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Heightened autoantibody immune response to citrullinated calreticulin in bronchiectasis: Implications for rheumatoid arthritis

Alex Clarke[a], Elizabeth Perry[a,b], Clive Kelly[c], Anthony De Soyza[d], Kate Heesom[e], Leslie I. Gold[f], William Ollier[g], David Hutchinson[a,h,1], P. Eggleton[a,*,1]

[a] Institute of Biomedical and Clinical Science, University of Exeter Medical School, Exeter, UK
[b] Rheumatology Department,Musgrove Park Hospital, Taunton, Somerset, UK
[c] Rheumatology Department, Queen Elizabeth Hospital, Gateshead, UK
[d] Institute for Cellular Medicine, Newcastle University & Sir William Leech Centre, Adult Bronchiectasis Service, The Freeman Hospital, Newcastle, UK
[e] Proteomics Facility, University of Bristol, Bristol BS8 1TD, UK
[f] Division of Translational Medicine, New York University School of Medicine, New York, United States
[g] Centre for Integrated Genomic Medical Research, The University of Manchester, Manchester, UK
[h] Rheumatology Department, Royal Cornwall Hospital, Truro, UK

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ABSTRACT

Calreticulin (CRT) and citrullinated (citCRT) are implicated in rheumatoid arthritis (RA) pathology. citCRT binds to RA shared epitopes (SE) on HLA-DR molecules with high affinity and triggers pro-inflammatory events in adjacent cells. The aim of the study was to detect the presence of citCRT prior to developing RA and evaluate if citCT is a target for autoantibodies in RA cohorts with and without lung disease. Antibodies were assessed by ELISA against native CRT, citCRT and general protein citrullination, in sera from 50 RA patients without lung disease, 122 bronchiectasis (BR) patients, 52 bronchiectasis patients with RA (BRRA), 87 asthma patients and 77 healthy controls (HC). Serum citCRT was detected by immunoblotting and mass spectrometry. Genomic DNA was genotyped for HLA-DRB1 alleles. Patients were assessed for DAS28, rheumatoid factor, and anti-cyclic citrullinated peptide antibodies. Extracellular citCRT was detected in BR patients sera prior to them developing RA. A citCRT SE binding peptide GEWKPR$_{261cit}$QIDNPDYK was identified. Anti-CRT antibodies were observed in 18% of BR patients with or without RA. Anti-citCRT antibodies were observed in ~35% of BR or RA patients, increasing to 58% in BRRA patients. In the RA alone patients 7/20 (35%) who were negative for RF and anti-CCP were anti-CRT antibody positive and had higher DAS28 scores than triple negative RA alone patients. Three of the four BR patients who developed RA over 18 months were anti-citCRT + ve SE^{+ve}. The detection of citCRT in BR and development of anti-citCRT in BR patients suggests citCRT antigens are early targets of antigenicity in these patients, especially in SE^{+ve} patients prior to the onset of RA.

1. Introduction

Rheumatoid arthritis (RA) is as an autoimmune disease of unknown aetiology, whereby tissue destruction within the joints is the end product of a progressive and chronic manifestation of immune tolerance breakdown over time. Inflammation in sites other than the joints, for example the lungs, eyes and heart may accompany or precede RA. A number of environmental factors contribute to the risk of developing RA, resulting in the generation of citrullinated peptides that can bind to the shared epitope amino acid sequence (SE) on HLA-DR molecules (Gorman and Criswell, 2002; Gregersen et al., 1987; Hedger et al., 1999; Holoshitz, 2010; Valenzuela et al., 1999). The cause of RA continues to be perplexing. There is credence in both neuronal signals (Sakane and Suzuki, 2000) and activation of inflammatory pathways by microorganisms (van Heemst et al., 2015) participating in immune tolerance breakdown. A clue to the origins of RA may be found in the serological profile that often precedes individuals developing the disease, namely the formation of rheumatoid factor (RF) comprised of IgM autoantibodies to IgG (Corper et al., 1997; Hutchinson et al., 2016) and anti-citrullinated peptide antibodies (ACPAs) (Vander Cruyssen et al., 2005).

There is evidence to suggest that chronic and persistent infections precede and contribute to the development of RA through the generation of enzymes e.g. peptidyl arginine deiminase (PAD) (Maresz et al.,

* Corresponding author. Present address: UCB Pharma, Slough, Berkshire, UK.
E-mail address: paul.eggleton@ucb.com (P. Eggleton).
[1] These two authors made an equal contribution to this study.

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2013). These enzymatic pathways can lead to post-translational modifications of host proteins causing ACPA production and RA development. The lungs are increasingly implicated as potential sites for autoimmunity in RA (Brusca et al., 2014; Perry et al., 2014a), due to a high load of bacterial antigens. Bronchiectasis is a respiratory disease where patients suffer from persistent chronic bacterial infections and have a high incidence of RF (Hilton and Doyle, 1978; Waller et al., 1971). Recently we have demonstrated that RA patients with BR have increased disease activity, severity and autoantibody positivity (Perry et al., 2015) and that RA autoantibodies are predictors of RA developing in BR patients (Perry et al., 2014b). Moreover, we have provided evidence that in BR, early breakdown of immune tolerance against a number of well known RA associated ACPAs occurs (Quirke et al., 2015).

Human PADs are responsible for self-citrullination and the citrullination of other proteins and the generation of ACPAs in tissues that are immunogenic in RA, particularly in patients who carry the SE. Several groups have detected extracellular CRT in RA patients (Ni et al., 2013; Tarr et al., 2010), where its presence is correlated with RA disease activity. The molecular mechanism of this association may be via interaction between CRT and the 5-amino acid sequence of the SE on the HLA-DRβ chain that results in nitric oxide pro-oxidative signalling in opposite cells (Ling et al., 2007). Moreover, in-vitro, citCRT binding to the SE appears to enhance these inflammatory functions compared to non-citCRT. Specifically, citrullination of arginine 205 enhances binding of CRT to the SE (Ling et al., 2013).

