Published in final edited form as:
Arthritis Rheum. 2012 May ; 64(5): 1338–1344. doi:10.1002/art.33472.

Increased Frequency of Complement *C4B* Deficiency in Rheumatoid Arthritis

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Abstract

Objective—To assess the Copy Number Variation (CNV) of complement *C4A* and *C4B* genes in patients with Rheumatoid Arthritis

Methods—DNA from 299 patients and volunteers were obtained and analyzed for CNV of total complement *C4, C4A*, and *C4B* genes. These results were analyzed by chi-square analysis and odds ratios calculated.

Results—Chi-square analysis revealed similar distribution patterns of total *C4* alleles in RA (n=160), non-RA (n=88) rheumatology patients and normal volunteers (n=51). There was no trend to *C4A* deficiency as in lupus. Significant differences in *C4B* distribution were observed in RA patients, where a ~ two-fold increase in the frequency (40%) of homozygous and/or heterozygous *C4B* deficiency (0 or 1 allele) was present relative to non-RA patients (21%) or healthy controls (22%). The *C4B* deficiency concentrated in the seropositive relative to seronegative RA patients (44% vs 31%). The odds of *C4B* deficiency were 2.99 (1.58-5.65, p=0.0006) in seropositive RA patients relative to non-RA controls. These findings were confirmed in a larger healthy control cohort yielding an odds ratio of 1.83 (1.21-2.76, p=0.0056). The association of SE with *C4B* deficiency was significantly greater in the seropositive RA patient population relative to non-seropositive RA controls (96% vs 54.5%, p<0.0001), suggesting that *C4B* deficiency interacts with the SE in the development of seropositive RA.

Conclusions—*C4B* CNV exhibits a relationship with RA that approximates that seen with *C4A* CNV and SLE. The concurrence of *C4B* deficiency and SE in seropositive RA can have broad implications for our understanding of RA pathogenesis.

Introduction

Rheumatoid Arthritis (RA) is a systemic inflammatory disease based in the synovium of affected joints, with a prevalence of 0.1-1% of the world's population (1). RA segregates into seronegative and seropositive patient populations on the basis of blood tests for Rheumatoid Factor (RF) or autoantibodies against citrullinated proteins (ACPA) (2-5). Seropositive patients typically express both RF and anti-CCP antibodies. Most (85%) ACPA

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Conflict of Interest: The authors report no financial support or other benefits from commercial sources for the work reported on in the manuscript, or any other financial interests that any of the authors may have, which could create a potential conflict of interest or the appearance of a conflict of interest with regard to the work.

positive RA patients have the 'shared epitope', a 5 amino acid sequence [(R/Q)(K/R)RAA] at position 70–74 in the HLA-DR β-chain (2,3). The shared epitope sequence appears to confer the risk of RA, as *HLA-DRB1* alleles not associated with RA differ at this site [Reviewed (6)]. Both seropositivity and the 'shared epitope' predict increased morbidity and mortality from RA (4,5). The association of RF with the shared epitope is only seen in the presence of ACPA (3). Given the role of RF in immune complex clearance, this suggests that RF is induced in response to ACPA-associated immune complexes (7,8). RF is associated with pathology through immune complex formation in the joint and at extra-articular sites (1).

The complement system is a group of plasma and membrane proteins involved in immunity as well as protection against autoimmunity. The proximal components of the antigen-antibody initiated classical pathway (C1, C4, C2) are important for the clearance of apoptotic cells and immune complexes (9-11). In the human, two classes of polymorphic C4 proteins exist, which are distinguished by the nature of their covalent linkage with the target cells or immune complexes through the highly reactive thioester carbonyl group: activated C4A tends to form an amide bond with amino groups on antigens; the activated C4B is strongly reactive towards hydroxyl groups on glycerols or glycosylated antigens (12-14). In *in vitro* hemolytic assays, purified C4B reacts about 4-times more efficiently than purified C4A (15). Although mice also have two classes of C4-like proteins, C4 and Slp (sex-limited protein), the functionality of Slp in the mouse complement pathways remains uncertain (16,17). Activated mouse C4 reacts biochemically like human C4B because it also consists of the orthologous histidine-1106 residue that facilitates the nucleophilic attack of thioester carbonyl group to form a covalent ester bond with substrate (14,18).

