

Antibodies to Citrullinated Vimentin are a Specific and Sensitive Marker for the Diagnosis of Rheumatoid Arthritis

Hannah Poulosom · Peter J. Charles

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Abstract

Background The last 5 years have seen the emergence and establishment of antibodies to citrullinated antigens as the diagnostic marker for rheumatoid arthritis (RA). Initially, these were detected using a synthetic peptide, which has undergone a number of modifications to give a diagnostic test with a sensitivity of 65–80% and a specificity of >95%. Antibodies to citrullinated vimentin were first described in 1994 as a highly specific marker for RA (anti-Sa). However, no easily performed assay for these antibodies has been available.

Methods We have examined the use of a ELISA-based assay with a mutated citrullinated vimentin (MCV) antigen (Orgentec, Mainz, Germany) to assess the diagnostic and prognostic utility of this antibody in RA.

Results Antibodies to MCV were detected in the sera of 74% RA patients (specificity 96%), 2% systemic lupus erythematosus, 14% Sjögren's syndrome, and 2% scleroderma. Anti-MCV was not detected in sera from healthy blood donors. There was no difference in the frequency of antibodies detected in RA patients with early (<2 years) or

chronic (>2 years) disease. There was no significant variation in anti-MCV antibody concentrations in early RA patients over a 52-week period. No significant change was observed with time between the two treatment groups of methotrexate alone or methotrexate plus infliximab.

Conclusions Antibodies to MCV are a specific and sensitive marker for the diagnosis of RA.

Keyword Mutated citrullinated vimentin · Anti-MCV · Autoantibodies · Rheumatoid arthritis

Introduction

Rheumatoid arthritis is a chronic systemic inflammatory autoimmune disease primarily characterized by a bilateral symmetrical polyarticular arthritis, which is often erosive. It is probably the most common autoimmune disease (about 1% of the world population), affects three times as many women as men, and usually appears in middle age. Although the etiology of RA remains unknown, it is widely accepted that multiple accumulative/compounding genetic and environmental 'hits' are required between the initiation of self-peptide recognition, subsequent loss of tolerance, and the development of autoimmunity. Disease onset is often insidious, initially affecting the small joints of the hands (proximal interphalangeal joints), feet, and wrists. Clinical course of the disease varies between individuals but may be mild, relapsing–remitting, or progressive.

The systemic complications and the severity of articular manifestations (with associated functional impairment) may have a debilitating and disabling impact on the patient, causing significant morbidity, reduced quality of life, and even premature mortality. It is therefore important that the disease is diagnosed and treated early to slow/stop joint

H. Poulosom · P. J. Charles
Division of Immunology, Hammersmith Hospitals NHS Trust,
London, UK

P. J. Charles
Kennedy Institute of Rheumatology, Imperial College,
London, UK

P. J. Charles (✉)
Division of Immunology, Charing Cross Hospital,
Fulham Palace Road,
London W6 8RF, UK
e-mail: peter.charles@imperial.ac.uk

damage, increase/maintain joint function, establish remission, and maximize quality of life. It has also been widely reported that, although the early stages of the disease are responsive to disease-modifying anti-rheumatic drugs (DMARDs), once the clinical disease is established, this effect is lost [1].

RA is currently diagnosed using the American College of Rheumatology (ACR) 1987 revised criteria that are primarily based on clinical parameters, the only serological criteria being IgM RF [2]. However, the criteria may be insufficient for the diagnosis of early RA, as they are based upon measurements for disease classification predominately featuring manifestations typical of later-stage disease. Diagnosis may be complicated by the insidious (and sometimes nonspecific) onset of the disease, resulting in delayed diagnosis, often to the detriment of the joint.

Numerous serological markers of RA have been described over the past half century including rheumatoid factor (RF) and antibodies against a range of citrullinated proteins including anti-perinuclear factor, anti-keratin antibody, anti-filaggrin antibody (AFA), and anti-Sa (vimentin) antibody. However, many others have been discarded in the routine clinical setting due to their lack of sensitivity, specificity, and/or laborious detection methods. An ideal marker would be sensitive, specific, detectable in early disease and prognostic, enabling clarification of disease subsets and the early identification of patients likely to develop severe disease and therefore requiring aggressive early treatment to prevent significant joint damage. Equally, patients with transient/mild arthritis would be spared unnecessary immunosuppressive therapy and its associated side effects, also representing a cost saving. The candidate marker would also be quantitative, to allow monitoring of disease (as in anti-dsDNA antibodies in systemic lupus erythematosus, SLE) and measurement of response to treatment.

