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The Complement System and Human Autoimmune Diseases

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Abstract

Genetic deficiency for early components of the classical complement activation pathway (especially C1q, r, s, and C4) are the strongest monogenic causal factors for the prototypic autoimmune disease systemic lupus erythematosus (SLE), but their prevalence is extremely rare. In contrast, isotype genetic deficiency for the acidic C4A and acquired deficiency of C1q by autoantibodies are frequent among patients with SLE. Here we review the genetic basis of complement deficiencies in autoimmune disease, discuss the complex genetic diversity seen in complement C4 and its association with autoimmune disease, provide guidance as to when clinicians should suspect and test for complement deficiencies, and outline the current understanding of the mechanisms relating complement deficiencies to autoimmunity. We focus primarily on SLE, as the role of complement in SLE is well-established, but will also discuss other informative diseases such as inflammatory arthritis and myositis.

I. INTRODUCTION – Complement pathways

The complement system is an ancient form of immune defense that has existed since the emergence of thioester proteins with opsonic functions in insects and worms (1, 2). Increasing evidence implicates complement in diverse biological processes in mammals ranging from modulation of immunity and tolerance and inflammatory and autoimmune diseases to intracellular signaling involved in metabolism. In all cases, appropriate balance of complement activation and inhibition is required to maintain homeostasis and

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health (3). Over-activation may lead to excessive inflammation and tissue damage while under-activation may impair pathogen clearance, lead to rampant infection, and possibly predispose towards autoimmune responses against self-antigens. So, while this review will focus on what is known and future directions for research regarding complement's role in autoimmune diseases with emphases on systemic lupus erythematosus (SLE) and the idiopathic inflammatory myopathies (IIM), it is written with circumspection and consideration for the fact that a small modification of the complement system can have far ranging ramifications in immunity and autoimmunity.

Complement was first described at the end of the 19th century as a soluble and heat-labile factor in the blood that efficiently lysed bacteria (4). Since then, the classical CP), alternative (AP), and lectin (LP) pathways of complement activation in immune defense have been elucidated (5–7). Figure 1 illustrates the three activation pathways of the complement system and positive feedback amplification leading to the release of anaphylatoxins, formation of complement C3 convertases, and the activation of a common terminal pathway (5, 8). Notably, there is a high degree of regulation at almost every step of the activation pathways to ensure rapid and robust destruction of foreign targets with minimal self-inflicted injury (3, 9, 10). Briefly, these pathways are respectively triggered by antigen/antibody complexes binding to C1q of the C1 complex (*classical pathway*), spontaneous hydrolysis of C3 (*alternative pathway*), or foreign carbohydrate moieties binding to mannose binding lectin (MBL) or ficolin (*lectin pathway*). Importantly, all three activation pathways lead to the generation of C3 convertases, C4b2a or C3bBb, and converge at the cleavage and activation of C3 and the amplification loop. In turn, C3b contributes to the formation of the C5 convertase C4b2a3b for the CP or LP, or $(C3b)_2Bb$ for the AP, which are essential for the activation of C5 and for the formation of the membrane attack complex (MAC) and direct lysis of target cells.

II. COMPLEMENT DEFICIENCIES AND IMMUNE DYSFUNCTION

A. Complement deficiency and infection susceptibility

Deficiencies of components of the complement classical pathway have been associated with increased susceptibility to infections caused by encapsulated bacteria (especially pneumonia and meningitis) and recurrent respiratory infections. Similar patterns of infection have been noted in patients with deficiencies of alternative pathway components. Reduced expression of mannose-binding lectin is associated with increased severity of infection by both bacteria and yeast. MBL deficiency has also been implicated in tuberculosis, malaria, and a variety of viral infections (11–13).

Frequent infections are also the predominant manifestation of deficiencies of C3, factors I and H, and the components of the membrane attack complex, C5-C8. Almost all reported patients with homozygous C3 deficiency have been infants or young children with severe bacterial infections (meningitis, pneumonitis, peritonitis, and osteomyelitis) (14). The reason for increased susceptibility to infection in cases of complement deficiency is easy to conceive: reduced complement availability impairs pathway activation, opsonization, and formation of MAC on the outer membranes of microbial invaders and leads to poor microbial killing. This phenomenon was demonstrated in a recent case report in which

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a patient with SLE, urticarial vasculitis, and acquired hypocomplementemia developed gonococcemia and septic shock (15). Reduced levels of complement components may also lead to the decreased generation of the anaphylatoxins C3a and C5a, which serve to recruit immune cells to the site of foreign pathogen invasion (16), and thus reduce immunocompetence. In addition to facilitating bacterial and fungal clearance, complement components including MBL are important in the innate immune response to viral infections (17), including upper respiratory infections such as the influenza (18, 19) and other viruses (20). It is known that viral infection temporarily weakens immunity and can open the door for bacterial superinfection (18, 21–23), and therefore complement deficiency may indirectly lead to increased susceptibility to bacterial infection in addition to the loss of its active role in pathogen killing.

B. Complement deficiency and autoimmunity

In addition to targeting invading pathogens with the MAC, the complement system is known to have a range of other immune and immunoregulatory roles, including: (1) the formation and release of the anaphylatoxins C3a and C5a, which serve to attract inflammatory cells to the site of complement activation, (2) opsonization and solubilization of native immune complexes (IC) composed of autoantigens and self-reactive antibodies, and (3) facilitating clearance of IC from the circulation by enabling the binding of complement receptor CR1 on erythrocytes or CR3 or CR4 on phagocytic myeloid cells to opsonized IC (24, 25). Thus, engagement of complement on IC helps reduce the potentially harmful sequelae associated with the presence of immunogenic autoantigen/antibody conglomerates and the subsequent generation of autoantibodies, which may then be deposited on and cause damage to otherwise healthy tissues (24, 26–31).