Gene copy-number variation (CNV) constitutes a major source of genetic variation but its genetic relevance was only recently recognized (19,20). Many inherent multi-allelic CNVs are highly complex, including continuous gene copy number variations, secondary sequence polymorphisms and the integration of mobile genetic elements (21-23). Genome Wide Association Studies relying on Single Nucleotide Polymorphisms (SNPs) and array comparative genomic hybridizations (aCGH) usually do not have the resolution power or are refractory to complex diversities of multi-allelic CNVs (19). The human complement *C4* gene complex is located within the central region of the major histocompatibility complex (MHC; also known as the HLA) on the short arm of the chromosome 6 and is a major genomic site of gene CNVs. Among different individuals, two to eight copies of *C4* genes in a diploid genome are frequently detectable, and each of these *C4* genes can code for a C4A protein or a C4B protein. In one-half to two-thirds of the general population, a human subject has two copies of *C4A* and two copies of *C4B* in a genome. The remainders have variable combinations of 0 to 6 copies of *C4A* and *C4B* genes (22,24-26). Such variations in *C4A and C4B* CNVs are not accurately detectable by GWAS approaches and aCGH and thus missed in most large scale disease-association studies, including those for RA (20,27).

C4 gene copy number (GCN) influences biosynthesis, as serum levels of C4 proteins parallel their gene copy-number (23,25). Thus, an individual can completely lack either *C4A* or *C4B*, but have a normal total number of *C4* alleles encoding the other *C4* isotype. This awareness has led to a refined understanding of the relationship between Systemic Lupus Erythematosus (SLE) and C4A, where a selective deficiency in *C4A* alleles has been demonstrated in SLE patients, without an association (e.g compensatory increase) with *C4B* gene copy-number (22). In this paper, we report that the reverse relationship exists in RA, with a selective *C4B* deficiency occurring at an increased frequency in seropositive RA patients. These data suggest that C4B deficiency may play a role in RA pathogenesis and/or phenotype.

Patients and Methods

Patients

The RA patients (n=160) in this study were followed at the Dartmouth-Hitchcock Medical Center (DHMC) in Lebanon, New Hampshire. Each of these patients met the diagnostic criteria defined by the American College of Rheumatology (formerly the American Rheumatism Association) 1987 criteria for RA and were followed by a board-certified rheumatologist. Seropositive RA (n=115, 74% female) was defined by the presence of a record of a positive test for rheumatoid factor (RF) or anti-citrullinated protein antibodies (ACPA) with an age range of 20-92. Seronegative RA (n=45) were comparable in age (69% female, 25-82 years old). Non-RA patients (n=88, 62% female, 19-80 years old) were obtained from the Rheumatology Clinic and spanned a number of diseases, the most common (>3 patients) being: psoriatic arthritis-13, ANCA-associated vasculitis-11; polymyalgia rheumatica-8; giant cell arteritis-8; myositis-7; Sjogren's Syndrome-5; osteoarthritis-5; systemic lupus erythematosus-4. A cohort of healthy male volunteers (n=51,19-59 years of age) was used as healthy controls. All patients and controls were of European ancestry.

Complement *C4A* and *C4B Gene* Copy-Number Variations (CNV) and HLA-DRB1 Typing

Following informed consent, peripheral blood was collected in PAXgene Blood DNA Tubes (Qiagen) and DNA isolated according to manufacturer's specifications. Total *C4, C4A* and *C4B* gene copy-numbers were determined by Southern blot analyses of *Taq*I, and *Psh*AI plus *Pvu*II restriction enzymes, as previously described (28,29). Briefly, genomic DNA samples were digested with *Taq*I, subjected to agarose gel electrophoresis and transferred to nylon hybridization membrane for Southern blot analyses. Using a probe corresponding to the *RP-C4* genomic region, the *Taq*I RFLP yields the copy-number of total *C4* genes. Using a C4d-specific probe, the *Psh*AI-*Pvu*II RFLP yields the relative copy-numbers of *C4A* and *C4B*. In the case of low quantity of genomic DNA and ambiguous results, quantitative real-time PCR experiments for copy-numbers of total *C4, C4A* and *C4B* were performed to obtain the missing data, or independently validate Southern blot results (30). *HLA-DRB1* typing was performed by the American Red Cross, Penn-Jersey Blood Services Region.

Statistical analysis

Statistical analysis was performed using JMP 8.0 (SAS Institute). Chi-square analyses were used to determine the differences of total *C4, C4A*, and *C4B* gene copy-numbers among groups. Odds ratios (OR) and 95% confidence intervals (CI) were calculated by analysis of 2 × 2 tables, using the Fisher's exact test for comparisons.