Rheumatoid Factor

RFs are immunoglobulins directed against the Fc portion of IgG. Although they may be of any immunoglobulin class, it is usually IgM that is measured in the diagnostic laboratory. IgA RFs have in the past been considered to be associated with erosive disease. Although first documented 60 years ago and of only moderate specificity, IgM RF remains the commonest assay for the diagnosis of RA (and the sole serological marker in the ACR revised 1987 criteria). Although IgM RF has a sensitivity of ~70%, it has a relatively low specificity as it may be detected in other rheumatic diseases, infections, and at low titer in the elderly healthy population [3]. RFs may be measured by particle agglutination, nephelometry/turbidometry, and ELISA, and methodological variation may also occur.

Citrullination

In the last few years, there has been an explosion of research into anti-citrullinated protein/peptide antibodies, including antibodies to filaggrin, fibrin, and vimentin, resulting in the commercially available anti-cyclic citrullinated peptide (CCP) antibody assay. More recently, the identification of an autoantibody against citrullinated alpha-enolase, a 47-kDa antigen abundant in RA synovium, has been published [4].

Citrullination (deimination) is a posttranslational protein modification characterized by the conversion of positively charged arginine amino acid residues into neutrally charged citrulline. When arginine is oxidized, a terminal nitrogen of the arginine sidechain is replaced by an oxygen molecule, with the reaction using one water molecule and releasing ammonia (NH₃) as a by-product. This process is performed by the calcium-dependent peptidylarginine deiminase (PAD) enzyme family, with certain isotypes being expressed in monocytes (PAD4) and macrophages (PAD4 and PAD2) [5], cells that are acknowledged to play a key role in the pathogenesis of RA and whose synovial numbers/populations correlate with rheumatoid joint inflammation [6]. A hypothesis for the role of cell death, PAD enzymes and anti-CCP antibodies in RA is discussed by Zendmen et al. [7].

Citrulline is not incorporated into proteins during protein synthesis as it is not coded for by DNA, so it is only expressed when converted from arginine by posttranslational modification. Citrullination causes protein unfolding and therefore alters protein structure [8] (as the neutral charge of citrulline increases the hydrophobicity), possibly resulting in aberrant recognition by the immune system.

Citrullination is upregulated by inflammation and found to increase immunogenicity of proteins in collagen-induced arthritis mouse models [9]. In transgenic mice, citrullinated peptides have been found to bind with 100-fold higher affinity to the RA-associated shared epitope HLA-DRB1*0401 antigen binding groove (positively charged peptide anchoring pocket P4) than corresponding arginine-containing peptides, which may result in activation of CD4⁺ T cells [10] and possibly cause loss of tolerance and initiation of autoimmunity.

Citrullinated antigens are thought to play a pivotal role in the pathogenesis of RA as they are expressed in inflamed joints and anti-citrullinated protein antibodies are present before clinical disease and are highly specific for RA [11]. Indeed, it has been demonstrated that anti-citrullinated protein antibodies are produced by plasma cells in the synovial tissue, as concentrations are more than sevenfold higher in synovial tissue than in serum [12]. However, although citrullination may play a role in the pathophysiology of RA, the process is not specific for the disease,

being detected histologically in RA synovium, polymyositis muscle, and chronically inflamed tonsillar tissue [13]. Nevertheless, the antibodies to citrullinated proteins continue to be the most specific serological markers of RA.