Deficiencies of various complement components therefore also have undeniable ramifications regarding autoimmunity. While C1q and C4 deficiency are the most strongly related to autoimmune disease (32–35), other complement components including C1r, C1s, C2, and C3 have also been implicated (31, 36, 37). The autoimmune phenotypes associated with the various complement deficiencies as well as literature pertaining to each is summarized in Table 1. The initial trigger for complement activation in various autoimmune diseases may be distinct: synovial proteins and C-reactive protein (CRP) in rheumatoid arthritis (RA) (38, 39); apoptotic cells, neutrophil extracellular traps containing DNA and modified DNA-binding proteins (40–44) and immune complexes in SLE (45); and possibly infection, malignancy, and certain medications in inflammatory myositis (46). In addition, regulation of the complement cascade has been shown to be altered in various autoimmune disorders including RA (47) and SLE (48).

Similar to the mechanisms by which insufficient complement activation leads to impaired antimicrobial immunity, C1, C2 or C4 deficiency can increase susceptibility to autoimmune disease through impaired opsonization and clearance of autoantigens and IC as well as through decreased formation of MAC on membranes of apoptotic cells (49–53). Decreased complement compromises phagocytes' response to immunogenic IC and apoptotic debris and increases the amount of time dendritic cells, T cells, and B cells are exposed to autoantigens (54–57). In addition, impairment of complement signaling may skew immunity

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towards more pro-inflammatory responses, thus contributing to the increased inflammation and immune reactivity seen in autoimmune disease (58, 59).

III. CLINICAL RELEVANCE OF COMPLEMENT IN AUTOIMMUNE DISEASE

This section will highlight the clinical signs and symptoms that suggest that clinicians should consider complement deficiency in their differential diagnosis. Complement deficiencies in general are under-diagnosed, in part because of the diversity in clinical presentation of these disorders, which varies based on the type of complement deficiency (60, 61).

A. Clinical findings suggestive of complement deficiency

History and physical examination features suggestive of complement abnormalities include multi-generational autoimmune disease, including nephritis, early onset of skin lesions resembling lupus rash, alopecia, photosensitivity, increased susceptibility to infection with encapsulated bacteria such as *Streptococcus pneumoniae* and *Neisseria meningitides,* increased susceptibility to viral infections, and angioedema (62, 63). The presentation of complement deficiency may vary across a spectrum from asymptomatic to invasive infection to severe rheumatic diseases resembling SLE. Examples of lupus-like rashes seen in various complement deficiencies are shown in Figure 2.

Clinicians should consider complement deficiencies in a patient presenting with hemolytic anemia, acute kidney failure, and thrombocytopenia concerning for hemolytic uremic syndrome (HUS). While typical HUS is preceded by an episode of bloody diarrhea most commonly caused by a *Shigella*-like toxin producing *E. coli* (64), there is also a rare, chronic, and severe form of HUS known as atypical hemolytic uremic syndrome (aHUS) (65). Atypical HUS is caused by genetic defects with a variety of mutations in genes of the complement pathway including complement factor H (CFH), membrane cofactor protein (MCP), Factor I, Factor B, and C3 (66). Reduced serum levels of complement C3 with normal levels of C4 have been reported in patients with atypical HUS, likely reflecting complement consumption as a result of AP activation (67). Knowing the genetic defect underlying aHUS is important for treatment. Atypical HUS is associated with a mortality rate of 20–25% and a morbidity of 48%, as pediatric patients typically progress to end stage renal disease (68).

B. Laboratory testing in cases of suspected complement deficiency

Laboratory evaluation in suspected complement deficiency should include C3, C4, and CH50 to not only assess C3 and C4 protein levels but also complement hemolytic activity. Functional screening for the complement system includes tests for the CP (CH50), the AP (AH50), and the LP. Low CH50 and normal AH50 suggest early classical complement component (C1, C2, and C4) deficiency. Low AH50 with normal CH50 suggests a deficiency of early AP factors (factor B, factor D, and properdin). Low AH50 and low CH50 suggest common terminal complement (C3, C5, C6, C7, C8, or C9) deficiency. If CH50 and AH50 are both normal and the clinician still suspects complement deficiency, MBL functional assay is indicated (16). Furthermore, clinicians should complete a serological

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work up for SLE including complete blood count (CBC), comprehensive metabolic panel (CMP), antinuclear antibody (ANA), anti-double-stranded DNA (anti-dsDNA), anti-Smith antibodies, and urinalysis. As SLE is extremely rare in children less than 5 years of age, findings suggestive of SLE in young patients should always trigger a deeper investigation into possible genetic causes and/or immunodeficiency.

C. Factors to consider when evaluating complement levels

a. C4 GCN and serum protein levels.—Physiologically, serum protein levels of complement C3 and C4 are correlated with each other very strongly. Immune-complex mediated consumption can lower the serum levels of C4 and C3, particularly during active disease in SLE. Hypocomplementemia in SLE is one of the established criteria for diagnosis of SLE (69–71). Serum levels of C4 and C3 fluctuate with disease activity in SLE, with concurrently low levels of C4 and C3 during disease relapses. Thus, C3 and C4 are useful biomarkers for monitoring disease activity and response to therapy.

Among SLE patients, there exist distinct profiles of C4 serum protein levels over time (72), dependent on the C4 genetic background and disease activities. Patients with low C4 gene copy numbers (i.e., with only two or three copies of total C4) tend to have consistently low levels of serum C4 even during disease remission (72, 73). Patients with medium to high copy number of C4 genes are more likely to demonstrate fluctuating serum levels of C4 and C3 that return to normal range during disease remission. Although low complement levels are seen in active lupus, especially lupus nephritis, it can be difficult to ascertain whether low complement levels are due to consumption during inflammation or due to an inherent isotype deficiency. Even more obfuscating, the two scenarios may coexist in one individual (74). A return to normal C3 but not C4 would imply low gene copy number (GCN) of C4 or other possibilities such as acquired deficiency of C1-inhibitor leading to unchecked turnover (75). An important difference between patients with low C4 due to low GCN versus those with high turnover is that the latter is accompanied by the presence of high levels of serum C4a, and/or high levels of cell bound C4d on the membranes of red blood cells, reflecting ongoing activation and consumption as the cause of hypocomplementemia.