Results

Increased frequency of C4B deficiency in Rheumatoid Arthritis patients

We examined the gene CNVs of complement *C4A* and *C4B* by Southern blotting in a population of healthy volunteers (n=51), non-RA (n=88) and RA (n=160) patients (Table 1). The distribution of total *C4* genes was examined by chi-square analysis (Figure 1). There was no statistically significant difference in total *C4* genes between these groups. The distribution of *C4A* and *C4B* gene copy-number was analyzed. A difference in *C4A* gene copy-number was observed between healthy controls and RA patients (p= .0117). This difference resulted from an increased frequency (67% vs. 45%) of healthy controls with 2 copies of *C4A* and was not seen between RA and non-RA patients. There was no obvious trend to *C4A* deficiency (0-1 allele) in RA, as reported with SLE patients (22).

In contrast, chi-square analysis of frequency distribution for *C4B* gene copy-number groups demonstrated significant differences with RA patients relative to both healthy controls (p=. 0364) and non-RA patients (p=.0058). The most apparent difference was the two-fold increase in *C4B* homozygous or heterozygous deficiency in RA patients (copy number =0 or 1; 64/160; 40%) relative to non-RA (21%) and healthy controls (22%). In the healthy control, non-RA and RA populations, there were 1,1, and 7 cases of homozygous deficiency and 10, 15, and 57 cases of heterozygous *C4B* deficiency, respectively.

The increased frequency of C4B deficiency segregates to seropositive Rheumatoid Arthritis patients

We examined the CNV of *C4, C4A* and *C4B* in our RA patient populations as a function of anti-CCP or RF seropositivity relative to non-RA patients (Figure 2). Chi-square analysis demonstrated no significant differences in the *C4* and *C4A* allelic frequency distribution as a function of seropositive status. Interestingly, homozygous or heterozygous deficiency of *C4B* concentrated in the seropositive RA population, as 44% (50/115) of seropositive patients had 0 or 1 copy of *C4B* genes in a diploid genome. Of these, 7 were completely *C4B* deficient, while 43 had 1 copy. In the seronegative patient population the frequency of *C4B* deficiency was 31% (14/45, all heterozygous - no homozygous C4B deficiency). Chi-square analysis of *C4B* distribution relative to the non-RA population yielded only statistically significant differences for the seropositive RA (p=0.0018).

The presence of C4B deficiency in seropositive RA: Odds-ratio (OR) analysis

We examined the odds of having *C4B* deficiency (0-1 copy) in the non-RA and RA populations relative to normal healthy controls. While there was no increase for *C4A*, the odds of *C4B* deficiency was statistically increased in our total RA [OR 2.42 (1.16-5.07); p=0.0187] and seropositive RA populations [OR 2.80 (1.31-6.00); p=0.0086], but not the non-RA and seronegative RA populations.

Use of the non-RA population as controls yielded a slightly stronger relationship. The OR for *C4B* deficiency in the total RA was 2.59 (1.41-4.76, p=0.0019). In the seropositive RA populations, the observed OR was 2.99 (1.58-5.65, p=0.0006). In each of these analyses, there were trends towards an increased OR for the seronegative RA patient population, but these did not reach statistical significance (p=0.35 and p=0.20 relative to healthy and non-RA controls respectively).

Finally, we examined these relationships using a different and larger (24, n=513) Ohio Caucasian control cohort (Table 2). The male percentages in the Ohio control cohort, total RA and seropositive RA populations were nearly identical (25%, 27% and 25% male). This analysis confirmed our finding with statistically significant increases in the frequency of *C4B* deficiency relative to a distinct control Caucasian population from Ohio.

C4B deficiency and the Shared Epitope (SE) are associated in seropositive RA

HLA-DRB1 typing demonstrated that 83% (95/115) of seropositive RA patients contained the SE (Table 3) a similar frequency to previous reports (3). Of the seropositive RA patients (n=50) with *C4B* deficiencies (0-1 allele), 48 were SE+ (96%). In the seropositive RA patients with ≥2 *C4B* alleles, the presence of the SE was significantly less, 76% (46/63, p<0.0009). In the 44 individuals with heterozygous *C4B* deficiency without seropositive RA, the shared epitope was present in 54.5% (24/44). A similar frequency of the SE (56.7%, 17/30) was found in the healthy volunteers/non-RA control subgroup with *C4B* deficiency. Thus, the frequency of the SE in *C4B* deficient without seropositive RA was no greater than the frequency of the SE in an unselected general Caucasian population (31,32).

