes of anti-$\beta_2$GPI antibodies in domain IV, as we reported previously (12). Although anti-$\beta_2$GPI antibodies are reported to direct to domain I (13) or domain V (14) as well, it should be considered that a certain polymorphism alters the conformation of the molecule, affecting function or antibody binding at a distant site.

We previously reported that, in a group of British Caucasian subjects, the Val$^{247}$ allele was significantly more frequent in primary APS patients with anti-$\beta_2$GPI antibodies than in controls or in primary APS patients without anti-$\beta_2$GPI antibodies (15), but the importance of the Val$^{247}$ allele in patients with APS is still controversial. In this study, we analyzed the correlation between the $\beta_2$GPI Val$^{247}$ allele and anti-$\beta_2$GPI antibodies in the Japanese population. We also investigated the reactivity of anti-$\beta_2$GPI antibodies to recombinant Val$^{247}$ $\beta_2$GPI and Leu$^{247}$ $\beta_2$GPI, using a series of monoclonal anti-$\beta_2$GPI antibodies and IgGs purified from sera of patients with APS. Finally, to investigate the difference in anti-$\beta_2$GPI binding to those variants, we conformationally optimized to domain V and the domain IV–V complex of $\beta_2$GPI variants at position 247, referring the crystal structure of $\beta_2$GPI.

## PATIENTS AND METHODS

**Patients and controls.** The study group comprised 65 patients (median age 38 years [range 18–74 years]; 57 women and 8 men) who attended the Hokkaido University Hospital, all of whom were positive for aPL (IgG, IgA, or IgM class aCL, and/or lupus anticoagulant). Thirty-four patients had APS (16 had primary APS, and 18 had secondary APS), and 31 patients did not have APS (24 had systemic lupus erythematosus [SLE], and 7 had other rheumatic diseases). Among all subjects, 19 had a history of arterial thrombosis, and 6 had venous thrombosis. Of the 31 patients with a history of pregnancy, 8 experienced pregnancy complications (some patients had more than 1 manifestation of pregnancy morbidity). Anti-$\beta_2$GPI antibodies were detected by enzyme-linked immunosorbent assay (ELISA) as $\beta_2$GPI-dependent aCL (16). IgG, IgA, or IgM class $\beta_2$GPI-dependent aCL were found in 30, 14, and 21 patients, respectively (some patients had >1 isotype), and 34 patients had at least 1 of those isotypes. Lupus anticoagulant, detected by 3 standard methods described previously (17), was found in 51 patients. The diagnoses of APS and SLE, respectively, were based on the preliminary classification criteria for definite APS (18) and the American College of Rheumatology criteria for the classification of SLE (19). Informed consent was obtained from each patient or control subject. The control group comprised 61 healthy individuals with no history of autoimmune, thrombotic, or notable infectious disease.

**Determination of $\beta_2$GPI gene polymorphism.** Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) using a standard phenol–chloroform extraction procedure or the DnaQuick kit (Dainippon, Osaka, Japan). Polymorphism assignment was determined by polymerase chain reaction (PCR) followed by allele-specific restriction enzyme digestion (PCR–restriction fragment length polymorphism) using *Rsa* I (Promega, Southampton, UK) as described previously (15).

**Purification of patient IgG.** Sera from 11 patients positive for IgG class $\beta_2$GPI-dependent aCL were collected. The mean (±SD) titer of aCL IgG from these patients was 29.0 ± 21.5 IgG phospholipid (GPL) units (range 12.4 to >98 GPL units). IgG was purified from these sera using a protein G column and the MAbTrap GII IgG purification kit (Pharmacia Biotech, Freiburg, Germany), as recommended by the manufacturer.

**Monoclonal anti-$\beta_2$GPI antibodies.** Two types of anti-$\beta_2$GPI monoclonal antibodies were used. Cof-19, Cof-20, and Cof-21 are mouse monoclonal anti-human $\beta_2$GPI antibodies obtained from immunized BALB/c mice, directed to domains V, III, and IV of $\beta_2$GPI, respectively. These monoclonal antibodies recognize the native structure of human $\beta_2$GPI (12).

EY1C8, EY2C9, and TM1G2 are IgM class autoimmune monoclonal antibodies established from patients with APS (20). These antibodies bind to domain IV of $\beta_2$GPI, but only after interaction with solid-phase phospholipids or with a polyoxygenated polystyrene surface. EY1C8 and EY2C9 were established from a patient whose genotype of $\beta_2$GPI was heterozygous for Val/Leu$^{247}$. The genotype of the patient with TM1G2 was not determined.

**Preparation of recombinant $\beta_2$GPI.** As previously reported, genes were expressed in *Spodoptera frugiperda* Sf9 insect cells infected with recombinant baculoviruses (12). A full-length complementary DNA of human $\beta_2$GPI coding Val$^{247}$ was originally obtained from Hep-G2 cells (21), and the valine residue was replaced by leucine, using the GeneEditor in vitro Site-Directed Mutagenesis System (Promega, Madison, WI). The sequence of the primers for a mutant Val$^{247}$→Leu (GTA→TTA) is as follows: 5′-GCATCTTGTAAATTACCTGTGAAAAAAG-3′. A DNA sequence of the mutant was verified by analysis using ABI Prism model 310 (PE Applied Biosystems, Foster City, CA).

**Binding assays of monoclonal anti-$\beta_2$GPI antibodies and purified IgGs to the recombinant $\beta_2$GPI (cardiolipin-coated plate).** The reactivity of a series of monoclonal anti-$\beta_2$GPI antibodies and IgG fractions (purified from the sera of APS patients positive for IgG class anti-$\beta_2$GPI) against 2 $\beta_2$GPI variants was investigated using an ELISA. ELISAs were performed using a cardiolipin-coated plate as previously reported (16) but with a slight modification. Briefly, the wells of Sumilon Type S microtiter plates (Sumitomo Bakelite, Tokyo, Japan) were filled with 30 $\mu$l of 50 $\mu$g/ml cardiolipin (Sigma, St. Louis, MO) and dried overnight at 4°C. After blocking with 2% gelatin in phosphate buffered saline (PBS) for 2 hours and washing 3 times with 0.05% PBS–Tween, 50 $\mu$l of 10 $\mu$g/ml recombinant $\beta_2$GPI and controls were distributed and incubated for 30 minutes at room temperature. Wells were filled with 50 $\mu$l of serial dilutions of monoclonal antibodies (Cof-19–21, EY1C8 and EY2C9, and TM1G2) or purified patient IgG (100 $\mu$g/ml), followed by incubation for 30 minutes at room temperature. After washing 3 times, 50 $\mu$l of alkaline phosphatase–conjugated anti-mouse IgG (1:3,000), anti-human IgM (1:1,000), or anti-human IgG (1:6,000) was distributed and incubated for 1 hour at room temperature. The plates were washed 4 times, and 100 $\mu$l of 1 mg/ml *p*-nitrophenyl phosphate disodium (Sigma) in 1*M* diethanolamine buffer (pH 9.8) was distributed. Optical density (OD) was read at 405 nm, with reference at 620 nm. One percent fatty acid–free bovine serum albumin (BSA) (A-6003; Sigma)–PBS was used as sample diluent and control.

**Binding assays of monoclonal anti-$\beta_2$GPI antibodies to recombinant $\beta_2$GPI (polyoxygenated plate).** Anti-$\beta_2$GPI antibody detection assay using polyoxygenated plates was performed as previously reported (22), with minor modifications. Briefly, the wells of polyoxygenated MaxiSorp microtiter plates (Nalge Nunc International, Roskilde, Denmark) were coated with 50 $\mu$l of 1 $\mu$g/ml recombinant $\beta_2$GPI in PBS and incubated overnight at 4°C. After blocking with 3% gelatin–PBS at 37°C for 1 hour and washing 3 times with PBS–Tween, 50 $\mu$l of monoclonal antibodies, diluted with 1% BSA–PBS, were distributed and incubated for 1 hour at room temperature. The following steps were taken, in a similar manner.

**Conformational optimization of domain V and the domain IV–V complex in human $\beta_2$GPI variants at position 247.** A conformation of domain V in the valine variant at position 247 was first constructed from the crystal structure of the leucine variant (implemented in Protein Data Bank: 1C1Z) (23). Replacement of leucine by valine at position 247 was performed using the Quanta system (Molecular Simulations, San Diego, CA), and the model was optimized by 500 cycles of energy minimization by the CHARMm program (24), with hydrophilic hydrogen atoms and TIP3 water molecules (25). Molecular dynamics simulation (5 psec) of the model was then performed with 0.002 psec time steps. The cutoff distance for nonbonded interactions was set to 15Å, and the dielectric constant was 1.0. A nonbonded pair list was updated every 10 steps. The most stable structure of each domain in the dynamics iterations was then optimized by 500 cycles of energy minimization. The final structures of domain V consisted of 2,616 atoms, including 603 TIP3 water molecules, and had a total energy of $-1.63 \times 10^4$ kcal/mole with a root-mean-square force of 0.869 kcal/mole.