Other markers of RA were assayed by indirect immunofluorescence (IIF); however, these assays were subjective, qualitative, and laborious. Anti-perinuclear factor antibody (APF) was first described in 1964 and was characterized as granules around the nucleus of differentiating buccal mucosal epithelial cells. However, there were problems with substrate availability as only ~5% of donors were able to provide suitable cells. The antibody had a sensitivity of 55–70% and a specificity of ~95%. In 1979 [14], anti-keratin antibody (AKA) was noted by its staining of the epithelial cells of rat esophagus stratum corneum. The antibodies had a sensitivity of 40–60% and a specificity of 90–99%. The antigen was identified in 1993 as filaggrin [15] (filament-aggregating protein). The high correlation between AKA and APF antibody was explained in 1995, when they were found to be autoantibodies against the same antigen, filaggrin [16]. However, there were problems with the reproducibility of the protein used in the anti-filaggrin antibody assay, which only had about 50% sensitivity for RA. It was later determined that AFA was targeting citrullinated residues on the filaggrin epitope [17]. Further investigation revealed that, during epithelial cell differentiation, filaggrin polypeptides are dephosphorylated and about a fifth of the arginine residues are citrullinated—a process which also occurs in the esophageal stratum corneum, explaining the autoantibody reactivity previously described in RA [18]. Antibodies to the deiminated alpha and beta chains of fibrin have also been documented as markers of RA. In early RA, the autoantibody has a sensitivity of 56–61% (rising to ~70% in late RA), specificity 93–99%, PPV ~94%, and may be detected in ~29% of seronegative patients [3, 19].

Anti-CCP Antibody

Anti-CCP antibodies were first described in 1998 by Schellekens et al. [20], with the peptide being selected for its reactivity with sera from RA patients. The anti-CCP antibody (CCP2) assay uses a synthetically formed cyclic citrullinated peptide, which shows no homology to tissue proteins and was generated using RA sera to screen citrullinated peptide libraries (14 million peptides). The cyclic structure (formed with disulfide bonds) provides optimal exposure of the citrulline residues, enhancing recognition of the peptide by the autoantibodies and therefore increasing the sensitivity of the assay. The benefit of synthetic peptide production and purification is that it is cheap and reproducible, with homogeneous citrullination of the peptides [7].

The anti-CCP2 assay has been reported with a sensitivity as high as 81% and specificity 91%, and it was also found to identify RF-negative (seronegative) patients and offer prognostic value [21]. Anti-CCP2 is reported to have a better prognostic value than previous assays [22]. However, while IgM RF concentrations have been found to decrease with treatment (DMARDs with/out infliximab), anti-CCP antibody concentrations do not appear to change [7], limiting the use of anti-CCP as a marker of disease activity and precluding its application as an indicator of response to treatment. There is an increasing evidence that anti-CCP antibodies are also detected in patients with other connective tissue diseases including Sjögren's syndrome, systemic lupus erythematosus, systemic sclerosis, mixed connective tissue disease, and idiopathic inflammatory myositis [23, 24].

Several studies have observed the detection of anti-CCP antibodies before disease onset. One study showed that anti-CCP antibodies and IgA RF may be detected years before the clinical onset of RA, using healthy blood donors who subsequently went on to develop the disease. Anti-CCP antibodies were detected in nearly 10% of the patients at 9 years before disease onset, rising steadily to ~30% at 2 years and 40% at 1 year before diagnosis [25]. A study of patients with undifferentiated disease in an early arthritis clinic found that 22% were anti-CCP antibody positive. At 3 years follow-up, anti-CCP antibodies demonstrated a PPV 93% and NPV 75% [26]. Similarly, others have demonstrated that patients positive for both the RA shared epitope (SE; HLA-DRB1*0404/0401) and anti-CCP antibody have an odds ratio (OR) of 66.8 for the development of RA or at least an OR 25.1 if they are SE-/CCP+ (compared to OR 1.9 SE+/CCP- and OR 1.0 for SE-/CCP-) [27].

It has been suggested that although anti-CCP-antibody-positive and -negative RA patients present with similar disease phenotypes, they progress differently, with anti-CCP-antibody-positive patients having more swollen joints and increased radiological damage than anti-CCP-antibody-negative patients at follow-up [28, 29]. This is coupled with greater disease activity and increased loss of function in RA [30].

Overall, anti-CCP antibodies are a useful marker for aiding the diagnosis of early RA, especially in seronegative (RF negative) patients, and aid in the identification of patients needing aggressive treatment for future erosive disease.