The frequency for the concurrence of SE with *C4B* deficiency was significantly greater in the seropositive RA patient population relative to non-seropositive RA controls (96% vs 54.5% $p<0.0001$). The same level of statistical significance was seen using non-RA/healthy volunteers as a comparator ($p<0.0001$). The almost absolute co-existence of *C4B* deficiency and SE in seropositive RA far exceeds the frequency of their associations in non-seropositive RA and controls. One probable interpretation for such phenomenon is that *C4B* deficiency and the SE interact in the induction of seropositive RA.

Discussion

We report that the frequency of homozygous and/or heterozygous C4B deficiency is increased in our Caucasian RA population and is restricted to seropositive RA patients. The frequency of *C4B* deficiency (0-1 allele) in the seropositive RA population (44%) is twice that seen in ethnically-matched controls and non-RA patients from Northern New England, yielding a statistically significant odds ratio (OR), ranging from 2.8-2.99. This OR was not influenced by abnormal distributions in our control populations as the frequency of *C4B* deficiency in our healthy control and non-RA populations were nearly identical (21.6% and 20.5%). Using a different control Caucasian population from Ohio, in which a greater frequency (29.6%) of *C4B* deficiency was observed, a statistically significant odds ratio for heterozygous *C4B* deficiency was greater in both the total RA (1.58) and seropositive (1.83) RA populations. There were no significant differences in total *C4* or *C4A* distribution seen in the seropositive patients.

The magnitude of the relationship between *C4B* deficiency with seropositive RA (OR 1.83) parallels that relationship between *C4A* deficiency with SLE where a very similar OR (2.024) was seen using the same Ohio controls (22). In each disease, the presence of C4 deficiency might shape disease induction as well as phenotype, given the role of C4 in B cell tolerance (11) in addition to its established activity in immune complex clearance. Immune complex clearance might be particularly important for the association *C4B* deficiency, given data suggesting that ACPA appear prior to the development of RF (8). The different natures of their covalent linkage [C4A: amino vs C4B: hydroxyl] (12,13,18) with the target cell/immune complex may have a role in this association. In this regard, certain citrullinated proteins in immune complexes associated with ACPA may have a reduced availability of amino groups from arginine residues, since certain autoantigens undergo extensive citrullination (33,34). This would reduce the binding of C4A, potentially leading to a greater dependence on C4B in the process of immune complex clearance. This model would predict a greater role for C4B relative to C4A in maintaining immune tolerance and removal of citrullinated antigens as well (11). Delayed or defective clearance of citrulllinated antigens in C4B deficient subjects would be predisposed to the generation of ACPA and subsequent IgM-RF induction, leading to rheumatoid arthritis (7,8).

The *C4A* and *C4B* genes reside in the Class III region of the MHC, in which linkage disequilibrium with HLA class II and class I polymorphic variants has been shown to exist. Results of this study raised the possibility of multiple genetic factors in the MHC, e.g., C4B deficiency and haplotypes coding for shared epitopes of HLA-DRB1, in conferring the disease susceptibility of seropositive rheumatoid arthritis. HLA-DRB1 typing of the seropositive RA population demonstrated a high frequency (96%) of the shared epitope in patients with *C4B* deficiency. This association did not appear to be due to linkage disequilibrium, as it was not seen in *C4B* deficient individuals without seropositive RA. Rather, these data are consistent with the interpretation that *C4B* deficiency and the shared epitope interact in the development of seropositive RA. Such an interaction would be consistent with the hypothesis that *C4B* deficiency favors either the breaking of tolerance to citrullinated antigens or the handling of ACPA immune complexes discussed above. As

mentioned earlier, GWAS studies are unable to detect *C4A* and *C4B* CNVs (27). Confirmative study will require accurate genotyping of large cohorts of RA patients for *C4B* gene CNV and *HLA-DRB1* alleles.

In addition to a potential role in RA pathogenesis, *C4B* deficiency might shape disease phenotype and severity in RA, potentially through its role in immune complex clearance. In this regard, 50% of patients with RA and accompanying Felty's syndrome (n=24) were found to have a *C4B* null allele by allotyping (35). There were no patients with Felty's Syndrome in our RA cohort. Ultimately, examination of the disease severity and/or phenotype as a function of *C4B* deficiency in a RA patient cohort will be the necessary first step in addressing this question.