Molecular models of a domain IV–V complex (leucine and valine variants at position 247) were further constructed by considering the location of the oligosaccharide attachment site in domain IV, the location of epitopic regions of the Cof-8 and Cof-20 monoclonal antibodies, the junction between domains IV and V, and molecular surface charges of both domains. These models were again optimized by molecular dynamics simulation and by energy minimization as described above. The final structures of the complex in the leucine and valine variants consisted of 3,773 and 3,778 atoms, respectively, including hydrophilic hydrogen atoms and 806 and 808 TIP3 water molecules, respectively, and had total energy of $-2.07 \times 104$ and $-2.03 \times 104$ kcal/mole with a root-mean-square force of 0.985 and 0.979 kcal/mole, respectively.

**Statistical analysis.** Correlations between the allele frequencies and clinical features such as the positiveness of $\beta_2$GPI-dependent aCL were expressed as odds ratios (ORs) and 95% confidence intervals (95% CIs). *P* values were determined by chi-square test with Yates' correction. *P* values less than or equal to 0.05 were considered significant.

## RESULTS

**Val/Leu$^{247}$ polymorphism of $\beta_2$GPI and the presence of $\beta_2$GPI-dependent aCL.** As shown in Table 1, the Leu$^{247}$ allele was dominant in the population of healthy Japanese individuals, compared with Caucasians, which is consistent with a previous report (26). Japanese patients with anti-$\beta_2$GPI had a significantly increased frequency of the Val$^{247}$ allele, compared with Japanese patients without anti-$\beta_2$GPI ($P = 0.0107$) or Japanese controls ($P = 0.0209$).

**The binding of autoimmune anti-$\beta_2$GPI to recombinant Val$^{247}$ and Leu$^{247}$ $\beta_2$GPI.** Representative binding curves using cardiolipin-coated plates and polyoxygenated plates are shown in Figure 1. Regardless of the type of plates, Cof-20 bound equally to valine and leucine variants of $\beta_2$GPI (Figures 1a and c), in any concentration of Cof-20. The binding curves of Cof-19 and Cof-21 were similar to that of Cof-20 (results not

Table 1. Frequency of the Val$^{247}$ allele of $\beta_2$GPI in patients with APS*

| Group | Japanese | British Caucasians |
|---|---|---|
| Patients with anti-$\beta_2$GPI | 23/68 (33.8)† | 48/56 (85.7)‡ |
| Patients without anti-$\beta_2$GPI | 9/62 (14.5) | 39/58 (67.2) |
| Controls | 23/122 (18.9) | 55/78 (70.5) |

* Values are the number (%). $\beta_2$GPI = $\beta_2$-glycoprotein I; APS = antiphospholipid syndrome.
† $P = 0.0107$ versus patients without anti-$\beta_2$GPI (odds ratio [OR] 3.01, 95% confidence interval [95% CI] 1.26–7.16), and $P = 0.0209$ versus controls, by chi-square test (OR 2.15, 95% CI 1.09–4.23).
‡ $P = 0.204$ versus patients without anti-$\beta_2$GPI (OR 2.92, 95% CI 1.16–7.39), and $P = 0.0396$ versus controls, by chi-square test (OR 2.51, 95% CI 1.03–6.13).

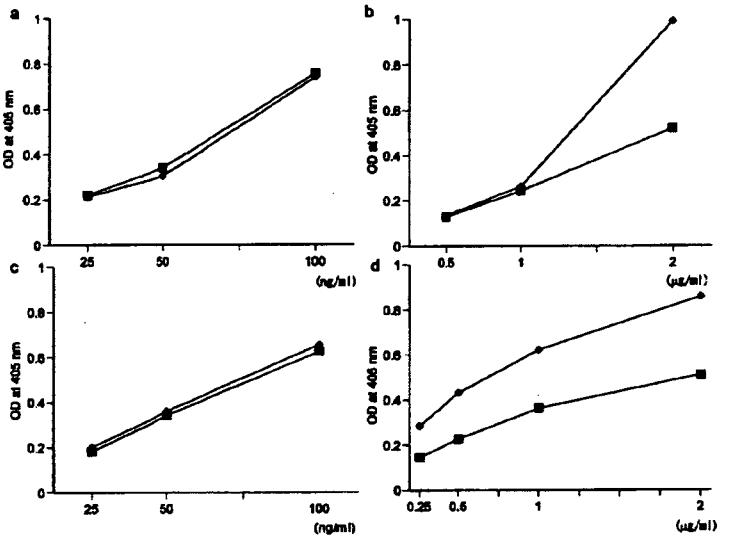


Figure 1. Representative binding curves of monoclonal anti-$\beta_2$-glycoprotein I (anti-$\beta_2$GPI) antibodies to recombinant valine/leucine$^{247}$ $\beta_2$GPI. a, Binding curve of Cof-20 using cardiolipin-coated plate. b, Binding curve of EY2C9 using cardiolipin-coated plate. c, Binding curve of Cof-20 using polyoxygenated plate. d, Binding curve of EY2C9 using polyoxygenated plate. Binding to Val$^{247}$ $\beta_2$GPI and Leu$^{247}$ $\beta_2$GPI are indicated with diamonds and squares, respectively. OD = optical density.

shown). In contrast, EY2C9 showed stronger binding to Val$^{247}$ $\beta_2$GPI than to Leu$^{247}$ $\beta_2$GPI (Figures 1b and d). EY1C8 and TM1G2 also showed stronger binding to Val$^{247}$ $\beta_2$GPI. Figure 2a shows the binding of the monoclonal antibodies, on cardiolipin-coated plates, in the following concentrations: for Cof-19–21, 100 ng/ml;

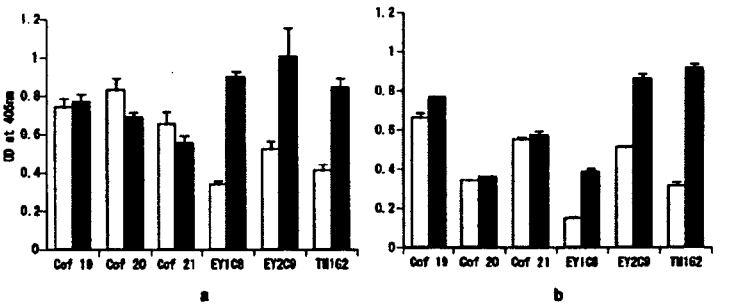


Figure 2. Reactivity of anti-$\beta_2$-glycoprotein I (anti-$\beta_2$GPI) antibodies to $\beta_2$GPI variants. a, The binding of monoclonal anti-$\beta_2$GPI antibodies to the recombinant valine/leucine$^{247}$ $\beta_2$GPI was investigated using enzyme-linked immunosorbent assay (ELISA) on cardiolipin-coated plates. Concentrations of antigens and antibodies were as follows: for recombinant $\beta_2$GPI, 10 $\mu$g/ml; for Cof-19–21, 100 ng/ml; for EY1C8 and EY2C9, 2 $\mu$g/ml; for TM1G2, 5 $\mu$g/ml. b, The binding of monoclonal anti-$\beta_2$GPI antibodies to the recombinant Val/Leu$^{247}$ $\beta_2$GPI was investigated using ELISA on polyoxygenated plates. Concentrations of antigens and antibodies were as follows: for recombinant $\beta_2$GPI, 1 $\mu$g/ml; for Cof-19–21, 50 ng/ml; for EY1C8 and EY2C9, 2 $\mu$g/ml; for TM1G2, 5 $\mu$g/ml. Results were presented as the optical density (OD) at 405 nm. Open columns indicate binding activity to Leu$^{247}$ $\beta_2$GPI, and solid columns indicate binding activity to Val$^{247}$ $\beta_2$GPI. Bars show the mean and SD.