Autoantibodies to non-citCRT have been frequently detected in several autoimmune diseases (Eggleton et al., 2000; Sanchez et al., 2003; Sanchez et al., 2000; van den Berg et al., 1998), but infrequently, if at all, in RA patients sera (Jorgensen et al., 2005; Routsias et al., 1993). Goëb et al. (2009) **demonstrated post-translational protein modifications of potentially deiminated peptides on calreticulin,** Given the high proportion of RA patients who are ACPA-positive, we were interested to know if CRT autoantibodies were directed against citCRT or native CRT in RA patients, supporting the notion that citrullination of selective proteins (e.g. CRT, vimentin, filaggrin, enolase) is a determinant of antigenicity in RA. Mechanistically, antibodies against citCRT might be formed to prevent citCRT binding to the SE^{+ve} subjects and/or act as a marker of the excessive generation of citCRT. Here we studied RA patients with and without BR, to assist in our understanding of the prevalence of citCRT in individuals and the autoantibody response to native and citCRT in association with other risk factors (lung disease, smoking, SE, RF).

2. Patient and methods

2.1. Antibodies and reagents

Antibodies against human CRT were purchased from Cambridge Biosciences (SPA-601) and Thermofisher (PA3-900). Horseradish peroxidase (HRP)–conjugated secondary antibodies were obtained from Sigma (A6029, A0545 and H5278). The enzyme-linked immunosorbent assay (ELISA) substrate 3,3,5,5-tetramethylbenzidine (TMB) was obtained from KPL (52-00-01). High binding 96-well plates were acquired from Greiner (665061). Anti-human citrulline antibody was purchased from Abcam (ab 100932). Peptidyl arginine deiminase 4 (PAD4) was obtained from Sigma (P1584-25UN). Fluorescent secondary antibodies used were IRDye800 (Li-Cor 925-32213) and IRDye680 secondary antibodies (Li-Cor 926-68072).

2.2. Patients and samples

Sera were selected from an RA and BR patient biobank generated by rheumatology and respiratory consultants obtained from their specialist clinics within four hospitals across the UK as previously described (Perry et al., 2015; Perry et al., 2014b; Quirke et al., 2015) and Table 1. Ethical approval was obtained at all centres participating in this study (multi centre ethics – IRAS 12324) and conforms to the provisions of the Declaration of Helsinki in 1995. Four seropositive BR patients (positive for CCP and RF), were of particular interest as they subsequently developed RA 12–18 moths post serum collection.

We identified adult patients (> 18 years) with HRCT proven symptomatic non-cystic fibrosis BR reported by an independent radiologist and a history of ≥2 respiratory infections per year determined by a clinician who met the 2010 American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) criteria for RA (Perry et al., 2015; Perry et al., 2014b; Quirke et al., 2015). Patients with any other form of lung disease in addition to BR were specifically excluded from the study. This included all those with established interstitial lung disease, asthma or advanced emphysema. All participating centres employed these criteria. In addition to patients with BR and co-existent RA (bronchiectasis-rheumatoid arthritis overlap syndrome − BRRA), we recruited 50 RA patients without lung disease (RA alone), 87 asthma patients (lung disease control) and 77 HCs. RA only patients met the ACR/EULAR criteria (Aletaha et al., 2010) for RA but had no clinical or radiological evidence of any lung disease. All disease cohorts were assessed for anti-CCP and RF (Table 2).

2.3. Preparation of citrullinated calreticulin

Purified human recombinant CRT, expressed from *E.coli* BL21 was citrullinated *in vitro* with 2 units of PAD4 from rabbit skeletal muscle (Sigma P1584) in reaction buffer (0.1 M Tris-HCl, pH7.6, 10 mM $CaCl_2$, 5 mM dithioerythritol) for 16 h at 37 °C (25) and confirmed by mass spectrometry and immunoblotting.

2.4. Immunoblotting of sera for citrullinated plasma proteins

Serum samples of equal protein concentration were prepared in sample buffer containing 84 mM dithiothreitol and run on 8–16% SDS-PAGE tris-glycine gradient gels. (Bio-Rad, prod no 4561105). Potential proteins on the gel were transferred to a nitrocellulose membrane (prod no 1704158) using a Trans-Blot® Turbo™ Transfer system (Bio-Rad prod no 1704155). Blots were incubated with protein-free blocking buffer (Pierce 37572) for 1 h at room temperature (RT) and incubated with 1:1000 dilutions of anti-citrulline and anti-CRT antibodies overnight at 4°C. After washing in Phosphate Buffered Saline (PBS) with 0.1% v/v Tween 20 (PBST), three times, proteins were detected with 1:15000 dilutions of appropriate fluorescent secondary antibodies prepared in blocking buffer for 1 h at RT, followed by infrared antibody imaging (Li-Cor Odyssey).

2.5. Mass spectrometry analysis and detection of serum peptidylcitrulline peptides

Serum aliquots (1 μl) from selected subjects according to their disease status BR > BRRA seroconverts (BR patients who later developed RA), RA and BRRA or asthma patients and HC were run on SDS-PAGE gels, stained with Coomassie blue. Protein bands in the region of 37–50 KDa were excised from gels and subjected to in-gel tryptic digestion using a DigestPro automated digestion unit (Intavis Ltd.). The resulting peptides were fractionated using an Ultimate 3000 nanoHPLC system in line with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). Peptides were ionized by nano-electrospray ionization. Tandem mass spectra were acquired using an LTQ- Orbitrap Velos mass spectrometer controlled by Xcalibur 2.1 software (Thermo Scientific) and operated in data-dependent acquisition mode.