In conclusion, we report the novel finding that *C4B* CNV exhibits a clear relationship with RA. This CNV manifests itself as the presence of *C4B* deficiency in over 40% of the seropositive RA population, but does not appear to result from linkage disequilibrium. This association approximates that seen between *C4A* CNV and SLE (22). Given this frequency and the strength of this relationship, our finding has broad implications for our understanding of RA. Not only might *C4B* deficiency play a role in disease pathogenesis/phenotype, its functional roles in host defense suggest potential contributions to the safety and efficacy of antibody-based therapeutics.

Acknowledgments

This work was supported by grants from the National Institutes of Health R01AR49834 (W.F.C.R.), the American College of Rheumatology Research and Education 'Within Our Reach' program (W.F.C.R.), and NIAMS grant R01AR054459 (C.Y.Y.). Dr. Zan was supported by NIH Training Grants (T32 AR07576, T32 AI07363) and the Hitchcock Foundation. This paper is subject to the NIH Public Access Policy. The technical assistance of Emilie Shipman is acknowledged.

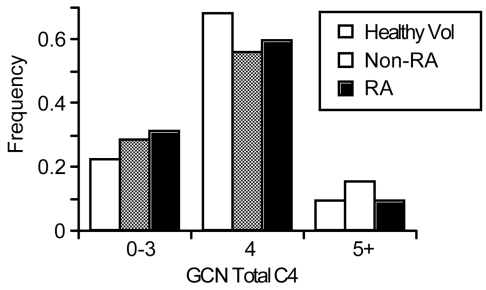
References

1. Firestein, GS.; Budd, RC.; Mcinnes, I.; Ruddy, S. Kelley's Textbook of Rheumatology. 8th. Saunders Company; 2008.
2. van der Helm-van Mil AH, Verpoort KN, Breedveld FC, Huizinga TW, Toes RE, de Vries RR. The HLA-DRB1 shared epitope alleles are primarily a risk factor for anti-cyclic citrullinated peptide antibodies and are not an independent risk factor for development of rheumatoid arthritis. Arthritis Rheum. 2006; 54:1117–21. [PubMed: 16572446]
3. Irigoyen P, Lee AT, Wener MH, Li W, Kern M, Batliwalla F, et al. Regulation of anti-cyclic citrullinated peptide antibodies in rheumatoid arthritis: contrasting effects of HLA-DR3 and the shared epitope alleles. Arthritis Rheum. 2005; 52:3813–8. [PubMed: 16320316]
4. Farragher TM, Goodson NJ, Naseem H, Silman AJ, Thomson W, Symmons D, et al. Association of the HLA-DRB1 gene with premature death, particularly from cardiovascular disease, in patients with rheumatoid arthritis and inflammatory polyarthritis. Arthritis Rheum. 2008; 58:359–69. [PubMed: 18240242]
5. Mewar D, Coote A, Moore DJ, Marinou I, Keyworth J, Dickson MC, et al. Independent associations of anti-cyclic citrullinated peptide antibodies and rheumatoid factor with radiographic severity of rheumatoid arthritis. Arthritis Res Ther. 2006; 8:R128. [PubMed: 16859535]
6. Feitsma AL, van der Helm-van Mil AH, Huizinga TW, de Vries RR, Toes RE. Protection against rheumatoid arthritis by HLA: nature and nurture. Ann Rheum Dis. 2008; 67(3):iii61–iii63. [PubMed: 19022816]
7. Winchester R. A golden anniversary: recognition that rheumatoid arthritis sera contain autoantibodies specific for determinants on native IgG molecules. J Immunol. 2007; 178:1227–8. [PubMed: 17237366]