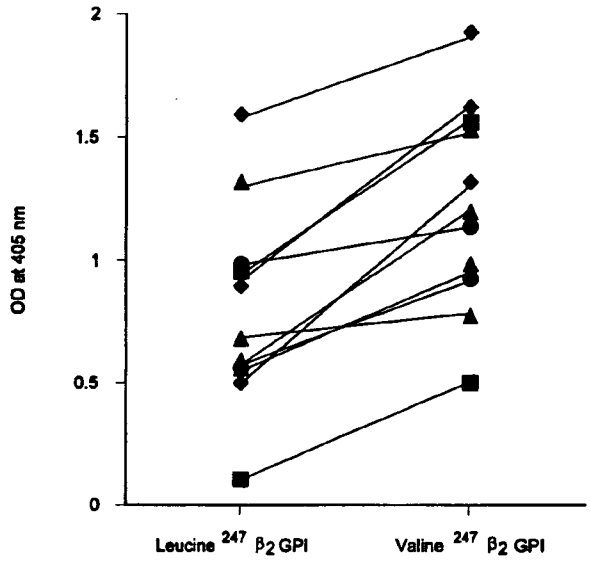


Figure 3. Reactivity of purified IgG from patients (100 μg/ml) to recombinant Val/Leu$^{247}$ $\beta_2$-glycoprotein I ($\beta_2$GPI) (10 μg/ml), presented as the optical density (OD) at 405 nm. Squares, circles, and triangles indicate patients homozygous for the Leu$^{247}$ allele, homozygous for the Val$^{247}$ allele, and heterozygous for the Val/Leu$^{247}$ allele, respectively. Diamonds indicate patients whose genotypes were not available.

for EY1C8 and EY2C9, 1 μg/ml; and for TM1G2, 2.5μg/ml. In contrast with the close reactivity of Cof-19, Cof-20, and Cof-21 between Val$^{247}$ $\beta_2$GPI and Leu$^{247}$ $\beta_2$GPI, autoimmune monoclonal antibodies (EY1C8, EY2C9, and TM1G2) showed higher binding to Val$^{247}$ $\beta_2$GPI than to Leu$^{247}$ $\beta_2$GPI. The autoimmune monoclonal antibodies also showed a higher binding to Val$^{247}$ $\beta_2$GPI directly coated on polyoxygenated plates (Figure 2b). IgG in sera collected from 11 patients (100 μg/ml) also showed higher binding to Val$^{247}$ $\beta_2$GPI than to Leu$^{247}$ $\beta_2$GPI on cardiolipin-coated plates, regardless of the patients' genotypes (Figure 3).

**Conformational alteration by leucine replacement by valine at position 247.** Each domain V conformation in 2 variants at position 247 is shown in Figure 4a. The root-mean-square deviations for matching backbone atoms and equivalent atoms in the leucine and valine variants were 0.76 and 1.11 Å, respectively. The largest shift was observed at Val$^{303}$, one of the residues located on the backbone neighboring position 247. The shift seemed to be caused by weak flexibility of side chains consisting of Val$^{247}$, Pro$^{248}$, and Val$^{249}$ and the electrostatic interactions between Lys$^{250}$, Lys$^{251}$, Glu$^{307}$, and Lys$^{308}$.

The molecular models of the IV–V complex in leucine and valine variants are shown in Figure 4b. The root-mean-square deviations for matching these backbone atoms and equivalent atoms were 1.72 and 2.03 Å, respectively. Electrostatic interactions and hydrogen bonds between Asp$^{193}$ and Lys$^{246}$/Lys$^{250}$, Asp$^{222}$ and Lys$^{305}$, and Glu$^{228}$ and Lys$^{308}$ appeared in the IV–V complex, but the interaction between Glu$^{228}$ and Lys$^{308}$ was disrupted by the leucine replacement by valine, because direction of the Lys$^{308}$ side chain was significantly changed in the complex. As a result, Trp$^{235}$ of domain IV, located on the contact surface with domain V, was slightly shifted.

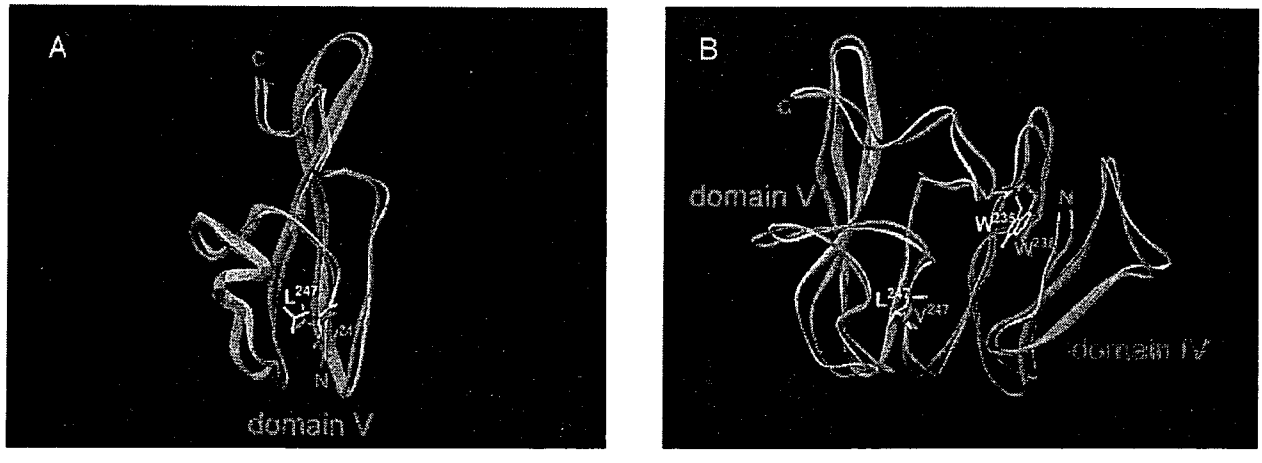


Figure 4. Conformational alterations in domain V (A) and in the domain IV–V complex (B), replacing leucine by valine at position 247. Structure of the valine (light blue) and leucine (white) variants was shown by a ribbon representation with the secondary structure.

## DISCUSSION

This study shows the positive correlation between the Val$^{247}$ $\beta_2$GPI allele and anti-$\beta_2$GPI antibody production in a Japanese population, confirming the correlation observed in a British Caucasian population in our previous report (15). A positive correlation between the Val$^{247}$ allele and the presence of anti-$\beta_2$GPI antibodies was also reported in Asian American (26) and Mexican patients (27). However, this correlation was not observed in other American populations (26) or in patients with thrombosis or pregnancy complications in the UK (28). This discrepancy may be the result of the difference in the frequency of the Val$^{247}$ allele among races, or the difference in the background of investigated patients. Another possibility is that the relationship between the Val$^{247}$ allele and thrombosis in Caucasians may be controversial due to underpowered studies or to differences in the procedure used to detect anti-$\beta_2$GPI antibodies. Methods for the detection of anti-$\beta_2$GPI antibodies differ among laboratories. For example, cardiolipin-coated plates or oxygenated plates are used in some methods, whereas unoxygenated plates are used in others. In addition, bovine $\beta_2$GPI is used instead of human $\beta_2$GPI in some assays. The antibodies used for standardization also differ, although monoclonal antibodies such as EY2C9 and HCAL (29) have been proposed as international standards of calibration materials.

$\beta_2$GPI is a major target antigen for aCL, and, according to our previous investigation, B cell epitopes reside in domain IV and are considered to be cryptic and to appear only when $\beta_2$GPI interacts with negatively charged surfaces such as cardiolipin, phosphatidylserine, or polyoxygenated polystyrene surface (7), although other studies indicate that the B cell epitopes are located on domain I (13) or domain V (14). According to another interpretation for the specificity of aCL, increment of the local antigen density on the negatively charged surface also contributes to anti-$\beta_2$GPI detection in ELISA (8,30). Studies on the crystal structure of human $\beta_2$GPI revealed that the lysine-rich site and an extended C-terminal loop region on domain V are crucial for phospholipid binding. Position 247 is located at the N-terminal side of domain V, and, around this position, Lys$^{242}$, Ala$^{243}$, and Ser$^{244}$ were suggested to play a role in the interaction between domains IV and V (9,23,31).

Although the Val/Leu$^{247}$ polymorphism may not be very critical for the autoantibody binding, the amino acid substitution at this point was revealed to affect the affinity of monoclonal aCL established from patients with APS and that of purified IgG from patients positive for $\beta_2$GPI-dependent aCL. We conformationally optimized to domain V and the domain IV–V complex of $\beta_2$GPI variants at position 247, referring the crystal structure of $\beta_2$GPI. IgG aCL was screened using the standardized aCL ELISA, in which both the Leu$^{247}$ and the Val$^{247}$ allele of $\beta_2$GPI are contained as antigen. Although biochemical characteristics and structure are similar between valine and leucine, the replacement of Leu$^{247}$ by Val$^{247}$ leads to a significant alteration in the tertiary structure of domain V and/or the domain IV–V interaction (Figure 4). It is likely that the structural alteration affects the affinity between anti-$\beta_2$GPI autoantibodies and the epitope(s) present on its molecule. One explanation for this phenomenon is that this $\beta_2$GPI polymorphism affects the electrostatic interaction between domain IV and domain V or the protein–protein interaction, resulting in differences in the accessibility of the recognition site by the autoantibodies, or the local density of $\beta_2$GPI.