The raw data files were processed and quantified using Proteome Discoverer software v1.4 (Thermo Scientific) and searched against the UniProt Human database (131351 entries) using the SEQUEST algorithm. Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.8 Da. Searches were performed with full tryptic

Table 1
Patients and disease control demographics.

Parameter	Asthma	Bronchiectasis (BR)	Rheumatoid Arthritis (RA)	BRRA
Number	87	122	50	52
Median Age (IQR)	60 (17)	49.97 (26.7)	65.61 (14.8)	65.98 (12.8)
Females, n (%)	69 (79.3)	82 (67.2)	36 (72)	38 (73.1)
DAS28-CRP	NA	NA	2.590	3.51
Lung Disease duration years (IQR)	32 (22)	13 (25)	NA	9 (14.5)
RA Disease duration years (IQR)	NA	NA	12.5 (13)	20 (22.5)
CRP μg/L (IQR)	5 (2)	5 (3.3)	3.55 (5.7)	11 (13.5)
Smoking ever%	42.5	39.3	56	42.3

Abbreviations: CRP = C-reactive protein; DAS28 = Disease Activity Score in 28 joints; IQR = Interquartile range.

digestion and a maximum of 1 missed cleavage was allowed. The reverse database search option was enabled and all peptide data was filtered to satisfy false discovery rate (FDR) of 5%. To identify potentially citrullinated peptides, a mass shift of +0.984 Da at R was included as a variable modification in the search.

2.6. Spectral abundance

The total number of mass spectrometry spectra matched to citrullinated- and non-citrullinated forms of the CRT peptides was used to semi-quantify the citrullinated peptide abundance (Lundgren et al., 2010).

2.6.1. Measurement of citrullinated serum protein levels by ELISA

Citrullinated proteins levels in sera were semi-quantified using a capture ELISA. 96-well plates were coated with 50 μl of 1:10 dilution of sera from each test cohort in sodium carbonate buffer, pH 9.6. The plates were left to incubate at 37 °C for 1 h. The plate was then washed four times with PBST. Remaining binding sites were blocked with 5% v/v BSA in distilled water for 30 min at 37 °C. Wells were washed a further four times with PBST. Next, 100 μl of a 1:2000 dilution of anti-citrulline antibody in PBST was added to each well, followed by a two hour incubation at 37 °C. The wells were washed as described above, and a secondary HRP-conjugated antibody was added to the plate, and incubated for 1 h at 37 °C. The wells were washed in PBST once more, and 100 μl TMB was added to each well. The reaction was developed for 30 min at RT in the dark and was then terminated by adding 50 μl of 2 M H_2SO_4 to each well. The optical density at 450 nm (OD_{450nm}) of each sample was measured on a BMG Labtech FLUROstar™ plate reader.

2.6.2. Anti-CRT and anti-citCRT ELISA

The concentration of anti-CRT antibodies was determined through capture ELISA. 96-well plates were coated overnight with 50 μl of native and PAD-treated CRT (at a concentration 2.7 μg/ml–0.14 μg/well) at 4 °C. The wash > block > wash steps (mentioned previously) were performed and a 1:250 dilution of patient serum (from each test cohort) in PBST was added to individual wells of the plate for two hours at 37 °C. After another washing stage with PBST, a 1:2000 dilution of anti-human IgG antibody was added to the plate and left to incubate for one hour at 37 °C. Following a final washing stage, 100 μl of TMB was added to each well and left in the dark for 15 min. At this point, the reaction was stopped by adding 50 μl of 2N H_2SO_4 to each well, and the OD_{450nm} obtained. The anti-CRT/anti-citCRT OD_{450nm} values/test sample were transformed into ELISA units (EU) from the average OD_{450nm} obtained from a lung disease control cohort of asthma patient sera (n = 87) using the formula: $(OD_{(sample)}/OD_{(AvgAsthma)}) \times 100$).

2.7. Genotyping of human leukocyte antigen-DRB1 alleles in BR subjects

Each BR patient had genomic DNA extracted from peripheral leukocytes using standard procedures. The DNA concentrations were then measured and diluted using sterile Tris–EDTA buffer (Qiagen) to 100 ng/μL. HLA-DRB1 alleles were determined in all cases and controls using a commercially available semi-automated system using PCR sequence specific oligonucleotides and LABScan3D Luminex technology for high resolution DRB1 typing. Sixty percent of BR subjects were positive for SE alleles. Of this cohort 50% were DRB1_0401; 40% DRB1_0101; 6.7% DRB1_0404; 3.3% DRB1_0102 and 1.7% DRB1_0405.

2.8. Protein Modelling method

The amino acid sequence of CRT was obtained from the uniprot database and then submitted to a phyre2 search to get the models in 3D. The UCSF Chimera protein viewing software (http://www.rbvi.ucsf.edu/chimera/) was used to visualize the citrullinated sites on the CRT molecule.

2.9. Statistical analyses

Continuous variables are expressed as median ± interquartile range (IQR). Fisher's exact test, the Mann–Whitney *U* test and multivariate analysis were used to compare categorical variables and outcome measurements between the two groups of patients with RA and BR and the Kruskal–Wallis test to compare variables between greater than two groups assessing antibody populations. Comparisons of diagnostic specificity and sensitivity were made between cohorts. All statistical tests were two-sided and P-values < 0.05 were considered statistically significant. Spearman non-parametric correlations between datasets were assessed. Calculations were performed using GraphPad software.

Table 2
Serum antibody positivity in patients possessing RF and antibodies against CCP.