In other autoimmune rheumatic disease such as myositis, complement levels may be depressed to the low “normal” range, but this is often not clinically remarkable and may go undetected if baseline levels are not known. However, there are readily detectable complement activation products in the circulation and deposited on the membrane of circulating blood cells that can provide laboratory evidence of disease activity (76–79). The clinical utility and availability of this test is not universal, however, and is often limited to the research rather than the clinical arena.

b. Immune factors contributing to acquired deficiency of complement.—The presence of C3 or C4 nephritic factors or autoantibodies against C3bBb and C4b2a can impair dissociation of complement molecular complexes and thus affect regulation of the AP and CP C3 convertases, respectively, leading to low levels of C3. Clinically, genetic or acquired deficiency of C1 inhibitor would also lead to excessive turnover and very low levels of complement C4.

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In addition, complement C3 is a strong acute phase protein whose expression may be stimulated by many cytokines, including TGFβ. Infections are known to stimulate immune cell function and cytokine secretion, and thus can cause fluctuations in C3 independent of autoimmune disease activity. The only known cytokine that stimulates the expression of complement C4 is IFNγ, which may also be stimulated by infection (especially viral) (80).

c. Non-immunologic sources of complement protein level variation.—The primary site of biosynthesis for most complement proteins in humans is the liver, but adipose tissues and tissue-resident myeloid cells including macrophages also synthesize complement proteins for local defense (81). Therefore, liver disease and obesity can affect complement levels in a manner independent of their correlation with autoimmune disease. The body mass index (BMI) of an individual strongly correlates with serum C3 level and oftentimes levels of C4, as well (82, 83). As male subjects tend to have higher BMI than female subjects, it is also natural to observe slightly higher serum complement C3 and C4 levels in males than females (82, 84).

Because of variations in GCN and gene size dichotomy plus the influence of body mass indices, circulating cytokines, and medications, the baseline levels of complement C4 and C3 vary greatly among different human subjects. It is prudent to establish complement profiles *in each patient* with data acquired during disease remissions and disease flares and to compare levels across time to accurately interpret the patient's current complement levels and to apply appropriate therapies.

IV. COMPLEMENT IN HUMAN SYSTEMIC LUPUS ERYTHEMATOSUS

Human SLE is an autoimmune disease characterized by the generation of autoantibodies against nuclear and cytoplasmic antigens accompanied by complement activation with dramatic longitudinal fluctuations of serum C4 and C3 levels and immune-mediated tissue injury (85). The etiology and pathogenesis of SLE are complex and involve multiple genetic risk factors and environmental triggers for disease onset, progression, and response to therapy.

A. Complement genetic deficiency and SLE

Not surprisingly given complement's role in immune complex formation and antigen clearance, homozygous or complete deficiencies for early components of the CP (i.e., C1q, r, s, C4A and C4B, and C2) are amongst the strongest genetic risk or causal factors for human SLE, although their incidences are extremely rare (33). As a result of the strength of the associations between complement deficiencies and lupus, the role of the complement system in autoimmune disease is the best studied in lupus. Importantly, cases of complement deficiencies provide important insights into the pathogenic mechanisms of human lupus (Table 1).

a. Genetic deficiency of C1q—C1q consists of 18 polypeptides with hexamers of A, B and C chains intertwined together into a bouquet arrangement (Figure 3, panel A) (86). Missense or nonsense mutations in one of the A, B or C chains can disrupt the structure of

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C1q and render the protein non-functional. C1q also forms a complex with two C1r and two C1s subunits, and impaired interactions between these can cause functional deficiency (87).

Clinical presentations of C1q deficiency.: Genetic deficiency of C1q is rare, with only 74 known cases (88, 89). A total of 17 nonsense mutations have been identified to cause C1q genetic deficiency (87, 90–94). The mutations that have been associated with disease are listed under their genetic location in C1qA, C1qB, and C1qC (Figure 3, panel B). Many patients described were of European or Middle Eastern ancestry. Homozygosity was almost exclusively the result of consanguineous marriages. While clinical presentations among C1q-deficient patients varied considerably, two common symptoms are overwhelmingly associated with a genetic deficiency of C1q: (a) SLE or lupus-like disease occurred in 88% of patients, and (b) recurrent bacterial infections in 41% of patients (87, 89–91, 95, 96).

As for SLE and lupus-like disorders, many patients had disease onset in early childhood. The range was 6 months to 42 years (37). The female to male sex ratio was close to 1:1. Many patients with C1q-deficiency died at a young age secondary to septicemia or renal failure. Among the C1q-deficient patients with SLE or lupus-like disease, cutaneous disorders, especially photosensitivity, were prominent with a frequency of 84%. Glomerulonephritis and neurologic disease affected about 30% and 19% of patients, respectively.

C1q deficiency leads to poorer clearance of apoptotic cells and increased exposure to potentially immunogenic autoantigens (97–100). Cai et al showed that UV damage-induced apoptosis caused C1q binding to nucleolar DNA and that C1q allowed C1r/s to degrade the nucleosomes, presumably decreasing their availability to trigger autoimmune responses (100). Furthermore, impairments in this pathway have been directly observed *in vitro* using cells from patients with SLE, with poorer C1q binding and impaired clearance of apoptotic cell debris by phagocytes (101). C1q was not sequenced, so it was not known if these results were due to C1q genetic deficiency, but the logical conclusion from this work is that C1q-mediated clearance of apoptotic debris is critical to prevent autoimmunity from developing and that this process can be disrupted by a lack of C1q or poor function of C1q *in vivo*, which might be inherited or acquired.