8. Nielen MM, van SD, Reesink HW, van de Stadt RJ, van der Horst-Bruinsma IE, de Koning MH, et al. Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. Arthritis Rheum. 2004; 50:380–6. [PubMed: 14872479]
9. Atkinson, JP.; Yu, CY. Genetic susceptibility and class III complement genes. In: Lahita, RG.; Buyon, JP.; Koike, T.; Tsokos, GC., editors. Systemic Lupus Erythematosus. 5. Amsterdam: Elsevier Academic Press; 2011. p. 21-45.
10. Manderson AP, Botto M, Walport MJ. The role of complement in the development of systemic lupus erythematosus. Annu Rev Immunol. 2004; 22:431–56. [PubMed: 15032584]
11. Carroll MC. The role of complement and complement receptors in induction and regulation of immunity. Annu Rev Immunol. 1998; 16:545–68. [PubMed: 9597141]
12. Law SKA, Dodds AW, Porter RR. A comparison of the properties of two classes, C4A and C4B, of the human complement component C4. EMBO J. 1984; 3:1819–23. [PubMed: 6332733]
13. Isenman DE, Young JR. The molecular basis for the differences in immune hemolysis activity of the Chido and Rodgers isotypes of human complement component C4. J Immunol. 1984; 132:3019–27. [PubMed: 6609966]
14. Yu CY, Belt KT, Giles CM, Campbell RD, Porter RR. Structural basis of the polymorphism of human complement component C4A and C4B: gene size, reactivity and antigenicity. EMBO J. 1986; 5:2873–81. [PubMed: 2431902]
15. Awdeh ZL, Alper CA. Inherited structural polymorphism of the fourth component of human complement. Proc Natl Acad Sci USA. 1980; 77:3576–80. [PubMed: 6932037]
16. Nonaka M, Nakayama K, Yeul YD, Takahashi M. Complete Nucleotide and Derived Amino Acid Sequences of the Fourth Component of Mouse Complement (C4). J Biol Chem. 1985; 260(20): 10936–43. [PubMed: 2993295]
17. Sepich DS, Noonan DJ, Ogata RT. Complete cDNA sequence of the fourth component of murine complement. Proc Natl Acad Sci USA. 1985; 82:5895–9. [PubMed: 3862104]
18. Dodds AW, Ren XD, Willis AC, Law SKA. The reaction mechanism of the internal thioester in the human complement component C4. Nature. 1996; 379:177–9. [PubMed: 8538770]
19. McCarroll SA, Altshuler DM. Copy-number variation and association studies of human disease. Nat Genet. 2007; 39:S37–S42. [PubMed: 17597780]
20. Craddock N, Hurles ME, Cardin N, Pearson RD, Plagnol V, Robson S, et al. Genome-wide association study of CNVs in 16,000 cases of eight common diseases and 3,000 shared controls. Nature. 2010; 464:713–20. [PubMed: 20360734]
21. McCarroll SA. Copy number variation and human genome maps. Nat Genet. 2010; 42:365–6. [PubMed: 20428091]
22. Yang Y, Chung EK, Wu YL, Savelli SL, Nagaraja HN, Zhou B, et al. Gene copy number variation and associated polymorphisms of complement component C4 in human systemic erythematosus (SLE): low copy number is a risk factor for and high copy number is a protective factor against European American SLE disease susceptibility. Am J Hum Genet. 2007; 80:1037–54. [PubMed: 17503323]
23. Yang Y, Chung EK, Zhou B, Blanchong CA, Yu CY, Füst G, et al. Diversity in intrinsic strengths of the human complement system: serum C4 protein concentrations correlate with *C4* gene size and polygenic variations, hemolytic activities and body mass index. J Immunol. 2003; 171:2734–45. [PubMed: 12928427]
24. Wu YL, Yang Y, Chung EK, Zhou B, Kitzmiller KJ, Savelli SL, et al. Phenotypes, genotypes and disease susceptibility associated with gene copy number variations: complement C4 CNVs in European American healthy subjects and those with systemic lupus erythematosus. Cytogenet Genome Res. 2008; 123:131–41. [PubMed: 19287147]
25. Saxena K, Kitzmiller KJ, Wu YL, Zhou B, Esack N, Hiremath L, et al. Great genotypic and phenotypic diversities associated with copy-number variations of complement C4 and RP-C4-CYP21-TNX (RCCX) modules: A comparison of Asian-Indian and European American populations. Mol Immunol. 2009; 46:1289–303. [PubMed: 19135723]
26. Blanchong CA, Zhou B, Rupert KL, Chung EK, Jones KN, Sotos JF, et al. Deficiencies of human complement component C4A and C4B and heterozygosity in length variants of RP-C4-CYP21-

TNX (RCCX) modules in Caucasians: the load of RCCX genetic diversity on MHC-associated disease. J Exp Med. 2000; 191:2183–96. [PubMed: 10859342]