Parameter	HC N = 79	Asthma n = 87	Bronchiectasis n = 122	RA n = 50	BRRA n = 52
RF + ve n (%)	9 (11.40)	16 (18.4)	41 (33.6)	28 (56)	47 (90.4)
Median RF IU/ml (IQR)	< 7	7 (4)	10.4 (7)	21.95 (60)	65.00 (107)
aCCP–2 + ve n (%)	0	0 (0)	4 (3.3)	23 (46)	47 (90.4)
aCCP–2 U/ml (IQR)	1	1 (0.6)	1.2 (1.2)	5.75 (243)	195 (293)

Abbreviations: aCCP = anti-cyclic citrullinated peptide antibodies; Interquartile range; RF = rheumatoid factor.

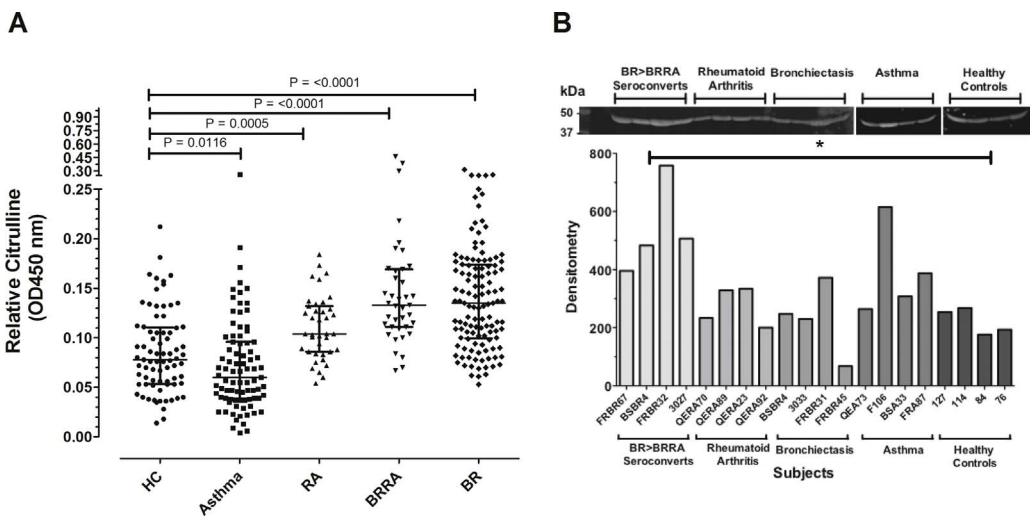

Fig. 1. Citrullinated proteins are detected to varying degrees in all serum samples. (**A**) Relative levels of citrulline proteins in serum samples from 77 HC, 86 asthma, 39 RA, 39 RA patients with BR and 121 BR patients alone by ELISA. Each dot represents an individual subject and the median and IQR for each cohort is shown. (**B**) Representative immunoblots of serum (1 μg/lane) probed with an anti-citrulline antibody from serum samples from s, Asthma, RA, BRRA) and BR. The intensity of citrullinated proteins detected in each serum sample was assessed by densitometry and shown as individual histograms.

3. Results

3.1. Citrulline proteins are abundantly expressed in blood serum

Different reactivity patterns were observed among RA patients with or without BR, asthma patients or HC, as shown in Fig. 1**A**. The BR, BRRA and RA patients all had consistently higher OD measurements for citrullination compared to HC ($P < 0.0001$ for BR and BRRA vs HC and $p < 0.005$ for RA vs HC). To determine the apparent molecular weight of citrullinated proteins in individual sera, we selected several sera from each cohort and screened them by immunoblotting. The blots revealed a single band of citrullinated proteins in all samples of a relative molecular mass (M_r) of between 37 and 50 kDa (Fig. 1**B**). Densitometry plots confirmed BR-seroconverts prior to developing RA, appeared to have a significantly higher amount of citrullinated proteins ($P < 0.05$) than the healthy control cohort. No other statistical significance was observed between any of the other cohorts tested.

3.2. Detection of citCRT in BR sera by immunoblotting and mass spectrometry prior to developing RA

The current interest in the pathophysiological roles of citCRT and its implications in RA, led us to probe our patient sera for evidence of its presence. As CRT is normally an intracellular endoplasmic reticulum chaperone, its abundance as an extracellular protein in serum is relatively rare, between 0- 20 ng/ml. Purified CRT migrates on an SDS-PAGE gel under reducing conditions with a M_r of 60 kDa due to the proteins high negative charge and pI of 4.6. This was outside the M_r zone (37–50 kDa) of citrullinated proteins we had identified above. However mass spectrometry data of the citrullinated bands (Fig. 2**A**) confirmed that peptides of the NH_2 terminal and proline-rich segments of CRT were present in our 37–50 kDa samples. The presence of CRT was validated by immunoblotting the sera protein bands with an anti-CRT antibody. The antibody detected in most of the RA patient samples a band at the expected size for intact CRT (60 kDa). But in two of our four BR patients who were anti-CCP^{+ve}, we detected CRT of a M_r of ~45 kDa (Fig. 2**B**). The membrane was blotted with anti-citrulline and anti-CRT antibody, which confirmed that these lower molecular weight CRT fragments were citrullinated (Fig. 2**C**). A citCRT fragment present in the serum of one of the BR patients (FR32) was detected by mass spectrometry prior to the individual developing RA. The CRT peptide contained citrullinated Arg_{261} within the sequence $GEWKPR_{261cit}QIDNPDYK$, a region of the P-domain of CRT that is in close proximity to Arg_{205}. Moreover, the same BR patient was positive for SE on HLADRB1_0101 and had positive serology markers of inflammation for CRP (27 μg/L), RF (14.3 IU/ml) and anti-CCP (340 U/ml). We were also able to detect citCRT peptides by immunoblotting in two other CCP^{+ve} BR sera tested from patients who went on to develop RA withing 12–18 months.