C1q deficiency is also associated with heightened levels of IFNα in the blood and cerebrospinal fluid and affects whether immune complexes are preferentially taken up by phagocytes rather than dendritic cells *in vitro* (102), which may be why plasmacytoid dendritic cells (pDC) from patients with C1q deficiency demonstrate higher IFNα production (59). As pDC are primary antigen presenting cells, increased uptake of immune complexes and stimulation of IFNα signaling is a plausible mechanism for increased adaptive immune activation and autoimmune responses seen in patients with C1q deficiency. Indeed, work by Hosszu et al suggests that locally synthesized C1q regulates dendritic cell (DC) differentiation and function in a way that negatively impacts self-tolerance (97, 103, 104). As IC clearance is decreased in a dose-dependent manner in C1q deficiency (55), it is also plausible that low C1q not only results in prolonged exposure to IC, but also to C1q itself, which may explain in part why patients with SLE are noted to frequently test positive for antibodies against C1q and other complement components (105, 106).

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Therapy and potential cure of SLE with C1q-deficiency.: Plasmapheresis or infusion of fresh frozen plasma (FFP) can restore C1q activity in C1q-deficient patients temporarily and ameliorate lupus disease symptoms. However, complement activity drops off rapidly and frequent treatments are necessary (90, 107). Furthermore, FFP infusions bring with them their own risk of infection and thrombotic complications and are therefore a far from ideal therapy.

Unlike most other complement components, the primary site of biosynthesis for C1q is not in the liver but rather in myeloid cells including macrophages, monocytes, and dendritic cells, which originate from the bone marrow. Proof of concept that complement function can be reconstituted through bone marrow transplant has been shown in mouse models. Bone marrow transplantation (BMT) of hematopoietic stem cells from wild-type mice has been shown to be effective in treating C1q deficient animals (108, 109). BMT of allogenic hematopoietic stem cells therapy (HSCT) has been performed in three C1q deficient patients to date, two of which were successful and one of which resulted in the patient's death (110, 111). The two successes demonstrate that allogenic hematopoietic stem cell transplant in humans can potentially restore complement function and eliminate an important factor contributing to lupus disease. However, there is a risk of considerable side effects, such as post-transplant lymphoproliferative disease, reactivation of latent viruses, and graft versus host disease. The two cases with excellent outcomes received bone marrow from siblings with matched HLA, which minimized graft versus host disease. Unfortunately, HLA-matched related donors are not always available. However, with the advent of CRISPR/cas9 technology for gene editing, correction of C1q deficiency using engineered autologous hematopoietic stem cells to cure SLE could become a reality.

b. Genetic deficiency of C1r or C1s—Deficiencies in sub-components of the C1 complex, C1r and C1s, were among the earliest reported linking complement deficiency with human glomerulonephritis or a lupus-like disease (112–115). A total of 20 cases of C1r and/or C1s deficiency have been reported, which includes 12 cases of C1r deficiency from eight families, and 8 cases of C1s deficiency from five families.

Clinical presentation of C1r or C1s deficiency.: Among the C1r/C1s deficient subjects studied, all but three had recurrent bacterial, viral, or fungal infections (85%). Many patients died at young ages due to severe infections. Thirteen subjects developed SLE or a lupus-like disease (65%). The female to male ratio among C1r/C1s deficient subjects with SLE was 1.5 to 1. Mortality at a young age due to fulminant infections likely explains the slightly lower frequency of lupus disease association. Most patients had severe cutaneous lesions. Eight patients had renal disease due to lupus nephritis (40%). The prevalence of anti-nuclear antibodies (ANA) among patients with SLE was only about 60% (116).

Whole exome sequencing of a multiplex family with SLE identified monogenic lupus with homozygous deficiency of C1r. A homozygous, single T-nucleotide deletion at coding sequence position 1332 of complement C1R (c1332delT) was found in all four SLE patients studied (117). This deletion resulted in the synthesis of a truncated protein without the serine proteinase domain in C1r. Of note, the same homozygous mutation was also detected in a 9-year-old female sibling who remained asymptomatic at the time of study. A heterozygous

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mutation was present in each of the four parents plus two other healthy siblings. Extensive screening of 300 patients with non-familial SLE, plus 1706 healthy subjects and 1618 patients with Behçet's disease, all from Turkey, did not detect this mutation. Thus, the c1332delT in C1R of this Turkish family was a private, recessive mutation that caused SLE with high penetrance. Gene expression profiling using PAXgene RNA revealed enhanced type I interferon stimulated gene expression signature in patients with SLE but not the healthy subjects or carriers. Such IFN-I gene expression signature persisted among patients even without active disease (117), which is in contrast to findings in C4 deficiency will be discussed below (118).

Two patients with C1r deficiency, a female with disease onset at 13 years old and a male with disease onset at 7 years old, were further studied at the National Institutes of Health in the US (117). The female patient had neurologic and cognitive impairments and proteinuria with renal biopsy showing mesangial proliferative nephritis. Laboratory results showed that she had a strong neutrophil signature as defined by enhanced neutrophil extracellular trap (NET) formation when induced by bacterial lipopolysaccharide (LPS), and marked increase of low-density granulocytes (CD10+, CD14-low, CD15+) in PBMC. Her brother presented with proteinuria and a renal biopsy showed acute proliferative glomerulonephritis. Clinical laboratory tests revealed a significant increase in serum levels of cytokines and chemokines such as IL-2, IL-7, IL-10, IL-13, MCP-1, and MIP-1α and spontaneous and LPS-induced NET formation.

c. C2 or C3 deficiency

C2 deficiency.: Among individuals of European descent, C2 deficiency occurs with an estimated prevalence of 1/20,000, which probably accounts for <1% of SLE patients. There are two types of C2 deficiency (119, 120), caused either by nonsense mutations leading to the absence of protein biosynthesis (type 1, the predominant cause of deficiency) or by missense mutations C111Y, S189F, and G444R (121, 122) (type 2, only ~10% of C2 deficiency). One example of type 1 deficiency is a 28-bp deletion that generates a premature stop codon (123). This 28-bp deletion is present in the HLA haplotype with A10 (A25) and B18 in the class I region; BF-S, C2Q0, C4A4, and C4B2 in the class III region; and DRB1*15 (DR2) in the class II region. A second type 1 C2 deficiency is associated with the haplotype HLA A3, B35, DR4, BF-F, C2Q0, C4A3, and C4A2 (124).