Anti-Sa Antibody

IgG anti-Sa antibody was first described in 1994 as a ~50-kDa protein detected in human spleen, placental extract, and rheumatoid synovium [31]. Assayed by IIF, it was found to be highly specific for RA (~99%) with a PPV of ~97%, although the mean sensitivity was only ~43% [32].

Anti-Sa antibody was detected at a lower prevalence in early RA than established disease [33] and found to be predictive of severe disease [34]. Sa has been identified as citrullinated vimentin, with anti-Sa autoantibodies only found to target citrullinated vimentin and not the native form [35]. Vimentin is an intermediate filament, one of the cytoskeletal proteins providing structural support in mesenchymal cells. The protein is normally uncitrullinated but converted during cell death and tissue inflammation. Anti-Sa antibodies were found to be predictive of poor outcome in RA, characterized by more rapid development and more severe joint damage (a finding not independently predicted by anti-CCP antibodies) [36].

Anti-MCV Antibody

The anti-mutated citrullinated vimentin antibody ELISA is a recently developed assay for the detection of IgG antibodies to a vimentin-based peptide. It may therefore be suggested that the assay is a development of the anti-Sa antibody IIF assay. Anti-MCV antibody has been reported with a sensitivity of 65–82%, specificity of 80–95%, PPV 90%, and NPV 78.8% [37–41]. It has also been reported as positive in 70% of RF-positive polyarticular onset juvenile idiopathic arthritis patients [42], 7.3% of psoriatic arthritis patients (with radiological characteristics) [43], and 20% of healthy elderly individuals (at the manufacturer's cut-off of 20 U/ml) [40]. Anti-MCV antibody concentrations have also been reported as correlating with disease activity score (DAS28), prognostic for disease severity, and being treatment sensitive, enabling their use in monitoring response to therapy [37].

In a recent study, we evaluated anti-MCV antibody for the diagnosis of RA. We examined the diagnostic sensitivity and specificity of anti-MCV antibody in RA, determined whether concentrations changed over time with different treatments [methotrexate (MTX) alone or in combination with infliximab (MTX/INF)] and whether the antibody correlated with anti-CCP antibody.

Materials and Methods

Sensitivity and Specificity

Sixty-eight clinically diagnosed RA patients with established disease (>2 years duration) and 24 patients with early RA (<2 years duration) were evaluated for anti-MCV antibody to establish its diagnostic sensitivity in RA. To determine the specificity, 92 patients with RA (a clinical cohort consisting of a spectrum of RA starting DMARD therapy) were analyzed together with 50 healthy controls (NHC; blood donors) and a cross-section of other connec-

tive tissue diseases including 50 systemic lupus erythematosus patients, 50 Sjögren's syndrome patients (SS), and 50 scleroderma patients (Scl).

Longitudinal Study and Effects of Different Treatments

In the longitudinal study, 22 patients with RA of <2 years duration were studied over four time points (pretreatment and 6, 18, and 52 weeks) to determine variation in anti-MCV antibody concentrations over time. This cohort was randomly split into two different treatment groups: 11 patients were treated with MTX alone and 11 with a combination of MTX/INF.

Correlation Between Anti-MCV and Anti-CCP Antibodies

Pretreatment and at 6 and 18 weeks posttreatment, 22 early RA patients of <2 years duration were also investigated for anti-CCP antibody (Immunoscan RA, Euro-Diagnostica, Malmö, Sweden).

Anti-MCV Antibody and Anti-CCP Antibody ELISAs

Commercially available ELISAs for the detection of anti-MCV antibody (kindly provided by Orgentec Diagnostica GmbH) and anti-CCP antibody (Immunoscan RA, Euro-diagnostica, Sweden; second generation) were performed according to the manufacturer's instructions using the automated BEST 2000 ELISA processor. The methods for the two ELISAs were very similar, only varying in incubation times. Briefly, serum samples were diluted (1:100 for MCV and 1:50 for CCP) and incubated in an antigen-coated microtiter plate together with kit standards and controls at room temperature. The plates were then washed thrice to remove unbound protein before the addition of an anti-human IgG antibody conjugated to horseradish peroxidase. After incubation, the unbound conjugate was washed away and the plates incubated with 3,3',5,5'-tetramethylbenzidine substrate. The resulting color development was halted with the addition of acid and the optical density of each well measured at 450 nm. The manufacturer's cut-off of 20 U/ml for anti-MCV antibody and 25 U/ml for anti-CCP antibody was used for analysis, unless otherwise stated.