3.3. Determination of Arg susceptibility for conversion to citrulline by PAD4 in CRT

To determine which Arg in CRT are targeted for citrullination by PAD4, we treated recombinant human CRT with human PAD4 (Fig. 3**A–B**). CRT contains eight Arg and we confirmed by mass spectroscopy that at least 6 out of the 8 Arg had been efficiently citrullinated (Fig. 3**C**), including Arg_{205} and Arg_{261}. In our model of CRT we have only depicted 6 of the 8 Arg, as two of them are in a carboxy terminal non-crystallisable region of the protein. However, we did not detect citrullinated forms of these peptides after PAD treatment. Employing a spectral analysis approach we were able to semi-quantify the Arg that were most susceptible to citrullination by PAD4 (Fig. 3**D**). This revealed that the Arg residues; Arg_{19}, Arg_{56} in the N-terminal segment of globular head region of CRT and Arg_{205} Arg_{261} of the P-domain were most susceptible to citrullination by PAD4.

3.4. Assessment of anti-calreticulin antibodies against native and citCRT in RA patients with and without BR

Initially ELISA titres comparing HC vs. unselected RA (RA patients with and without BR) were analysed. IgG anti-CRT antibody levels were not significantly elevated in unselected RA (median 119 EU/ml) compared with HC (median 110 EU/ml) sera. Whereas, antibody levels to citCRT in unselected RA (median 135 EU/ml) did differ significantly from HC (74 EU/ml). Sixty percent of the unselected RA sera were anti-citCRT positive compared with 7% of the controls (Fig. 4**A**). When the EU values for anti-CRT were subtracted from the anti-citCRT for each cohort, a similar significant number of unselected RA patients (51%) remained positive for anti-citCRT compared to only 5% of sera, indicating the anti-CRT response was anti-citCRT specific.

To further investigate the citCRT-specific antibody response in 122 BR patients alone and 51 BRRA patients and 49 RA alone patients without BR we evaluated the antibody response to CRT and citCRT in each cohort of patients and 75 controls. Eighteen percent of BR patients had antibodies to native CRT, but 35% of these sera were positive for anti-citCRT. Similarly, 19.6% of BRRA sera were anti-CRT positive, but this increased to 58% against citCRT. Using the ninety-fifth percentile of controls as a cut-off, no RA alone patients without lung disease had anti-CRT antibodies, but 37% of these patients had anti-citCRT antibodies (Fig. 4**B & C**). Subtraction of the EU values for anti-CRT against native CRT from EU values observed for antibody against citCRT, IgG

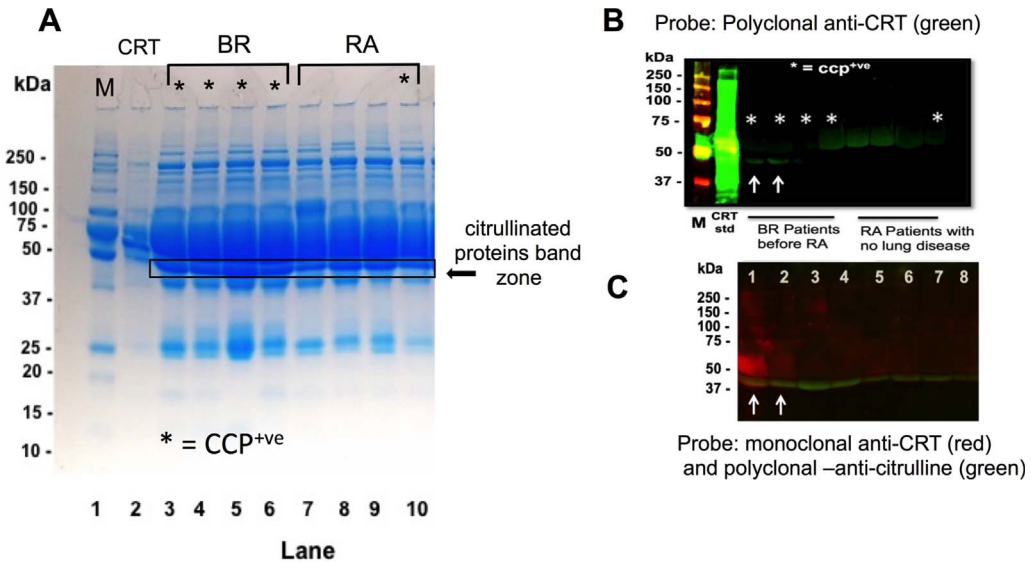

Fig. 2. Detection of CRT and citrullinated proteins in serum of BR and RA patients sera. (**A**) Profile of serum proteins showing equal loading and indicating the band of citrullinated proteins. (**B**) Detection of 58 kDa bands of CRT (green) in several subjects with RA and BR, but in some BR patients seropositive for CCP prior to developing RA a lower molecular weight isoform of CRT (~47 kDa) is evident in some BR patients (depicted by arrows). (**C**) the same serum samples were probed for both citrulline (green) and CRT (red) and in two CCP + ve BR patients there appeared to be CRT present in citrullinated protein band. (depited by arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

antibodies specific for citCRT were observed in 51% of RA alone, 47% of BRRA, 25% of BR patients and only 4% of controls. The sensitivity and (specificity) of anti-citCRT in BRRA, RA and BR was 58% (96%), 39% (96%), 36% (96%) compared to normal controls respectively.