Unlike C1 or C4 deficiency, the penetrance of C2 deficiency on SLE is only about 10%. Like other risk factors for SLE, there is a female predominance. C2-deficient SLE patients tend to have early childhood onset but a milder disease course with prominent photosensitive dermatologic manifestations, speckled ANA [a pattern common for the Ro (SSA) antigen], and a family history of SLE. Anti-DNA antibody tests are usually negative, and severe kidney disease is rare.

C3 deficiency.: C3 deficiency is generally associated with severe infections (125). Despite its prominent role in the complement functions of opsonization, phagocytosis, and clearance of immune complexes, C3 deficiency has *not* been found to associate with increased SLE

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predisposition except in Japanese subjects, with five out of six patients identified as C3 deficient being diagnosed with SLE (125–127).

d. Genetic diversity and deficiency of C4.—Figure 4 summarizes the unusual diversity seen in human C4 genetics. Panel A depicts the genetic locations for constituents of the C3 convertases—C4 and the enzymatic components C2 and factor B—in the class III region of the human major histocompatibility complex (MHC or HLA) on the short arm of the chromosome 6 (128–130). Panel B of Figure 4 illustrates the segmental duplications with one to five modules of the RP-**C4**-CYP21-TNX (RCCX) in haplotypes of the HLA (83, 131–136). Each duplicated segment, which includes C4 as well as CYP21, can be 30.6 or 24.2 kb in size. Panel B further depicts the exon-intron structures and the highly unusual gene size dichotomy of human C4 genes explaining this size variation (137). While each C4 gene consists of 41 exons (138), the long gene contains a 6.4 kb endogenous retrovirus, HERV-K(C4), in the ninth intron (137, 139, 140). In most ethnic groups, the majority (approximately 75%) of C4 genes are long genes. The single exception is in African American populations, in which only 60% of all C4 genes are long (141). The benefit or detriment of long versus short genes and the physiologic relevance of the coexistence of both isoforms of C4 genes are not yet well understood, though it has been proposed that the retrovirus may be a source of anti-sense mRNA that may be useful for antiviral defense and regulation of gene expression (139, 142).

Polymorphisms of C4A and C4B genes and proteins.: Figure 5 shows the molecular basis of isotypic changes for the acidic C4A and the basic C4B proteins (138, 140, 143). The boxed sequence in Panel A is specific for *Psh*AI restriction enzyme cleavage, which has been used as a tool to distinguish the A and B isotypes of C4 through restriction fragment length polymorphisms (RFLP) (135, 144, 145). Panel B describes the functional implications of the biochemical differences between the two isotypes. In short, C4A is thought to be superior at binding amino groups, including those present in antibodies that contribute to immune complexes, while C4B tends to bind hydroxyl groups more readily, which may explain the finding that it is superior at binding to and triggering the lysis of red blood cells *in vitro* [70].

Complete C4A and C4B deficiency.: A total of 28 individuals with a *complete* genetic deficiency of both C4A and C4B have been reported (146–150). These C4-deficient subjects come from 19 families, characterized by 16 different HLA haplotypes and with European, African, and Asian ancestries. Among those subjects with complete C4 deficiency, 22 (78.6%) were diagnosed with SLE or a lupus-like disease, and four others had renal disease such as glomerulonephritis. The female to male ratio was 1:1. Early disease onset, a severe photosensitive skin rash, presence of anti-Ro/SSA, and high ANA titers were common. The frequency of sepsis or severe recurrent infections was 29%.

Almost all completely C4 deficient patients were homozygous with HLA class I and class II markers for both copies of chromosome 6, suggesting consanguinity. The molecular basis of complete C4 deficiency has been determined in 15 cases (146–150). All nonsense mutations except one were private mutations that were only observed in the patient's family or local

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community. The exception was a 2-bp insertion in exon 29 (codon 1232) of *C4A* that has an allelic frequency of 1 to 3% among Europeans (151–153).

In a European family from Austria and Northern Italy with multiplex lupus-related mortality and low serum C4 levels, the culprit was a heterozygous, recurrent haplotype with HLA-A30, B18 and DR7 that segregated with two defective and short C4B genes (C4B-C4B/SS) with identical mutations at the donor splice site of intron 28 (gt→at) (154). Notably such duplicated splice-junction mutations were found previously in two other families with complete (homozygous) C4 deficiency, vasculitis, and lupus nephritis who resided in the same geographic area (150). Such phenomena suggest that C4 hypocomplementemia caused by heterozygous deficiencies could increase the risk of SLE.

C4A or C4B isotype deficiency.: Similarly, in complement C4 isotype deficiency—especially that of C4A, which binds amino groups (and thus immune complexes) better than does C4B (155, 156)—reduced ability to opsonize immune complexes has been suggested as a putative trigger for autoimmune responses and disease (157, 158). In addition, in comparison with other complement components, C4 exhibits substantial heterogeneity in gene copy number (GCN) variation among different individuals, gene size dichotomy with long and short genes, DNA sequence and protein polymorphism, biochemical and serological properties, and expression levels, all of which may contribute to C4's association with autoimmune conditions such as systemic lupus erythematous (SLE). Importantly, this layered system of diversity in innate immunity has not been fully appreciated in the past and is distinct to complement C4, suggesting critical and varied roles for this molecule in immune defense. This complex diversity will be discussed in depth in later sections of this review.