Statistical Analysis

The statistical analyses were performed using the GraphPad Prism version 4 statistical package (GraphPad Software, San Diego, CA, USA). For analyzing the specificity of anti-MCV antibody (RA versus each disease and healthy control group), a two-tailed Mann–Whitney *U* test was used. For evaluating anti-MCV antibody concentrations over time and

the effects of different treatments, two tests were used—repeated measures ANOVA to compare median antibody concentrations over the whole time period and Mann–Whitney *U* test to measure between consecutive time points. To examine the correlation between quantitative anti-MCV antibody and anti-CCP antibody measurement, Spearman’s rank coefficient was used.

Results

Sensitivity and Specificity

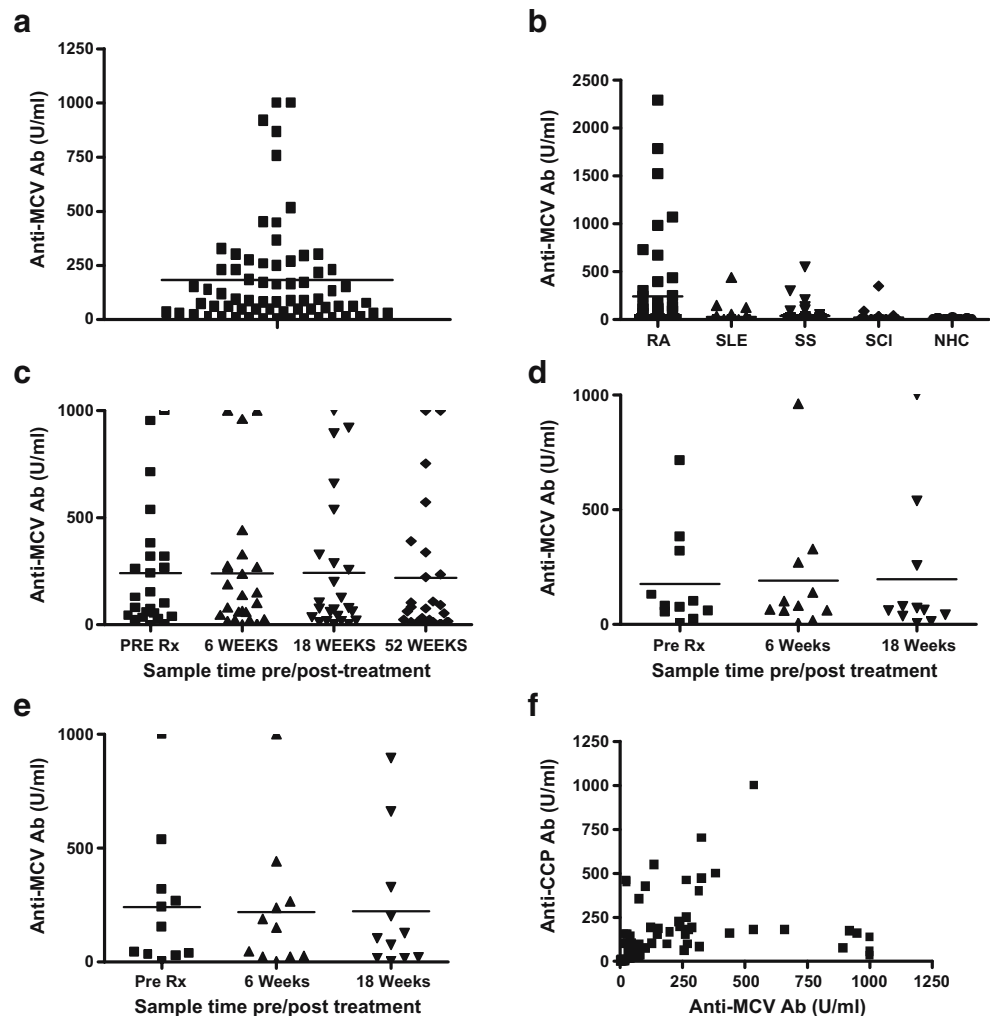
At a cut-off of 20 U/ml, sera from 55 out of 68 patients with established RA (>2 years duration) were positive for anti-MCV antibody (median 86 U/ml, 25% percentile 33.5, 75% percentile 241; Fig. 1a), giving a sensitivity of 81%. In the early RA patient group (<2 years duration), 22 of 24 sera were anti-MCV positive, with a sensitivity of 92%. Combining the data of these two RA cohorts, anti-MCV antibody had a sensitivity of 84% in RA.