27. Fernando MM, Boteva L, Morris DL, Zhou B, Wu YL, Lokki ML, et al. Assessment of complement C4 gene copy number using the paralog ratio test. Hum Mutat. 2010; 31:866–74. [PubMed: 20506482]
28. Chung EK, Wu YL, Yang Y, Zhou B, Yu CY. Human complement components C4A and C4B genetic diversities: complex genotypes and phenotypes. Curr Protoc Immunol. 2005:13.8.1–13.8.36.
29. Chung EK, Yang Y, Rupert KL, Jones KN, Rennebohm RM, Blanchong CA, et al. Determining the one, two, three or four long and short loci of human complement *C4* in a major histocompatibility complex haplotype encoding for C4A or C4B proteins. Am J Hum Genet. 2002; 71:810–22. [PubMed: 12224044]
30. Wu YL, Savelli SL, Yang Y, Zhou B, Rovin BH, Birmingham DJ, et al. Sensitive and specific real-time PCR Assays to accurately determine copy-number variations (CNVs) of human complement *C4A, C4B, C4-Long, C4-Short* and RCCX modules: Elucidation of *C4* CNVs in 50 consanguineous subjects with defined HLA genotypes. J Immunol. 2007; 179:3012–25. [PubMed: 17709516]
31. Meyer JM, Evans TI, Small RE, Redford TW, Han J, Singh R, et al. HLA-DRB1 genotype influences risk for and severity of rheumatoid arthritis. J Rheumatol. 1999; 26:1024–34. [PubMed: 10332964]
32. Korendowych E, Dixey J, Cox B, Jones S, McHugh N. The Influence of the HLA-DRB1 rheumatoid arthritis shared epitope on the clinical characteristics and radiological outcome of psoriatic arthritis. J Rheumatol. 2003; 30:96–101. [PubMed: 12508396]
33. Raptopoulou A, Sidiropoulos P, Katsouraki M, Boumpas DT. Anti-citrulline antibodies in the diagnosis and prognosis of rheumatoid arthritis: evolving concepts. Crit Rev Clin Lab Sci. 2007; 44:339–63. [PubMed: 17558653]
34. van Beers JJ, Raijmakers R, Alexander LE, Stammen-Vogelzangs J, Lokate AM, Heck AJ, et al. Mapping of citrullinated fibrinogen B-cell epitopes in rheumatoid arthritis by imaging surface plasmon resonance. Arthritis Res Ther. 2010; 12:R219. [PubMed: 21182780]
35. Clarkson R, Bate AS, Grennan DM, Chattopadhyay C, Sanders P, Davis M, et al. DQw7 and the C4B null allele in rheumatoid arthritis and Felty's syndrome. Ann Rheum Dis. 1990; 49:976–9. [PubMed: 2270969]



Chi-square p values

	Healthy vs non-RA	Healthy vs RA	Non-RA vs RA
Total C4	0.34	0.48	0.36
C4A	0.18	***0.0117***	0.37
C4B	0.86	***0.0364***	***0.0058***

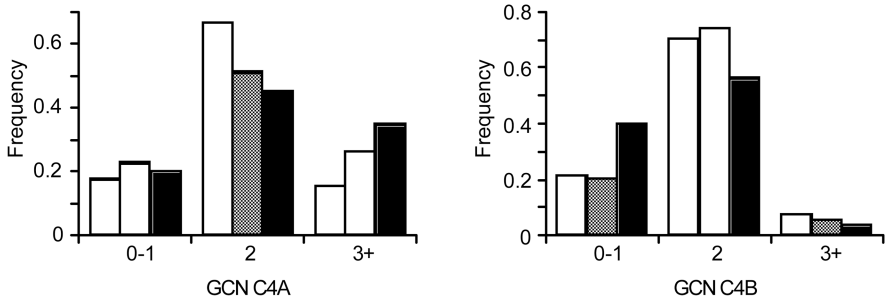
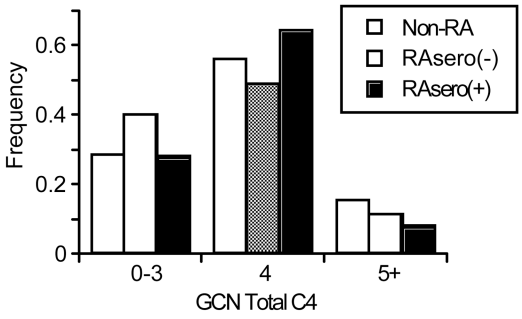


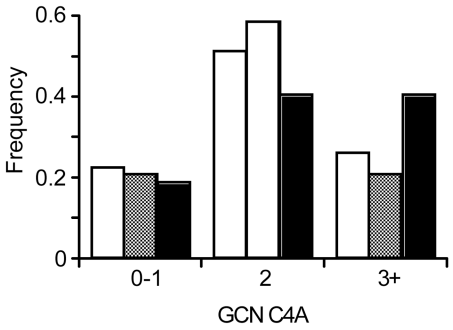
Figure 1. Distribution patterns of *C4, C4A, and C4B* gene copy number (GCN) among healthy controls, non-RA and RA patients

Chi-square analysis show emboldened values for statistically significant differences in distribution of *C4A (p=0.0117)* and *C4B (p=0.0364)* in the healthy volunteers relative to RA populations. Between the Non-RA (n=88) and RA (n=160) populations, significant differences were only seen for C4B (p=0.0058).