Another possible explanation of the correlation between the Val/Leu$^{247}$ polymorphism of $\beta_2$GPI and anti-$\beta_2$GPI antibodies is T cell reactivity. Ito et al (32) investigated T cell epitopes of patients with anti-$\beta_2$GPI autoantibodies by stimulating patients' PBMCs with a peptide library that covers the $\beta_2$GPI sequence. Four of 7 established CD4+ T cell clones reacted to peptide fragments that include amino acid position 244–264, then position 247 is included among the candidate epitopes. Arai et al (33) found preferred recognition of peptide position 276–290 by T cell clones from patients with APS. They also found high reactivity to peptide 247–261 in one patient. We speculate that a small alteration in the conformation arising from the valine/leucine substitution at position 247 may affect the susceptibility to generate autoreactive T cell clones in patients with APS.

Our results in this study indicate that the Val/Leu$^{247}$ polymorphism affects the antigenicity of $\beta_2$GPI for anti-$\beta_2$GPI autoantibodies, and that the Val$^{247}$ allele can be a risk factor for having autoantibodies against this molecule. Therefore, the Val/Leu$^{247}$ variation of $\beta_2$GPI may be crucial for autoimmune reactivity against $\beta_2$GPI. We further show the significance of the Val/Leu$^{247}$ polymorphism of $\beta_2$GPI in the strength of the binding between $\beta_2$GPI and anti-$\beta_2$GPI autoantibodies. The significance of antigen polymorphisms in the production of autoantibodies or in the development of autoimmune diseases is not well understood. To our knowledge, this report is the first to present a genetic polymorphism of

autoantigen directly affecting its interaction with autoantibodies.

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# Multiple Autoimmune Diseases after Autologous Stem-Cell Transplantation

**TO THE EDITOR:** Hematopoietic stem-cell transplantation can be an effective treatment in patients with refractory systemic sclerosis.[1] We report on a 19-year-old woman with systemic sclerosis who underwent CD34+-selected autologous hematopoietic stem-cell transplantation in March 2001. Before the transplantation, the physical and laboratory findings showed no evidence of any other autoimmune diseases. After written consent was obtained from the patient, CD34+ hematopoietic stem cells were transplanted according to a method used for systemic sclerosis.[1] The dermal sclerosis improved immediately after transplantation, but thrombocytopenia and Graves' disease developed.

In June 2005, the patient was admitted to the hospital because of fever and edema. Blood tests revealed proteinuria (11.4 g per day) and new autoantibodies in the serum (Fig. 1A). On the sixth hospital day, paralysis developed on the left side as the result of a right cerebral infarction. Systemic lupus erythematosus with membranous-type lupus nephritis (Fig. 2) and the antiphospholipid-antibody syndrome were diagnosed; the patient was treated with prednisolone, warfarin, and cyclosporine. She is currently in clinical remission and is back at work.

During the early phases of immune reconstitution, residual lymphocytes undergo proliferation and expansion, a process controlled by regulatory T cells.[2,3] These cells, defined by the phenotype CD4+CD25+FOXP3+, are important in the prevention of autoimmunity. Interleukin-17–producing helper T (Th17) cells may play a role in the induction of autoimmunity.[4,5] In our patient, the level of serum interleukin-17, released mainly by Th17 cells, was elevated at the onset of the systemic lupus (Fig. 1B). Levels of *FOXP3* messenger RNA, a marker of regulatory T cells, were reduced, suggesting a deficiency of such cells (Fig. 1C). The findings in our patient suggest a role of both regulatory T cells and Th17 in the development of systemic lupus.

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1. Farge D, Passweg J, van Laar JM, et al. Autologous stem cell

**Figure 1 (facing page). Clinical and Laboratory Findings after CD34+-Selected Autologous Hematopoietic Stem-Cell Transplantation.**
Panel A shows the association between clinical events (including the onset of autoimmune thrombocytopenia [AITP], Graves' disease, systemic edema, and cerebral infarction) and changes in titers of each autoantibody. At the onset of edema, a serum sample from the patient contained anti-Sm, anti-Scl70, and anticardiolipin IgG antibodies (IgG-aCL), in addition to anti-DNA autoantibodies and lupus anticoagulant. The solid line indicates the modified Rodnan total skin thickness score (ranging from 0 to 51, with higher values indicating more thickness). Normal ranges for these levels are as follows: anti-Sm, 0 to 5.9 U per milliliter, anti-Scl70, 0 to 18.9 U per milliliter; and IgG-aCL, <1.3 U per milliliter. Panel B shows serum levels of interleukin-17, transforming growth factor $\beta$1 (TGF-$\beta$1), and interleukin-6. Normal ranges for these levels are as follows: TGF-$\beta$1, 30.95 to 38.65 ng per milliliter; interleukin-6, 0.54 to 1.10 ng per milliliter; and interleukin-17, not detected. Panel C shows changes in T cells, including the ratio of interferon-$\gamma$–producing CD4+ T cells (Th1) and interleukin-4–producing CD4+ T cells (Th2) and FOXP3 messenger RNA (mRNA) on peripheral-blood mononuclear cells. The solid squares indicate levels of CD19+ cells, and the circles indicate levels of CD4+CD25+ cells. Normal ranges are as follows: ratio of Th1 to Th2, 7.22 to 47.52; FOXP3 mRNA, 57.10 to 175.19 copies per glyceraldehyde-3-phosphate dehydrogenase (GAPDH) standard; CD19+, 9.24 to 17.01%; and CD4+CD25+, 5.66 to 10.24%. Calculations were made with the JMP statistical software package, version 5.0 (SAS Institute).

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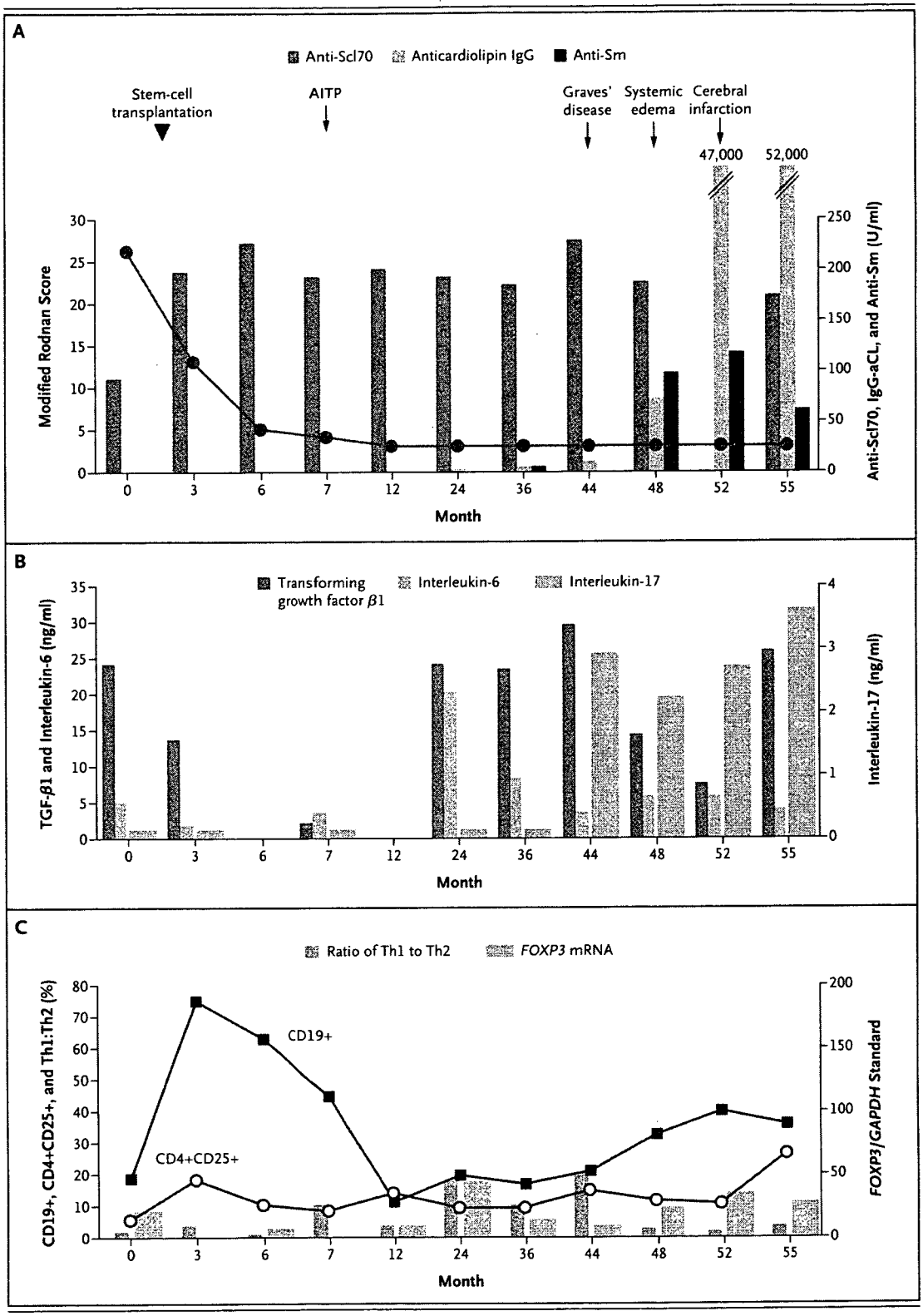
A
Anti-Scl70
Anticardiolipin IgG
Anti-Sm
Stem-cell transplantation
AITP
Graves' disease
Systemic edema
Cerebral infarction
47,000
52,000
Modified Rodnan Score
Anti-Scl70, IgG-aCL, and Anti-Sm (U/ml)
Month
B
Transforming growth factor β1
Interleukin-6
Interleukin-17
TGF-β1 and Interleukin-6 (ng/ml)
Interleukin-17 (ng/ml)
Month
C
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CD4+CD25+
CD19+, CD4+CD25+, and Th1:Th2 (%)
FOXP3/GAPDH Standard
Month