Anti-MCV antibody was detected in sera from all disease control groups (SLE 7/50=14%, SS 12/50=24%, Scl 8/50=16%) but only in one healthy control (which was equivocal at 20 U/ml). The specificity of anti-MCV antibody for RA was 87%. The widest range of anti-MCV antibody concentrations was detected in RA patients (median 27.5 U/ml, range 1–2,290 U/ml, SD 486.6 U/ml). Similar ranges were seen between the SLE, SS, and Scl groups (1–439, 3–549, and 1–348 U/ml, respectively; Fig. 1b). The majority of patients in these groups were anti-MCV negative. Although one NHC had a concentration of 20 U/ml (equivocal), the majority were <10 U/ml.

A comparison of anti-MCV values between RA and the other disease/control groups showed that values from RA patients were significantly different (RA vs SLE $p=0.0006$, RA vs SS $p=0.0051$, RA vs Scl $p=0.0002$, RA vs HC $p=<0.0001$ by two-tailed Mann–Whitney *U* test).

It is more useful clinically to have a diagnostic marker with high specificity, although this may compromise the sensitivity of the assay. To examine the effect of adjusting the cut-off value, we also analyzed the data at two higher

Fig. 1 The clinical utility of anti-MCV antibody measurement. The sensitivity of anti-MCV antibody in RA of <2 years duration (a), its specificity in early RA (<2 years duration) compared to disease and healthy controls (b), effect of treatment on anti-MCV antibody concentrations over the study period (c) in patients treated with MTX (d) or MTX/INF (e), and its correlation with anti-CCP antibody (f). *MCV Ab*, anti-mutated citrullinated vimentin antibody; *RA*, rheumatoid arthritis; *MTX*, methotrexate; *MTX/INF*, methotrexate and infliximab; *CCP Ab*, anti-cyclic citrullinated peptide antibody



values of 30 and 40 U/ml. By raising the cut-off, the specificity of the antibody was increased but to the detriment of the sensitivity (Table 1). Although the specificity could be increased to 92 and 96% at cut-off values of 30 and 40 U/ml, the sensitivity decreased to 78 and 74%, respectively. Therefore, although a cut-off of 40 U/ml resulted in a specificity comparable to anti-CCP antibody, the sensitivity was lower.

Longitudinal Study

Sera from 2 of 22 RA patients (<2 years duration) were anti-MCV negative at all four time points (pretreatment and at 6, 18, and 52 weeks posttreatment), and one patient was equivocal (20 U/ml) at pretreatment then negative at 6, 18, and 52 weeks. Two patients were anti-MCV positive at pretreatment but became negative after 18 weeks treatment. The spread of anti-MCV antibody concentrations across the four time points was similar (Fig. 1c). There was also no statistical difference between consecutive treatment points.

Response to Treatment

In patients treated with either MTX alone (Fig. 1d) or a combination of MTX/INF (Fig. 1e), there was no statistically significant change in anti-MCV antibody concentrations over the whole study period nor between individual time points.

Correlation Between Anti-MCV and Anti-CCP Antibodies

Anti-MCV antibody was detected more frequently than anti-CCP antibody over the study period (in two patients pretreatment, in three patients at 6 weeks posttreatment, and in four patients at 18 weeks posttreatment). At 18 weeks posttreatment, two patients were anti-CCP antibody positive but anti-MCV antibody negative. There was a moderate statistical correlation between quantitative anti-MCV and anti-CCP antibody measurement (Spearman $r=0.6049$; Fig. 1f).