Chi-square p values

	Non-RA vs RA(-)	Non-RA vs RA(+)
Total C4	0.39	0.20
C4A	0.70	0.10
C4B	0.37	***0.0018***



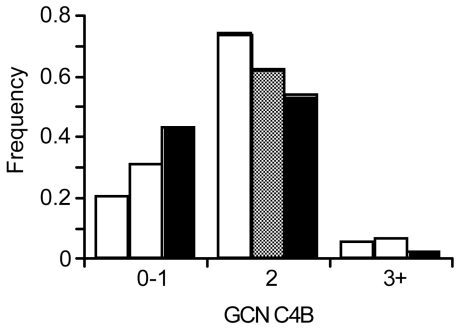


Figure 2. Distribution patterns of *C4, C4A, and C4B* gene copy number (GCN) among non-RA, seropositive (sero+) and seronegative (sero−) RA patients

Chi-square analysis relative to non-RA patients (n=88) show emboldened values for statistically significant differences *C4B (p=0.0018)* in the seropositive RA (n=115) population only. Statistically significant associations were not seen for C4A GCN in either analysis, nor was significance observed for *C4B* GCN between the non-RA and seronegative RA population (n=45).

Table 1

Distribution of *C4A* and *C4B* CNV Frequency*

	C4A/*C4B* Gene Copy Number Frequency (%)				
Patient	**0**	**1**	**2**	**3**	**4**
RA	1.2/4.4	19.4/35.6	46.3/56.3	3.1/29.4	2.5/0.6
Sero(+)	1.7/6.1	18.3/37.4	40.0/53.9	35.7/1.7	2.6/1.7
Sero(−)	0/0	22.2/31.1	62.2/62.2	13.3/6.7	2.2/0
Non-RA	1.1/1.1	22.5/21.3	49.4/73.0	23.6/2.2	3.4/2.2
Healthy	0/2	17.6/19.6	66.7/70.6	15.7/5.9	0/2.0
Ohio Cohort	0/2.7	17.3/26.9	56.3/63.3	21.6/6.8	3.3/0.2

* Frequency analysis of *C4A* and *C4B* gene copy number shown as percentage of each group. Number of individuals analyzed in each group: RA: n=160; Seropositive RA (Sero+) n =115; Seronegative RA (Sero−) n=45; Non-RA patients (non-RA) n =88; Healthy volunteers (Healthy) n=51. Ohio Cohort represents a separate, unrelated healthy control cohort [(n=517; 389 females, mean age ± SD: 38.6 ± 11.1 years old; and 128 males, 34.3±12.1 years old (22)]. All patients and volunteers are of European descent. No patient had more than 5 copies of either *C4A* or *C4B*.

Table 2

C4B Isotype Deficiency (0-1 copy of *C4B* gene) Relative to Ohio Control Cohort*

Patient	Odds Ratio (95% CI)	p value
Non-RA	0.61 (0.35-1.06)	0.0953
All-RA	1.58 (1.10-2.29)	0.0155
Sero(+)	1.83 (1.21-2.76)	0.0056
Sero(-)	1.07 (0.55-2.07)	0.87

* Odds ratios (OR) and 95% confidence intervals (CI) were calculated by analysis of 2 × 2 tables, using the Fisher's exact test for comparisons.

Table 3

C4B Deficiency and Shared Epitope Associated Only in Seropositive RA*

Group	SE positivity (%)	p value
Seropositive RA	94/113 (83%)	
C4B (<2)	48/50 (96%)	
C4B (≥2)	46/63 (76%)	p<0.0009
Non-Seropositive RA		
C4B (<2)	24/44 (55%)	p<0.0001
Non-RA/Volunteers	17/30 (57%)	p<0.0001

* Data shown represents SE frequency in seropositive RA patient population subgrouped on the basis of C4B deficiency p<0.0009. In the non-seropositive RA group, made up of healthy volunteers, non-RA and seronegative RA patients with C4B deficiency (n=44), the frequency of the SE was 55%, similar to that seen in the general population (31,32). Thus, the association of SE with C4B deficiency was significantly greater in the seropositive RA patient population relative to non-seropositive RA controls, 96% vs 55%, p<0.0001 and this was identical to the Non-RA/ Volunteer subgroup. In seronegative RA with *C4B* deficiency, 7/14 contained the SE consistent with the general population (31,32).