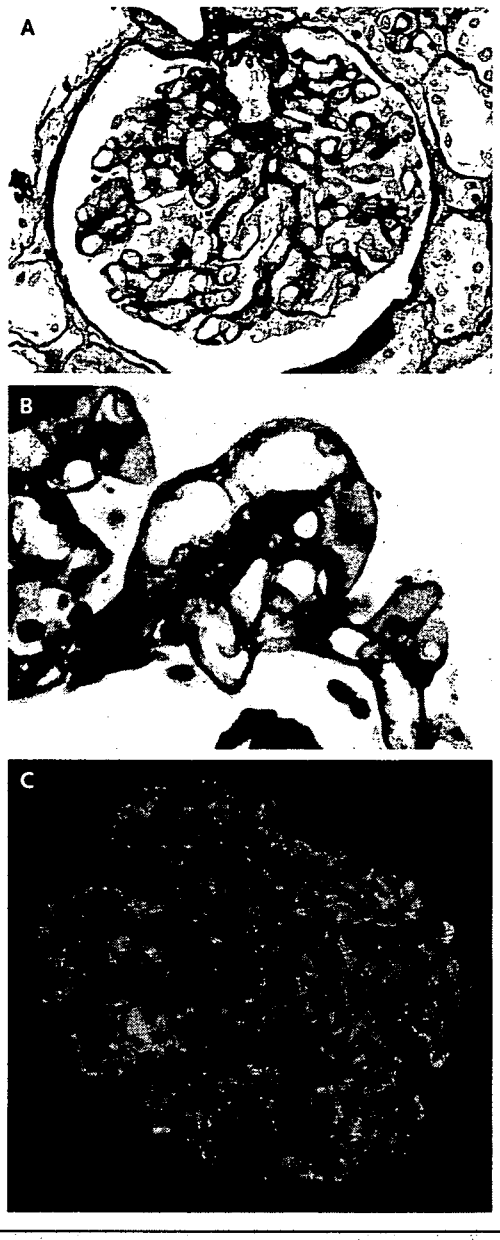


**Figure 2. Renal-Biopsy Specimens Showing Representative Lesions at the Onset of Lupus Nephritis.**

Renal-biopsy specimens show increased mesangial matrix (Panel A, periodic acid–Schiff staining), capillary-wall thickness and bubble appearance (Panel B, periodic acid–methenamine silver staining), and global granular immune deposits (Panel C, immunofluorescence staining).

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LETTERS

# Methotrexate-induced lung injury in patients with rheumatoid arthritis occurs with peripheral blood lymphocyte count decrease

**S Inokuma, H Kono, Y Kohno, K Hiramatsu, K Ito, K Shiratori, J Yamazaki, H Nakayama, H Shoda, Y Tanaka**

*Ann Rheum Dis* 2006;**65**:1113–1114. doi: 10.1136/ard.2005.045211

Pulsed methotrexate (MTX) treatment, of which the standard dose for Japanese patients with rheumatoid arthritis (RA) approved by the National Health Insurance System is 2–8 mg/week, is still the standard regimen. However, life threatening adverse reactions to this treatment, including lung injury, remain to be elucidated in detail.

We treated 11 patients with RA who developed lung injury during MTX treatment. Their clinical and laboratory features were examined retrospectively, focusing mainly on the changes in peripheral blood lymphocyte count.

The 11 patients (three men, eight women) had a mean (SD) age of 69.8 (7.8) years. All the patients had taken non-steroidal anti-inflammatory drugs, and nine had received steroids with a mean (SD) prednisolone dose of 6.6 (2.9) mg/day.

All of them responded well to MTX, when evaluated by American College of Rheumatology 20 core set.[1] The dose of MTX had been increased from 2.8 (1.4) to 7.1 (3.4) mg/week during 15.4 (17.8) months. At the onset of respiratory distress with a fever, plain chest radiography and/or computed tomography showed ground-glass opacities, mainly from the middle to upper central lung fields; the findings differed from those of rheumatoid pneumonitis which usually involves the periphery and results in structural derangement. The bronchoalveolar lavage fluid of five patients contained CD4+ T cells (70.7 (15.1)% of the total cells). No micro-organism was detected either in lavage fluid or sputum in any of the patients. Hypoxaemia was severe, with a mean (SD) $Pao_2$ of 47.1 (15.2) mm Hg. Serum C reactive protein (CRP) level was as high as 233 (136) mg/l, and serum $\beta_2$-microglobulin level was 4.94 (1.83) mg/l (normal range 0.8–1.7). Three patients underwent mechanical ventilation, and six received pulsed steroid treatment; a patient who received both of these treatments died 1 month later. After recovery in 10 patients, the opacities in chest radiography/computed tomography completely disappeared, and $Pao_2$ and serum CRP and $\beta_2$-microglobulin levels returned to normal.

The peripheral blood lymphocyte count was followed throughout from the start of MTX treatment to after the recovery from lung injury, and the counts at the start of MTX treatment, at arthritis remission, at the onset of lung injury, and after the recovery from lung injury were compared with each other (fig 1). The count did not change from the start of MTX treatment (1.91 (0.83)$\times 10^9$/l) to arthritis remission (1.77 (0.69)$\times 10^9$/l, $p = 0.293$), then decreased in all of the patients at the lung injury onset (0.56 (0.45)$\times 10^9$/l, $p<0.0001$ *v* arthritis remission; $p<0.0001$ *v* MTX treatment start), and re-increased to preinjury level in 10 patients who recovered (1.96 (1.06)$\times 10^9$/l, $p = 0.0002$ *v* lung injury onset; $p = 0.738$ *v* arthritis remission; $p = 0.880$ *v* MTX treatment start). In the patient who did not recover, the lymphocyte count did not re-increase after MTX administration was stopped. Other blood cell count changes did not correlate with the event.

The new finding in this study was the significant decrease in peripheral blood lymphocyte count concurrent with the lung injury. This observation was in agreement with our previous study that the lymphocyte count did not change during RA remission, but decreased only when various adverse reactions to MTX occurred.[2] Along with this, the increase in lymphocyte count in bronchoalveolar lavage fluid, particularly in the count of CD4+ T cells, in this study and in previous reports,[3 4] suggests that lymphocytes may participate in the injury. A high serum level of $\beta_2$-microglobulin at injury onset might be relevant to the changes of lymphocytes.

We conclude that a peripheral blood lymphocyte count decrease in a responder, concurrent with respiratory distress and fever after receiving MTX dose, strongly suggests MTX-induced lung injury, and an increase again to the preinjury level predicts recovery from the injury.

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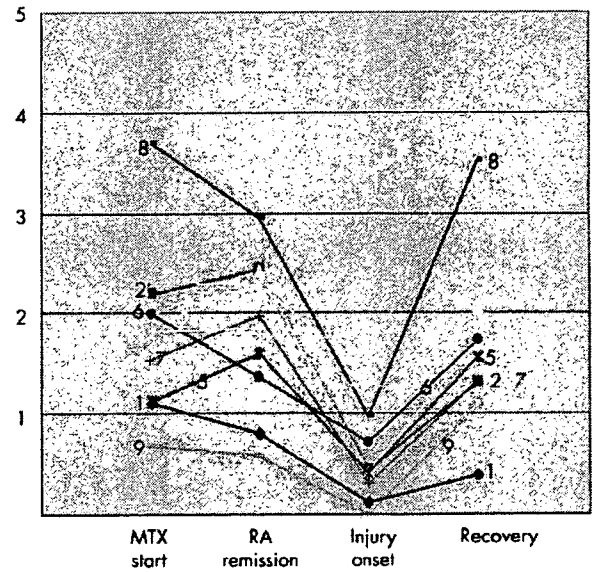


**Figure 1** Peripheral blood lymphocyte count ($10^9$/l) at the start of methotrexate treatment, at arthritis remission, at lung injury onset, and after recovery from lung injury. The numbers refer to the individual patients. Patient 2 eventually died.