3.5. Comparison of serum anti-CRT/citCRT levels with RF and anti-CCP

A Venn diagram was constructed to identify the common and exclusively generated antibodies based on disease serology. RF and anti-CCP was detected in 73% and 68% respectively in 100 RA patients with and without BR (Fig. 5). Anti-CRT has been proposed to be a useful biomarker of disease activity as assessed by DAS28 (Ni et al., 2013). We evaluated the detection of anti-CRT and anti-citCRT in association with the other two biomarkers to compare their biomarker usefulness. In 100 unselected RA patients only 9% of them were positive for anti-CRT antibodies. In contrast, 49% of unselected RA patients were positive for anti-citCRT, or a 5.4-fold increase. Many of these anti-citCRT individuals were also positive for both RF and anti-CCP (36 subjects). Interestingly, 7/23 (33%) unselected RA were negative for both RF and anti-CCP but positive for anti-citCRT; these anti citCRT positive only subjects tended to have the highest median DAS28 scores (DAS28 = 3.63 n = 7) compared to unselected RA subjects positive for all three biomarkers (DAS28 = 3.25; n = 36) or RA alone (DAS28 2.31; n = 9).

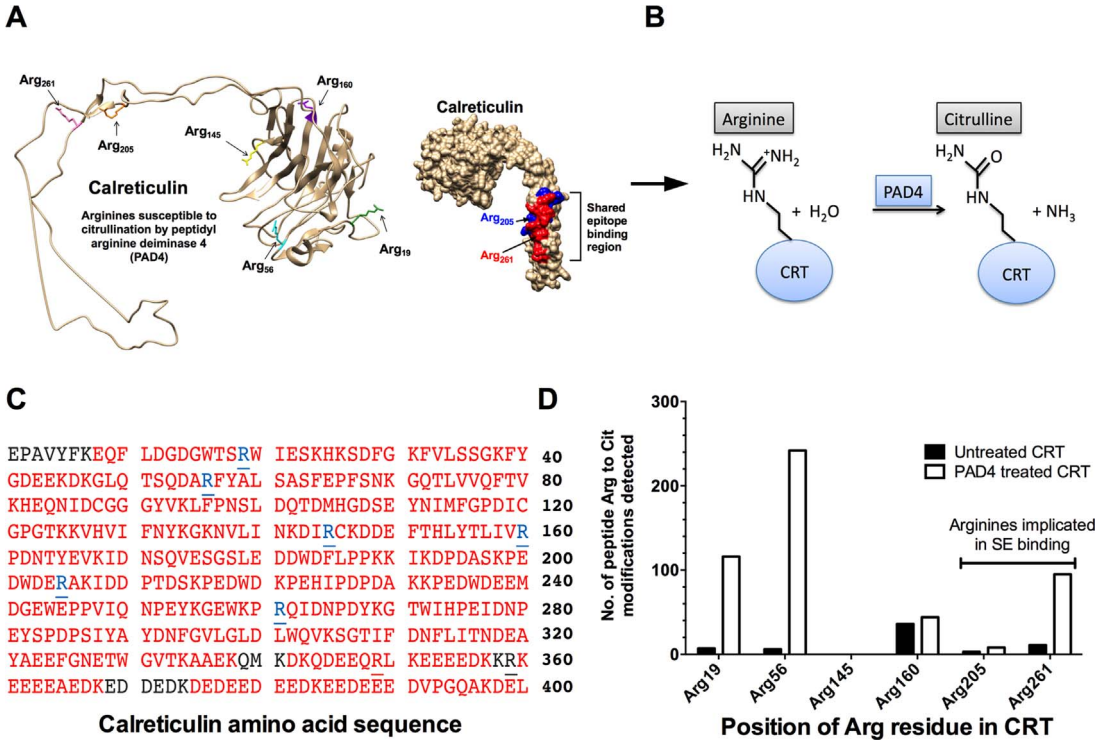

Fig. 3. Susceptibility of CRT to post translational modification by pedtidyl arginine deiminase 4 (PAD4). **(A)** CRT possesses eight Arg residues, six of which were identified by mass spectrometry to be liable to citrullination to varying degrees by PAD4 but only Arg261 was found to be citrullinated in a BR patient serum. **(B)** Overnight treatment of human CRT with PAD4, leads to citrullination of some Arg residues. (**C**) Mass spectrometry sequence coverage of CRT. Sequence in red indicates the identified sequence. Arginine residues are underlined. Arg residues that were modified to citrulline are shown in blue. (**D**) Mass spectrometry citrullinated peptide abundance pre- and post-PAD4-treatment of purified CRT. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