A retrospective chart review of medical histories for 32 patients with homozygous C4A deficiency, 87 patients with homozygous C4B deficiencies, and 120 patients without C4A or C4B deficiencies was performed in Helsinki University Hospital in Finland (159). Besides the increased prevalence of autoimmune disease such as SLE, it was found that patients with homozygous C4A deficiency had increased frequencies of lymphoma, celiac disease, and sarcoidosis. Moreover, patients with homozygous C4A or C4B deficiency tended to have a higher incidence of adverse drug reactions, especially to antimicrobials (159).

While complete or heterozygous genetic deficiency of C1 or both C4A and C4B are likely causal factors for SLE pathogenesis, the prevalence of these anomalies is extremely rare and not seen regularly in rheumatology or nephrology clinics. However, there are two common complement C4-associated risk factors that modulate susceptibility to and the clinical presentation of SLE. The first is the low copy numbers of total C4 genes or C4A genetic deficiency. The second is the generation of autoantibodies against neo-epitopes of complement proteins, which are readily observable in patients with lupus nephritis.

Interferon stimulated gene expression signatures in complement C4 isotype deficiency.: Type 1 interferon stimulated gene (ISG) expression signatures in the absence of infection have become recognized as a hallmark of many autoimmune diseases (160, 161). However, they are not conspicuous among patients with C4 (C4A) deficiency (or

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mice with C3 deficiency). In a study of SLE, ten patients with a C4 protein deficiency but normal C3 did not present with interferon α-induced gene expression signature (118). In this study, it was reasonably argued that patients with SLE who had persistently low C4 but normal C3 were likely to have a genetic deficiency (or low GCN) of C4, although this was not proven by a parallel genetic analysis. This observation was consistent with another study on patients with juvenile dermatomyositis (JDM), in which gene expression analyses using RNA microarray and real time PCR experiments of cDNA from peripheral blood transcripts revealed that patients who exhibited strong type I interferon-induced gene expression signatures had neither complement C4A genetic deficiency nor HLA-DR3 (Figure 6) (77).

One possible explanation for these findings is that lupus secondary to genetic C4 deficiency leads to activation of a distinct pathway not related to type I IFN signaling. In a murine model of lupus, mice exhibited strong type 1 interferon gene expression signature except when the mice had deficiencies of IgM, C3, or CD18. CD18 is the common chain for complement receptors CR3 and CR4. Thus, a pathway leading to elevated expression of interferon α or the presence of type 1 interferon induced gene expression signature in autoimmune rheumatic disease appears to require the presence of C4 (or C4A) in human, or C3, complement receptors, and natural IgM in mice (118). In the absence of C4, this pathway is less active and alternative mechanisms to promote inflammation and autoimmune disease must therefore be at the forefront of disease pathogenesis.

B. Gene copy number (GCN) variation of total C4 and C4A in SLE among different racial groups

a. C4 and C4A gene copy number among patients with SLE of European ancestry.—

The first comprehensive study to investigate the complement C4 genetic diversities in SLE of European decent and race-matched controls was published in 2007 (153). The investigators employed (a) regular genomic Southern blot analyses of *Taq*I and *Psh*AI-*Pvu*II digested DNA resolved by regular agarose gel electrophoresis to define the relative dosage of C4A and C4B genes, (b) long-range mapping by *Pme*I digested genomic DNA fragments resolved by pulsed field gel electrophoresis, and (c) C4A and C4B protein phenotyping by immunofixation to define protein polymorphisms (144, 145, 153). The primary study population included 1241 European Americans with 233 SLE patients and 356 first degree relatives, plus 517 unrelated healthy controls (Figure 7, panel A; Table 2). In comparison to healthy controls, the distribution of total *C4* and *C4A*, but not *C4B*, GCN was left-shifted in SLE patients. The risk associated with low C4 GCN was particularly evident among female SLE patients: 9.3% had only two copies of total *C4* genes, and 6.5% had a homozygous deficiency of *C4A*, compared to 1.5% and 1.3%, respectively, in healthy controls. The risk of SLE (odds ratio, OR) for a subject with only two copies of total *C4* genes in a diploid genome was 6.5 times greater (OR=6.5) than for those with three or more copies of *C4* genes. As for subjects with homozygous deficiency of *C4A* genes, the risk of SLE was nearly 6 times greater than those with one or more copies of *C4A* genes (OR=5.7) (153).

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Parallel increases in the frequency of heterozygous C4A deficiency (GCN=1), and moderately low copy number of total C4 (GCN=3) were also observed amongst patients with SLE, but these yielded smaller effect sizes or impact on disease risk (153). On the other hand, human subjects with high copy-numbers of total C4 or C4A genes were protected against SLE disease risk. The frequency of subjects with C4T≥5 was 6.0% in patients with SLE but 12.0% in controls (OR=0.47). Similarly, 15.3% of subjects with SLE had C4A≥3 in comparison with 23.8% of controls (OR=0.57). In the same study, family-based association tests further revealed that monomodular-short (mono-S) RCCX haplotypes with single C4B gene and the absence of C4A (i.e., deficiency) were a risk factor for SLE (Figure 7, panel B and red arrow). The mono-S haplotype with C4A deficiency is in strong linkage disequilibrium with HLA-DRB1*03:01 (also known as HLA-DR3) among Caucasian subjects (153).

The natural conclusion from these results is that low gene copy number of total C4 or C4A is a risk factor for, and high copy-number of total C4 or C4A is a protective factor against, SLE disease susceptibility in European-Americans (153). A replication study including 128 independent patients of Caucasian descent with SLE yielded similar results. It must be acknowledged, however, that linkage disequilibrium with HLA-DR3 confounds a causal analysis.