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## Analyses of ADAMTS13 activity and its inhibitor in patients with thrombotic thrombocytopenic purpura secondary to connective tissue diseases: Observations in a single hospital

Sirs,

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening disorder characterized by generalized platelet thrombi in arterioles and capillaries (1, 2). A severe decrease of a disintegrin and metalloprotease with thrombospondin type I motif 13 (ADAMTS13) activity and positive ADAMTS13 inhibitor have been considered to be characteristic features of classical TTP (3, 4). TTP has been described as a rare but severe complication associated with connective tissue diseases (CTDs) (5). In this study, we examined whether patients with TTP secondary to CTDs have a decreased ADAMTS13 activity and ADAMTS13 inhibitor.

Among the number of 1056 patients with CTDs hospitalized in Department of Allergy and Immunological Diseases, Tokyo Metropolitan Komagome Hospital from 1978 to 2004, 12 patients were diagnosed as having TTP with four or five of the pentad of TTP (Table I). The incidences of TTP secondary to CTDs were estimated as follows: 12 of 1056 patients (1.14%) as a whole, 3 of 53 (5.66%) in systemic sclerosis, 2 of 66 (3.03%) in vasculitic syndrome, 5 of 222 (2.25%) in systemic lupus erythematosus, 1 of 65 (1.54%) in myositis, and 2 of 132 (1.52%) in primary Sjögren's syndrome. All the patients were female, and none of them was pregnant. Ten patients had active CTDs. Anti-phospholipid antibody was detected in none of the nine patients examined. None of them received cyclosporine, antimalarial, ticlopidine, and clopidogrel before the development of TTP. In one patient, TTP developed at the diagnosis of CTD, and in 11 patients 0.2 to 16 years (median 1 year) after the diagnosis of CTDs. The mortality rates were 58% overall, and 88% and 0% in patients with and without neurological disorders, respectively.

Among the eight patients whose ADAMTS13 activity was measured (6, 7), three had a moderately decreased (3 to 25%), two a mildly decreased (25 to 50%), and three a normal activity (more than 50%); the ADAMTS13 inhibitor was detected at a low titer in only one patients. In five of the eight patients tested, unusually-large von Willebrand factor (VWF) multimers were clearly detected, and plasma VWF antigen levels were markedly high, ranging from 440% to 1400%.

In the present study, none of our patients had a severely decreased ADAMTS13 activity, indicating that the pathogenesis of TTP secondary to CTDs is not necessarily the same as that of classical TTP. Mannucci *et al.* (8) reported that patients with systemic lupus erythematosus and systemic sclerosis had low but detectable levels of ADAMTS13 without inhibitor, although they had no sign or symptom of TTP. On the other hand, Matsumoto *et al.* (9) reported that 10 of 43 patients with TTP secondary to CTDs had a severely decreased ADMATS13 activity and its inhibitor was detected in 13 of 27 patients tested. From these findings, it appears that a severely decreased ADAMTS13 activity caused by its inhibitor is involved in some patients with TTP secondary to CTDs, but a mildly to moderately decreased ADAMTS13 level can be a finding in CTD patients with or without TTP.

Particularly interesting was the result that unusually-large VWF multimers were detected in five of eight patients tested. In cases of CTDs, the damage of the endothe-

**Table 1.** Clinical features and measurement of ADAMTS13 activity, its inhibitor, ULVWFM, and VWF Ag in patients with TTP secondary to CTDs..

| | | Underlying CTDs | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient No. | Sex/age (years) | Disease | Activity | Positive autoantibody* | Treatment | TTP treatment | Outcome | ADAMTS13 activity (%) | Inhibitor (BU/mL) | ULVWFM | VWF Ag (%) |
| 1 | F / 28 | SLE | None | ANA, DNA | PSL 5 mg/day | PE, mPSL 64mg/day dipyridamole, aspirin | Recovered | NA | NA | NA | NA |
| 2 | F / 55 | SSc, PM | + | ANA, DNA, Scl-70, RF | PSL 1.4 mg/kg, d-PC | PE, dipyridamole, aspirin | Died | NA | NA | NA | NA |
| 3 | F / 67 | SjS | NA | ANA, RF | Non | PI | Died | NA | NA | NA | NA |
| 4 | F / 50 | MPA | + | MPO-ANCA, PR3-ANCA | PSL 1.2 mg/kg, CPA | PI | Recovered | NA | NA | NA | NA |
| 5 | F / 74 | SjS | + | ANA, RF, MPO-ANCA, PR3-ANCA | None | PI, PSL 0.8mg/kg | Died | 70 | < 0.5 | None | 440 |
| 6 | F / 83 | SSc | + | ANA | None | PE, dipyridamole, VCR PSL 1.2mg/kg | Died | 20 | < 0.5 | + | 500 |
| 7 | F / 68 | GS, MPA | + | RF, MPO-ANCA, GBM | PSL 1.2 mg/kg, CPA | PE | Died | 48 | < 0.5 | None | 720 |
| 8 | F / 42 | SLE, SjS | + | ANA- ds-DNA | PSL 0.8 mg/kg | PE | Recovered | 17 | < 0.5 | + | 560 |
| 9 | F / 25 | SLE, SjS | + | ANA, ds-DNA, RNP | PSL 1.2 mg/kg | PE | Died | 26 | < 0.5 | + | 1400 |
| 10 | F / 48 | SLE | + | ANA, RNP, Sm, SS-A, SS-B | PSL 1.2 mg/kg | PI | Recovered | 66 | < 0.5 | + | 560 |
| 11 | F / 33 | SLE | + | ANA, ds-DNA, SS-A | PSL 1.2 mg/kg | PE, PI | Recovered | 51 | < 0.5 | + | 680 |
| 12 | F / 72 | SSc | + | ANA, Scl-70, SS-A | PSL 0.6 mg/kg | ACE inhibitor | Died | 10 | 0.5 | None | 1000 |

ADAMTS: a disintegrin and metalloprotease with thrombospondin type I motif; TTP: thrombotic thrombocytopenic purpura; CTDs: connective tissue diseases; ULVWFM: unusually-large von Willebrand factor multimers; VWF Ag: von Willebrand factor antigen; F: female; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; PM: polymyositis; SjS: Sjögren's syndrome; MPA: microscopic polyangiitis; GS: Goodpasture's syndrome; NA: not available; ANA: anti-nuclear antibody; RF: rheumatoid factor; MPO: myeloperoxidase; ANCA: antineutrophil cytoplasmic antibody; PR3: proteinase-3; GBM: glomerular basement membrane; PSL: prednisolone; d-PC: d-penicillamine; CPA: cyclophosphamide; PE: plasma exchange; mPSL: methylprednisolone; PI: plasma infusion; VCR: vincristine; ACE: angiotensin converting enzyme

*ANA was measured by immunofluorescent antibody technique, RF by nephelometry, and other autoantibodies by enzyme-linked immunoassay.

lium of capillaries caused by angiitis, the deposition of immune complexes, or certain drugs may result in a defective utilization of ADAMTS13 and the subsequent accumulation of unusually-large VWF multimers in the circulation. Since a markedly high level of plasma VWF antigen observed in our study appears to reflect a proportional increase in plasma unusually-large VWF multimers, it is conceivable that a decreased enzyme-to-substrate (ADAMTS13/unusually-large VWF multimers) ratio results in an accumulation of undigested unusually-large VWF multimers, leading to TTP.

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**Annals of Internal Medicine** | REVIEW

# Meta-analysis: Diagnostic Accuracy of Anti–Cyclic Citrullinated Peptide Antibody and Rheumatoid Factor for Rheumatoid Arthritis

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**Background:** Rheumatoid factor (RF) and autoantibodies against cyclic citrullinated peptide (CCP) are markers that might help physicians diagnose rheumatoid arthritis.

**Purpose:** To determine whether anti-CCP antibody more accurately identifies patients with rheumatoid arthritis and better predicts radiographic progression than does RF.

**Data Sources:** MEDLINE through September 2006 and reference lists of retrieved studies and review articles.

**Study Selection:** Studies in any language that enrolled at least 10 participants and that examined the role of anti-CCP antibody and RF in the diagnosis or prognosis of known or suspected rheumatoid arthritis.

**Data Extraction:** Two authors independently evaluated studies for inclusion, rated methodological quality, and abstracted relevant data.

**Data Synthesis:** The DerSimonian–Laird random-effects method was used to summarize sensitivities, specificities, and positive and negative likelihood ratios from 37 studies of anti-CCP antibody and 50 studies of RF. The pooled sensitivity, specificity, and positive and negative likelihood ratios for anti-CCP antibody were 67% (95% CI, 62% to 72%), 95% (CI, 94% to 97%), 12.46 (CI, 9.72 to 15.98), and 0.36 (CI, 0.31 to 0.42), respectively. For IgM RF, the values were 69% (CI, 65% to 73%), 85% (CI, 82% to 88%), 4.86 (CI, 3.95 to 5.97), and 0.38 (CI, 0.33 to 0.44). Likelihood ratios among IgM RF, IgG RF, and IgA RF seemed to be similar. Results from studies of patients with early rheumatoid arthritis were similar to those from all studies. Three of 4 studies found that risk for radiographic progression was greater with anti-CCP antibody positivity than with IgM RF positivity.