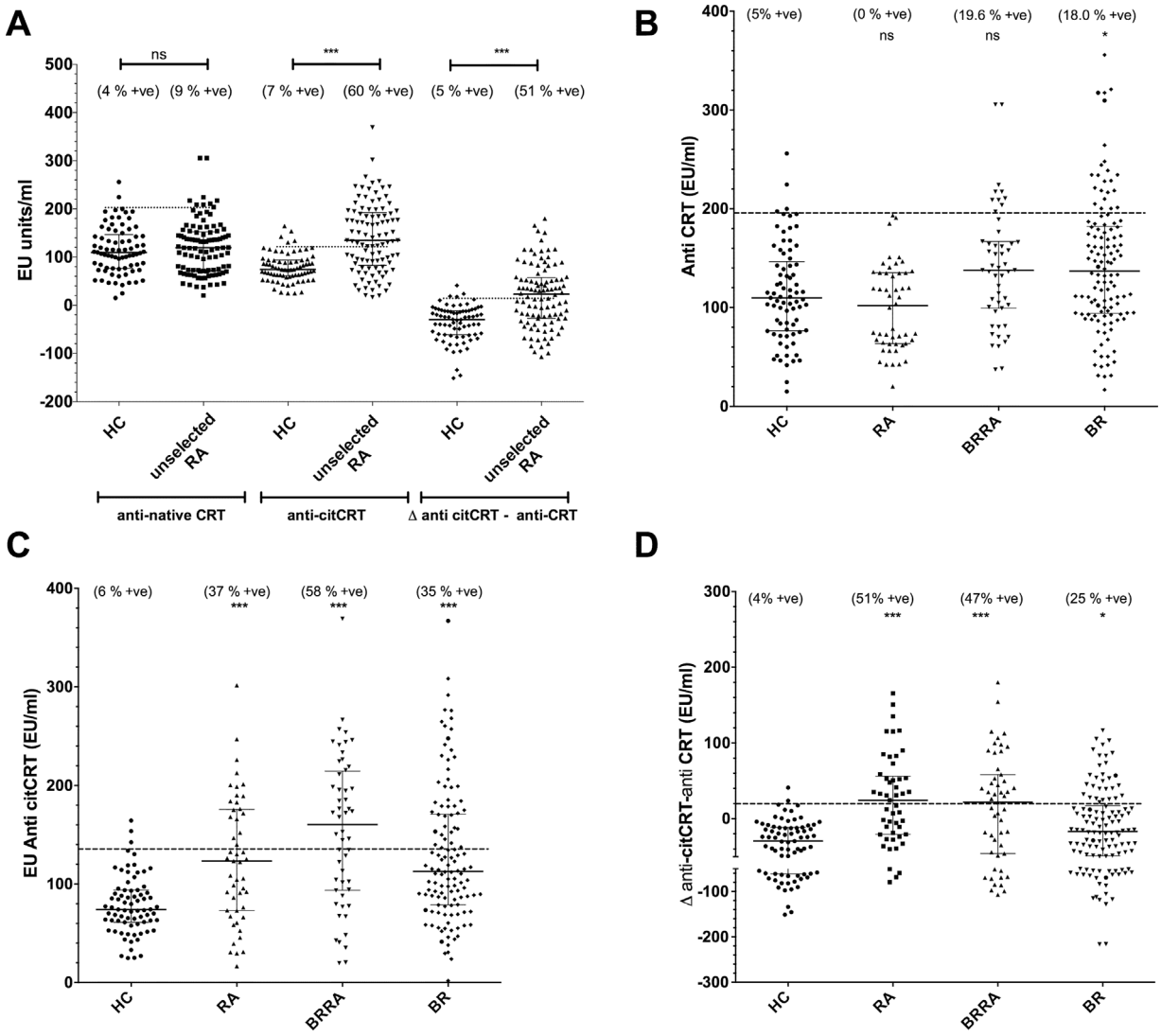

Fig. 4. ELISA analysis of anti-CRT and anti-citCRT in controls, RA with and without BR and in BR alone sera. (**A**) The ELISA detection of antibodies to CRT and citCRT in unselected RA versus age-sex matched controls. (**B**) Anti-CRT antibody titres in all groups. (**C**) Anti-citCRT antibodies in all groups screened and (**D**) specific antibodies against citCRT in all groups tested. The median and interquartile range of autoantibody levels for each cohort is shown. The cut-off values are shown as dashed lines across groups and were calculated based on the mean 95th percentile of the paired control sample values. Kruskal-Wallis analysis was used to calculate the p values of the differences between groups (ns = no significant difference, * = $p < 0.05$ and *** = $p < 0.0001$). The EU units/ml of the anti-CRT response were subtracted from the anti-citCRT to give the difference (Δ) or specific level of anti-citCRT antibody response.

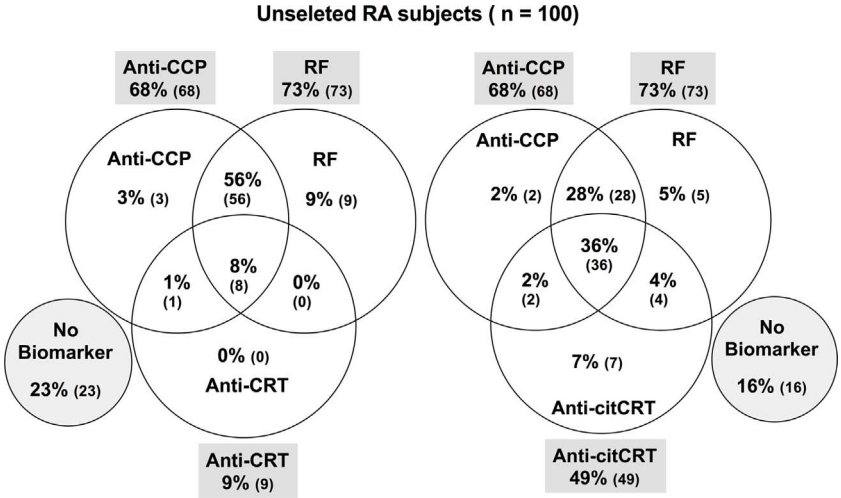

Fig. 5. Distribution of anti-cyclic citrulline peptide (anti-CCP), rheumatoid factor (RF) in association with either anti-calreticulin (anti-CRT) or anti-citrullinated calreticulin (anti-citCRT) in RA patients with and without BR. Data are shown as Venn diagrams, with the percentage of individuals with anti-CCP, RF and anti-CRT or anti-citCRT alone or in combination with each other. The total number of unselected patients is 100 and the raw number of patients for the cohort is shown in parenthesis.

4. Discussion

There is interest in the possible role of calreticulin (CRT) in the pathogenesis of RA, based on binding of CRT to the SE causing inflammation in opposite cells, that can be inhibited by anti-CRT antibodies (Ling et al., 2007). Moreover, in an *in vivo* model of collagen-induced arthritis, small mimetic peptides can compete with the SE-binding sites in the P-domain of CRT, inhibiting SE-activated signalling via the SE/CRT pathway, correlating with reduced bone damage in this arthritis model (Ling et al., 2015). To our knowledge extracellular citCRT or anti-citCRT antibodies have not been detected in RA patients blood circulation.