Table 1 Comparison of sensitivity and specificity of anti-MCV antibody at different cut-off values

Cut-off value	Sensitivity (%)	Specificity (%)
20 U/ml	84	87
30 U/ml	78	92
40 U/ml	74	96

Discussion

The development of a sensitive and specific biomarker for the diagnosis of RA, which could be detected in early disease and offer a prognostic indication for disease course, would enable RA patients to be identified, monitored, and treated appropriately to curtail disease morbidity and establish remission.

While citrullinated antibodies have been documented for some time and are widely accepted as being the most specific assays for the diagnosis of RA, the IIF methods were subjective and qualitative, and the substrates were problematic in their availability and standardization. The development of commercial peptide ELISAs and the advent of the anti-CCP assay has provided the most specific and quantitative marker available for RA. Although anti-Sa antibodies had excellent specificity, their sensitivity of ~43% was disappointing [32]. In this study, we evaluated a new commercial ELISA assay for the detection of antibodies against mutated citrullinated vimentin. At the manufacturer's cut-off of 20 U/ml, we found the sensitivity of anti-MCV antibody in established RA (>2 years duration) to be 81%. However, the sensitivity was found to be much higher in the early RA patient group (<2 years duration) at 92%. When we combined data from the two cohorts, the sensitivity was 84%. This was slightly higher than the 65–82% documented in previous studies [37–41] and makes detection of anti-MCV antibody in RA comparable to anti-CCP antibody [21].

Anti-MCV antibody was detected in all the disease control groups but only in one healthy blood donor (equivocal at 20 U/ml), with 24% of patients with Sjögren's syndrome, 16% of scleroderma patients, and 14% of SLE patients testing positive. However, there was a significant difference in anti-MCV antibody concentrations between the RA and disease control groups. The detection of anti-MCV antibody in SLE and Sjögren's syndrome [44] and the borderline positivity among healthy individuals [38] have also been noted by other researchers. The specificity of anti-MCV antibody was 87%, which broadly agreed with previously published reports [37–41]. This specificity is higher than RF (60–80%) but lower than anti-CCP antibody (>90%; dependent on patient cohort).

Due to the reduced specificity of the assay and the finding that up to 24% of patients with other connective tissue diseases were also positive for anti-MCV antibody, we reexamined the specificity when the cut-off values were raised. Although the specificity of the assay was improved when the cut-off was raised (92% at 30 U/ml, 96% at 40 U/ml), this was to the detriment of the sensitivity, which was 78% at 30 U/ml and 74% at 40 U/ml. At a cut-off of 40 U/ml, anti-MCV antibody was detected in only 2% of SLE and scleroderma patients, and 14% of Sjögren's syndrome patients (compared to 14, 16, and 24%, respectively, at the 20 U/ml cut-off).

Anti-MCV antibody concentrations were not found to change significantly over a period of 52 weeks nor were the changes treatment dependent. Although the antibody has been previously reported as being responsive to treatment (in patients also treated with methotrexate/anti-TNF α therapy) [37], we were unable to confirm this finding.

We have evaluated a commercially available quantitative ELISA for the detection of anti-mutated citrullinated vimentin antibody, a descendant of the anti-Sa antibody IIF assay. At the manufacturer's cut-off, anti-MCV antibody had a sensitivity of 84% and specificity of 87%, but when the cut-off was raised the specificity increased to 96% but to the detriment of the sensitivity (74%). Although this cut-off produced a specificity comparable to anti-CCP, the sensitivity was lower. Anti-MCV antibody concentrations were unresponsive to treatment (methotrexate or combination of methotrexate/infliximab). A moderate statistical correlation was demonstrated between the quantitative measurement of anti-MCV antibody against anti-CCP antibody. The results of this study indicate that antibodies to MCV are a specific and sensitive marker for the diagnosis of RA. Further studies need to be performed to analyze its relationship to anti-CCP antibody and to assess its value in the prediction of disease outcome and severity.

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