**Limitations:** Many studies had methodological limitations. Studies of RF were heterogeneous and had wide ranges of sensitivity and specificity.

**Conclusions:** Anti-CCP antibodies are more specific than RF for diagnosing rheumatoid arthritis and may better predict erosive disease.

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Rheumatoid arthritis is the most common autoimmune disease, affecting approximately 1% of the world's population (1). It causes persistent synovitis, pain, joint destruction, and functional disability. Because irreversible joint destruction can be prevented by intervention during the first months of disease, early diagnosis of rheumatoid arthritis is important (2–4).

Rheumatoid factor (RF) is an antibody directed against the Fc region of IgG that has been used as a diagnostic marker for rheumatoid arthritis. However, it is nonspecific and may be present in healthy elderly persons or in patients with other autoimmune and infectious diseases (5). Other rheumatoid arthritis–associated autoantibodies known to be specific for rheumatoid arthritis include antiperinuclear factor and antikeratin antibodies (6, 7). Because of rigorous technical requirements for their detection, antiperinuclear factor and antikeratin antibodies have never been widely used as markers for rheumatoid arthritis, despite their high specificity. The epitopes of their antigens are arginyl residues citrullinated by peptidyl arginine deiminase (8–10). Some enzyme-linked immunosorbent assays (ELISAs) use linear citrulline-containing peptides that have similar sensitivity to and higher specificity than RF for diagnosing rheumatoid arthritis (11). To improve sensitivity, assays that use cyclic citrullinated peptide (CCP) were developed to detect anti-CCP antibody (12).

In this systematic review, we summarize published data on the sensitivity, specificity, and likelihood ratios of RF and anti-CCP antibodies for diagnosing rheumatoid arthritis. We also summarize results of studies that assessed the associations of these markers with development and radiographic progression of rheumatoid arthritis.

## METHODS

### Data Sources and Searches

We developed a protocol for the review and followed standard reporting guidelines (13, 14). We searched MEDLINE for studies published in any language through September 2006 that examined autoantibodies against citrullinated proteins, rheumatoid factor, or both for the diagnosis of rheumatoid arthritis. Our searches (available

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***Context***

Are autoantibodies against cyclic citrullinated peptide (CCP) better serum markers for rheumatoid arthritis than rheumatoid factor (RF)?

***Contribution***

This meta-analysis of 86 studies found that the positive likelihood ratio for anti-CCP antibody was greater than that for IgM RF for identifying patients with rheumatoid arthritis (12.5 vs. 4.9). Sensitivity was similar for the 2 tests, although specificity of anti-CCP antibody (95%) was higher than specificity of IgM RF (85%).

***Cautions***

Fewer studies evaluated anti-CCP antibody than RF. There was possible publication bias for reporting positive findings regarding anti-CCP antibody.

***Implication***

Anti-CCP antibody positivity seems to be more specific than IgM RF positivity for identifying patients with rheumatoid arthritis.

*—The Editors*

on request) were based on combinations of the following index terms: *rheumatoid arthritis, antiperinuclear factor, antikeratin antibody, citrullinated protein, anti–cyclic citrullinated peptide, rheumatoid factor, sensitivity, specificity, mass screening, predictive value of tests, receiver-operating characteristic curve,* and *accuracy.* We also reviewed reference lists of retrieved studies and review articles.

### Study Selection

Two reviewers independently scanned abstracts that met the inclusion criteria. We included studies that evaluated the utility of assaying anti-CCP antibody or RF for diagnosis of known or suspected rheumatoid arthritis, enrolled at least 10 participants, were published after 1987, and provided enough data to allow calculation of sensitivity and specificity for diagnosis of rheumatoid arthritis. We used the 1987 revised American College of Rheumatology (ACR) criteria as the reference standard of rheumatoid arthritis (15). In general, we regarded reports of patients with symptom duration of less than 1 year as studies of early rheumatoid arthritis, although we also used the researchers' definitions of early rheumatoid arthritis.

### Data Extraction and Study Quality Assessment

We extracted data by using a standard form that included the demographic characteristics of the participants, inclusion and exclusion criteria, number of participants who were evaluated with the index test, and methods of antibody testing. Two investigators independently assessed the design of the studies by using previously developed quality criteria for studies of diagnostic tests (16–18). These assessments addressed the technical quality of the anti-CCP antibody test, technical quality of the RF test, application of the reference or index test, blinding of observers, description of the study sample, and cohort assembly. We used $\kappa$ coefficients to examine interrater agreement for our initial overall quality score (19) and resolved any item discrepancies through discussion.

### Data Analysis

We used a random-effects model to combine estimates of sensitivity, specificity, and positive and negative likelihood ratios (19–21). We planned analyses that were stratified by generation of anti-CCP antibody assay (first [anti-CCP1] second [anti-CCP2]) and by RF subtype (IgA, IgG, and IgM). We analyzed subgroups of relevant studies that included patients with early rheumatoid arthritis and that evaluated combination testing for anti-CCP antibody and RF. We conducted a stratified analysis for different threshold and measurement methods when we suspected heterogeneity among studies. We also conducted threshold analyses and metaregression to assess whether the threshold effect and heterogeneity among studies existed (22).

We examined funnel plots for diagnostic odds ratios to explore the possibility of publication bias (23). For analyses, we used MetaDiSc, version 1.1.4 (Hospital Universitario Ramón y Cajal, Madrid, Spain); Stata, version 8.2 (Stata Corp., College Station, Texas); and R, version 2.21 (R Foundation for Statistical Computing, Vienna, Austria).

### Role of the Funding Sources

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## RESULTS

### Search Results and Characteristics of Studies

We identified 302 reports, of which 86 met the inclusion criteria (11, 12, 24–106) (**Appendix Figure**, available at www.annals.org). Thirty-seven studies in 14 949 patients (11, 12, 24, 26, 29–38, 40–42, 44, 45, 47, 48, 50, 52, 54, 56, 58, 60–62, 64, 66, 67, 70, 74, 76, 97, 99, 100) reported on the diagnostic accuracy of anti-CCP antibody, whereas 50 studies in 15 286 patients (12, 24, 27, 29, 30, 32–37, 39, 40, 42–44, 47, 48, 50, 52, 54, 55, 60–62, 64, 66, 70, 72–74, 76, 80–85, 88–98, 100) reported on the diagnostic accuracy of RF.

**Appendix Table 1** (available at www.annals.org) (11, 24, 26, 29–38, 40–42, 44, 45, 47, 48, 50, 52, 54, 56, 58, 60–62, 64–67, 74, 76, 97, 99, 100) and **Appendix Table 2** (available at www.annals.org) (12, 24, 27, 29, 33–35, 37, 39–43, 45, 47, 48, 52, 65, 66, 72–74, 76, 80, 81, 88, 90–92, 94–98, 100) summarize the characteristics of the

included studies. In anti-CCP antibody and IgM RF studies, respectively, the median numbers of participants were 404 and 226, their median ages were 57 years and 53 years, and the median proportions of women were 59% and 68%. Studies of anti-CCP antibody that were published after 2000 usually addressed anti-CCP2 assays.

Characteristics of control groups varied. Among the anti-CCP antibody studies, 5 used patients with undifferentiated arthritis, 13 used patients with other rheumatic diseases, 1 used healthy persons, 1 used hepatitis C carriers, and 17 used a mix of healthy persons and patients with other diseases. Among the IgM RF studies, 5 used patients with undifferentiated arthritis, 16 used patients with other rheumatic diseases, 2 used healthy persons, 1 used hepatitis C carriers, 1 used patients with polymyalgia rheumatica, and 22 used a mix of healthy persons and patients with other diseases. Three studies did not report details on the control group.

### Study Quality

Only 1 study satisfied all criteria on our quality checklist. Twenty-two studies (30%) met at least 70% of the criteria, and 9 studies (10%) met fewer than 50% of the criteria. The $\kappa$ coefficient for interrater agreement was 0.92 on the quality score.

Most studies adequately described the technical aspects of assaying anti-CCP antibody and RF. In 86% (32 of 37) of anti-CCP antibody studies and 82% (41 of 50) of RF studies, the 1987 revised ACR criteria were used as the reference standard for rheumatoid arthritis. Most studies did not explicitly mention blinding of investigators to the clinical assessment or to the reference standard. Most studies (90%) enrolled patients with known or suspected rheumatoid arthritis. Characteristics of these patients were fully described in just over half of the studies. Enrollment was prospective in 18 of 37 anti-CCP antibody studies and 25 of 50 RF studies.