The data presented here, indicates that citCRT possibly breaks tolerance, giving rise to specific antibodies against citCRT observed in RA/BRRA. Our previous report, suggested extracellular CRT is found in ng/ml amounts in the synovia and serum of RA patients (Tarr et al., 2010). Neutrophils in both these sites are a source of both PAD4 and CRT. Typically, BR patients have high levels of neutrophil and macrophage infiltration in the pulmonary tissues, both rich sources of both PAD4 and 2 respectively, leading to the generation of RF and ACPAs (Perry et al., 2015; Perry et al., 2014b; Quirke et al., 2015). By studying BR, RA alone and BRRA patients, we sought to understand if citrullination of CRT occurred prior to RA disease development. We detected elevated levels of citrullinated proteins in the sera of BR, BRRA and RA patients, compared to HC and asthma patients. The increased citrullination in the sera of the BR and BRRA cohorts was mainly associated with lung disease rather than compounding factors such as smoking. (Perry et al., 2015; Perry et al., 2014b). Although, a previous study observed increased PAD2 and citrullination in lungs of smokers (Makrygiannakis et al., 2008).

In the current study, the abundance of citrullinated proteins appeared greatest in sera of the four non-smoking BR patients who subsequently developed RA, compared to RA patients alone. We employed mass spectrometry to identify some of these citrullinated proteins. We identified a citrullinated peptide of CRT at low frequency at position Arg_{261} in a BR patient who developed RA. A recent report found that the reduction of charge caused by the citrullination of Arg_{261} and Arg_{205} is an important physical characteristic that enhances the interaction of CRT with the SE (Ling et al., 2013). Our data supports the existence of citCRT P-domain peptides in a patient prior to them developing RA. This particular patient was also positive for the shared epitope HLADRB1_0101 allele a risk factor for developing RA.

Citrullinated Arg_{205} is believed to be the SE ligand, but in our limited mass spectrometry screen we did not detect this particular post-translational modification in serum. This may reflect that citCRT is bound to the SE on MHC class II cells. The treatment of human CRT with PAD4 provided evidence that CRT is susceptible to citrullination and is a mechanism of generation of ACPA responses in BRRA patients, RA alone patients, or both.

The citrullination of CRT results in the production of anti-citCRT antibodies. Whether these are produced to prevent citCRT binding to the SE and promoting inflammation in bystander cells is unknown. But commercial antibodies to CRT can block CRT binding to the SE (Ling et al., 2007). We and others, have detected anti-CRT IgG antibodies in RA and other autoimmune patients (Sanchez et al., 2000; Tarr et al., 2010; van den Berg et al., 1998). While others have implicated the concentration of serum CRT correlates with RA disease activity (Ni et al., 2013). Others have examined the generation of anti-CRT IgA antibodies in diseases where the mucosal immune system might be prevalent (Sanchez et al., 2008; Sanchez et al., 2003). In these latter studies, Sanchez and co-workers identified two immuno-dominant peptides in 75% of their coeliac patients that were recognized by anti-CRT IgA autoantibodies – the N-terminus peptide $GLQTSQDA_{\mathbf{Arg56}}F$ and C-terminus peptide $EQ_{\mathbf{Arg349}}LKEEEED$. Interestingly both these immunogenic peptides contained an arginine, one of which was Arg_{56}, a peptide we identified at susceptible to citrullination by human PAD4. This supports the notion that release of CRT from cells damaged in a mucosal environment, such as in the gut or lung as occurs in lung cancer (Liu et al., 2012) could trigger an autoimmune reaction.

Our data demonstrates that citCRT and not native CRT was the dominant autoantigen, particularly in BR patients. It is noteworthy that not all BR patients go on to develop RA and in this study only 60% of 122 BR patients investigated possessed a SE allele. We found anti-citCRT antibodies in 35–37% of patients with RA alone or BR alone, but notably this increased to 58% in BRRA. Given that citCRT appears to be a common antigenic target, three of our four anti-CCP positive BR patients who went on to develop RA (Perry et al., 2014b) were also HLADRB1 SE positive. The detection of antibodies to non-citCRT in only BR and BRRA patients may suggest an increase in antigenicity towards native CRT via epitope spreading.

While the detection of anti-citCRT antibodies generally correlated with RA patients who were either anti-CCP or RF positive, a potentially important subgroup of 14% of patients with RA alone exclusively had anti-citCRT autoantibodies and also had the highest mean DAS28 scores. These anti citCRT positive only subjects tended to have the highest median DAS 28 scores (DAS28 = 3.63 n = 7) compared to seronegative RA alone (DAS28 2.31; n = 9), although this did not meet clinical significance. Further work is needed to determine whether citCRT can potentiate inflammation in RA and is a risk factor for more severe disease as suggested by others (de Almeida et al., 2011; Fu et al., 2013). Studies have shown that peptide mimetics of the SE can bind to CRT in vivo and prevent erosive arthritis in mice by blocking the SE-CRT pathway (Ling et al., 2015) and our findings of anti-citCRT antibodies may help to identify patients in future studies investigating specific targeted treatments as described above.

Limitations of the study are important to note; we present cross sectional data only. Longitudinal studies in “high risk” patients that may develop RA are of great interest. Our RA alone population had no reported lung disease, nor simple radiographic markers of lung disease but future studies with high resolution CT scanning to confirm no subclinical lung involvement would be helpful. Notably we excluded patients with known interstitial lung disease and the anti-CRT antibody profiles in comparison to those with airways centred disease is also of great interest.

In conclusion, we present for the first time early insights into the possible role of CRT and anti-citCRT in BR and RA. The role for anti-citCRT autoantibodies in predicting the progression of RA and the potential therapeutic use of peptide mimetics of the SE that can bind to CRT in anti-citCRT positive RA patients will be of great interest.

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None of these authors have any conflicts of interest to declare.

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