Nevertheless, additional studies have yielded similar results. TaqMan-based real time PCR was employed to interrogate copy number variation (CNV) of C4A and C4B in 169 patients with SLE and 520 healthy controls in Kiel, Germany (162, 163). C4T GCN was calculated by summing GCNs of C4A and C4B. Low C4 GCN (C4T=2) was more common in subjects with SLE compared with controls: 6.9% versus 1.9%, respectively (OR=3.7, $p=6.8\times10^{-3}$). In addition, 4.3% of subjects with SLE demonstrated homozygous C4A deficiency (C4A=0), compared with only 0.8% of controls (OR=5.33, $p=7.7\times10^{-3}$). Intragroup analyses of German patients with SLE revealed that patients with homozygous C4A deficiency experienced a more severe disease course based on their requirement of cyclophosphamide therapy (OR=5.8, p=0.034). Patients with homozygous C4A deficiency were also diagnosed with SLE about ten years earlier than those without C4A deficiency (24 versus 34 years of age).

b. Low copy number of C4 and C4A deficiency in East Asians.—SLE affects all racial groups, but patients of East Asian and African ancestries and those of Hispanic ethnicity have more severe disease including a higher prevalence of renal complications (164). Although risk factors for SLE identified in European populations, such as C4A deficiency, are germane to other racial groups (165), there are substantial differences between races with respect to the frequency and effect sizes of risk factors. In a study including 999 patients with SLE and 1,347 healthy subjects of East Asian ancestry from Taiwan, Hong Kong, and the US, gene copy number variations of total C4, C4A, and C4B and their polymorphisms were determined by either TaqMan-based real time PCR (163) or by genomic Southern blot analyses and protein phenotyping (132, 144). In this study population, high GCN of total C4 (GCN≥5) and C4A (GCN≥3) were protective factors against SLE, whereas medium and low copy numbers of total C4 and C4A were risk factors for SLE (Table 2).

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Homozygous deficiency of C4A is infrequent among East Asian subjects but has a remarkable odds ratio of 12.4 for SLE disease susceptibility (p= 0.0015). The haplotype including HLA-DR3 (or DRB1*03:01) and mono-S C4 (with one copy of C4B and total C4A deficiency) is basically absent among East Asians. Instead, East Asian subjects with C4A deficiency usually present with bimodular LS (long-short) RCCX with *two* C4B genes (i.e., C4B-C4B, but no C4A), a haplotype that is linked to HLA DRB1*15:01 (or DR2), which is common amongst Chinese patients with SLE (132).

Comparisons of C4A GCN variations between European and East Asian healthy subjects and between patients with SLE revealed highly significant differences in the distributions of gene copy number groups for C4A (Figure 7, panel B). Notably, European subjects were more likely to have low copy numbers of C4A, which was especially evident when considering only subjects with SLE (Figure 7, right side of panel C). It is notable that mono-modular short-RCCX with a single short C4B gene (i.e., S or mono-S) is quite prevalent among White subjects but infrequent among East Asian subjects (red arrow in Figure 7, panel B). Other differences are seen, as well. For example, the most common haplotype for White subjects is bimodular LL with a frequency of 51% and for East Asian subjects is bimodular LS with a frequency of 39%.

In two independent studies of East Asian SLE in Han-Chinese and South Koreans, C4A and C4B gene copy numbers were also interrogated by TaqMan-based real time qPCR methods (163, 166, 167). Significantly lower GCN of C4A and total C4 were observed in SLE compared with race-matched controls. Again, a notable feature in those two East Asian populations was the low frequency of homozygous C4A deficiency in both SLE (≤1%) and healthy controls.

c. Low GCNs of complement C4 in SLE of Hispanic populations.—A slightly different version of TaqMan real time PCR methodology was used to determine C4 GCN for 427 patients with SLE and 301 matched healthy controls from São Paulo, Brazil (168). Subjects' ethnicity was not described, but Brazil is known to be a melting pot for European, African, indigenous, and Asian ethnicities. Mean copy numbers for total C4 (C4T) and C4A were statistically lower in subjects with SLE compared with controls. Low C4T GCN (C4T<4) and C4A deficiency (C4A GCN<2) more common in subjects with SLE compared to controls with odds ratios of 2.62 and 3.59, respectively. Homozygous deficiency of C4A was more common in subjects with SLE: 2.1% compared with 0.3% of controls (OR=6.49; p=0.053). It was noted that low gene copy number of C4B also appeared to be a moderate risk factor for SLE among the Brazilian subjects, which was not found in studies of either Europeans/European Americans or East Asians/Asian Americans outlined above. Furthermore, low GCN of C4A was associated with higher permanent disease damage indices and higher frequencies of serositis in subjects with SLE. Similarly, patients with lower GCN of C4B had a higher frequency of arthritis. An earlier publication from the same team found that juvenile onset patients with SLE had lower GCN of total C4, C4A, and C4B than adult-onset patients (169).

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C. APPLE clinical trial investigating complement in SLE

Leveraging biorepository samples and data from the clinical trial APPLE (atherosclerosis prevention in pediatric lupus erythematosus), Mulvihill and colleagues determined CNV of C4 genes in 200 children with SLE (cSLE) (170, 171). This three-year trial recruited patients between 10 and 21 years old for randomized atorvastatin or placebo-controlled therapy. Patients with ≥2 copies of C4B genes had 2.5 times the odds of having hypertension and higher diastolic blood pressure (Figure 8, panel A). C4B GCN≥2 was also associated with headaches and pericarditis, though potential mechanisms relating complement with these problems were not elucidated. Patients with ≥2 copies of C4B genes also appeared to respond more effectively to atorvastatin treatment with reduced progression of atherosclerosis as shown by slower increase in carotid intima media thickness, a surrogate marker of atherosclerosis, when compared with those treated with placebo (170).