Studies of RF showed a wide range of sensitivities and specificities (**Appendix Table 1**, available at www.annals.org). One study (35) reported very low sensitivity and specificity. In this study, 57% of control patients had conditions that can present with RF-positive arthritis (primarily the Sjögren syndrome or Wegener granulomatosis).

Laboratory techniques for measuring RF varied across studies. Fifteen studies used nephelometry, 16 used latex agglutination, and 16 used ELISA. Twenty-two studies used less than 20 U/mL as the cutoff value for negative test results, 11 used less than 40 U/mL as the cutoff value, and 17 did not report cutoff values.

### Diagnostic Accuracy of Anti-CCP Antibody and IgM RF, IgA RF, and IgG RF

The summary positive and summary negative likelihood ratios, respectively, were 12.46 (95% CI, 9.72 to 15.98) and 0.36 (0.31 to 0.42) for anti-CCP antibody and 4.86 (CI, 3.95 to 5.97) and 0.38 (CI, 0.33 to 0.44) for IgM RF (**Figure 1** and **Figure 2**). The pooled sensitivity and specificity were 67% (CI, 65% to 68%) and 95% (CI, 95% to 96%), respectively, for anti-CCP antibody and 69% (CI, 68% to 70%) and 85% (CI, 84% to 86%) for IgM RF. Data that were limited to studies of patients with early rheumatoid arthritis were similar to those from all studies (data available from the authors on request).

Studies published before 2000 tended to report high sensitivity and specificity for RF compared with studies published from 2000 onward. More recent studies reported favorable specificities for anti-CCP antibody. Summary likelihood ratios for studies that directly compared anti-CCP antibody and IgM RF (11, 12, 24, 26, 29–38, 40–42, 44, 45, 47, 48, 50, 52, 54, 56, 58, 60–62, 64, 66, 67, 70, 74, 76, 97, 99, 100) were similar to summary data from all studies. Positive likelihood ratios for anti-CCP antibody and IgM RF were 12.32 and 3.86, respectively. Negative likelihood ratios for anti-CCP antibody and IgM RF were 0.40 and 0.41, respectively. Positive and negative likelihood ratios for IgA RF and IgG RF seemed to be qualitatively similar to those for IgM RF (**Figure 3**). Stratified analyses for IgM RF showed no major differences for positive summary likelihood ratios or negative likelihood ratios across the strata of cutoff values and measurement methods (**Table**). The threshold effect for IgM RF is not statistically significant, and no covariate was statistically significant in the metaregression model.

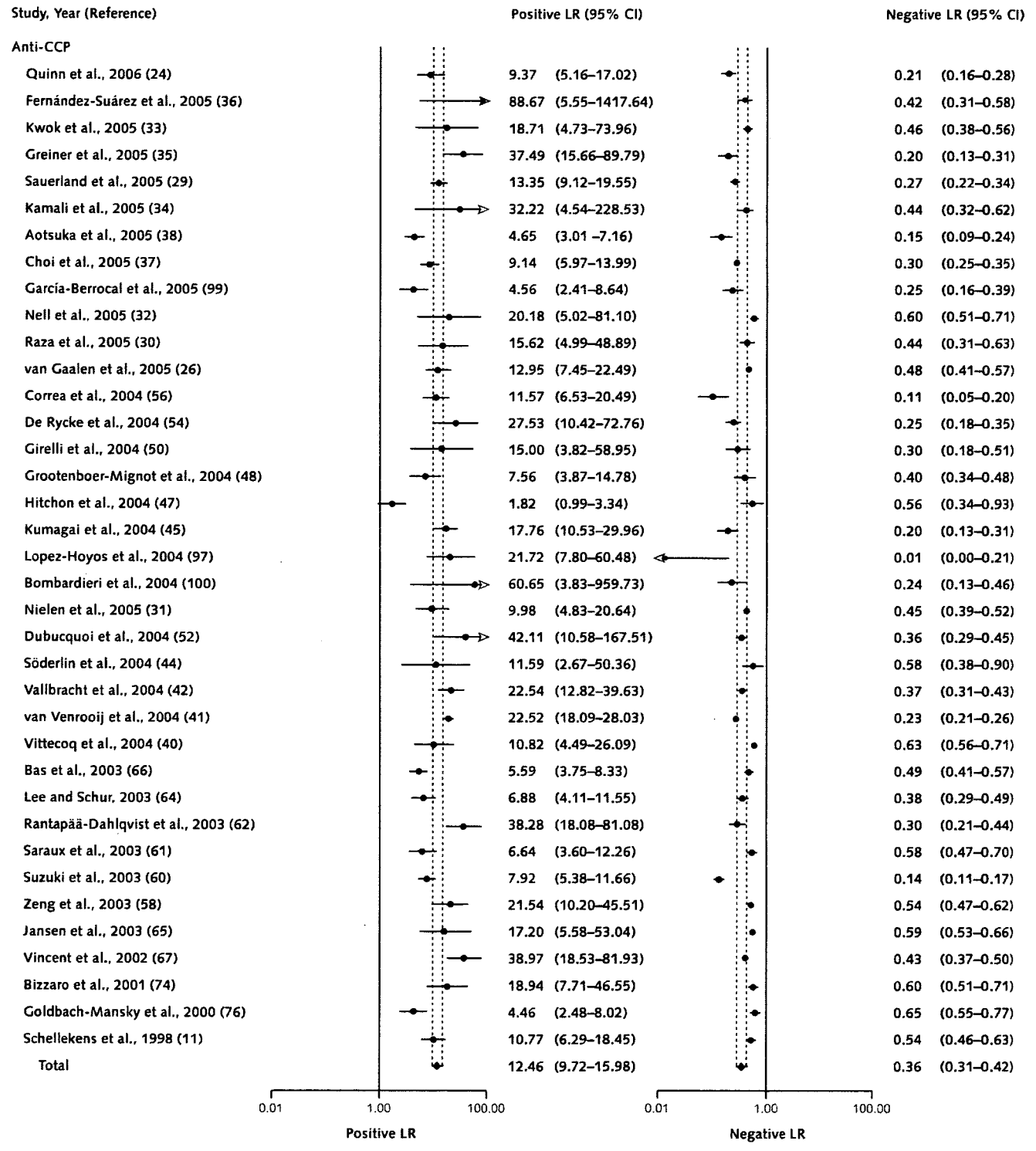
### Diagnostic Accuracy of Anti-CCP1, Anti-CCP2, and Both Anti-CCP Antibody and IgM RF

Twenty-nine studies in 11 821 patients (24, 26, 29–38, 40–42, 44, 45, 47, 48, 50, 52, 54, 56, 60, 62, 64, 97, 99, 100) assessed anti-CCP2, whereas 5 studies in 2098 patients (61, 66, 67, 70, 74) assessed anti-CCP1.

Although the sensitivities and specificities were similar to those in the anti-CCP1 studies, 3 studies (12, 58, 76) that used an in-house ELISA were excluded because incorporating them introduced a positive threshold effect and caused heterogeneity among the studies. The summary positive and negative likelihood ratios were 12.77 (CI, 9.62 to 16.94) and 0.32 (CI, 0.27 to 0.38), respectively, for anti-CCP2 and 13.03 (CI, 5.74 to 29.04) and 0.53 (CI, 0.46 to 0.61) for anti-CCP1 (**Figure 4**).

Six studies in 1753 patients (12, 30, 37, 50, 64, 74) simultaneously measured anti-CCP antibody and RF, whereas 8 studies in 2837 patients (12, 30, 37, 42, 50, 64, 70, 74) performed 1 of the tests only when the results on the other test were positive. For studies that required the presence of both anti-CCP antibody and IgM RF for a positive result, the summary positive and negative likelihood ratios were 15.72 (CI, 8.30 to 29.75) and 0.46 (CI, 0.35 to 0.61), respectively. For studies that considered a result positive if either anti-CCP antibody or IgM RF was detected, the positive and negative summary likelihood ratios were 4.32 (CI, 2.71 to 6.90) and 0.32 (CI, 0.25 to 0.42), respectively.

*Figure 1.* Likelihood ratio *(LR)* for autoantibodies against cyclic citrullinated peptides *(anti-CCP).*




## Prognostic Value of Anti-CCP Antibody and IgM RF

Appendix Table 3 (available at www.annals.org) summarizes the results of 5 studies of the association between rheumatoid arthritis and anti-CCP antibody. The odds ratio for rheumatoid arthritis was 16.1 to 38.99 for anti-CCP antibody positivity and 1.2 to 8.7 for RF positivity.

Fifteen studies examined associations between marker positivity and radiographic progression (Appendix Table 4,

*Figure 2.* **Likelihood ratio *(LR)* for autoantibodies against IgM rheumatoid factor *(RF)*.**