In the APPLE trial, serum complement protein levels also varied with the severity of disease sequelae. Longitudinal studies of serum complement protein levels in SLE patients have revealed three distinct serum complement profiles, as discussed previously (72, 73, 172, 173). As serum complement protein levels fluctuate, so do levels of complement degradation products like C4d. C4d can be found in the fluid phase or attached to membranes of red blood cells (RBC-C4d) and other cell types in the circulation (76, 78, 174). The deposition of C4d on cell surfaces can be monitored by flow cytometry using antibodies against C4d. It is suggested that levels of RBC-C4d reflect complement activation in the past 60 days, the levels of reticulocyte-C4d the past 2–3 days, and platelet-C4d the past 5 to 10 days. In the APPLE trial, patients with hypertension were found to have significantly higher serum levels of complement C3 and C4 than normotensive patients at the baseline and throughout the trial study period (Figure 8, panels B and C). The relatively higher complement C4 protein levels among patients with hypertension, as well as amongst patients with antiphospholipid (aPL) antibodies and thrombosis or recurrent pregnancy loss (RPL) (175) reveal the pro-inflammatory aspect of complement. Normal range or elevated levels of complement in these patients likely aggravated complement-mediated tissue damage.

D. Opposite profiles of complement in SLE and antiphospholipid syndrome

Antiphospholipid syndrome (APS) is defined by the persistence of aPL on two laboratory tests separated by 6–8 weeks and clinically by thrombosis or recurrent pregnancy loss (176, 177). Antiphospholipid antibodies are heterogeneous autoantibodies whose targets include β_2 glycoprotein I (β_2GPI), prothrombin, and phospholipids found in the cell membrane, including cardiolipin. Some of these antibodies can interfere with clotting *in vitro* assays and therefore have been dubbed lupus anticoagulants, though *in vivo* they predispose patients towards *thrombosis* rather than bleeding events. β_2GPI is an enigmatic plasma protein that consists of five short consensus repeats (SCR), which are characteristic of complement control proteins (178–180). Similar to ANA, aPL antibodies form immune complexes and fix complement (181, 182). Anti-phospholipid antibodies are detectable in ~30% of SLE patients, and 10–15% of SLE patients are diagnosed with APS (183–186).

Concurrence of thrombosis and SLE imposes a very high risk of recurrent pregnancy loss; 40% of aPL-positive patients with recurrent pregnancy loss had thrombotic SLE in

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one study (175). Remarkably, all aPL antibody-positive female patients with homozygous C4A deficiency experienced recurrent pregnancy loss. In contrast, female patients with homozygous C4B deficiency were protected from recurrent pregnancy loss (187). This phenomenon underscores the importance of C4A in the protection against the onset of autoimmune disease and the engagement of C4B in complement-mediated tissue injury. Similarly, genetic ablation of C1q, C4, C3, or C5 in a rodent model protected against aPL antibody-induced clinical symptoms such as fetal resorption (181, 188–193). While complement activation is important in the pathology of both APS and SLE, the genetic and immunological profiles of complement in these related diseases are very different (187). For example, the thrombo-inflammatory process in APS seems to drive not only complement activation and consumption (as is seen in SLE), but also higher production of component proteins, particularly C4 (187, 194).

In a cross-sectional study of 525 human subjects with aPL (187), 45% developed thrombosis, 56% had SLE, and 24% of female patients experienced recurrent pregnancy loss (Figure 8, panel D). Meticulous analyses of complement proteins among these aPL antibody-positive patients revealed significantly higher levels of C4 and C3 proteins among patients with thrombosis only, and lower levels of these proteins among patients with SLE only (Figure 8, panels E and F). Amongst all aPL antibody-positive patients, those with concurrent SLE but not thrombosis had significantly higher factor H and functional MBL levels (187, 195, 196). Patients with APS also demonstrated elevated levels of complement activation products such as the anaphylatoxins C5a and C3a and the MAC (181, 195). aPL antibody-positive patients with both SLE and thrombosis and subjects without either disease had intermediate C4 and C3 protein levels.

E. Autoantibodies to complement in SLE

There are many autoantibodies to complement proteins in SLE (197), which are mostly directed against neo-epitopes exposed after activation or inactivation of complement proteins or against multi-molecular complexes formed during the activation process. The binding of autoantibodies to complement can lead to a state of acquired complement deficiency and contribute to disease pathogenesis.

a. Autoantibodies to C1q—About 30% of SLE patients synthesize autoantibodies to C1q. The presence of C1q autoantibodies correlates with anti-dsDNA, nephritis, and low complement levels in about 75% of such patients (60, 198, 199). The development of these autoantibodies may be a secondary phenomenon that occurs when immune complexes (IC) bind C1q and activate the classical pathway (CP). Following CP activation, the C1 inhibitor strips the C1r and C1s proteases from the C1 complex, which is bound to Ab. C1q remains attached to the IC at the site of inflammation. Local proteases will next degrade IgG, C1q, and the autoantigen and generate multiple proteolytic fragments and neoantigens, to which novel autoantibodies may be generated. A large study was undertaken to assess the specificity of anti-C1q antibodies and their potential association with SLE manifestations and diagnostic tests (200). The authors confirmed an association between anti-C1q antibodies, low complement (C4 and C3), and anti-dsDNA antibodies. This combination also had the strongest serological association with the clinical development

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of renal disease. Anti-C1q antibodies were seen in 28% of all SLE patients but 68% of patients with renal disease. By contrast, the absence of anti-C1q antibodies makes a nephritis flare less likely (199).

b. Autoantibodies to C3 or C4

Immunoconglutinins were among the earliest complement-directed autoantibodies discovered (201). Immunoconglutinins are autoantibodies against solid-bound or processed fragments of C3. The IgG isotype of immunoconglutinins is strongly correlated with SLE disease exacerbation (202), increased anti-C1q IgG level and anti-dsDNA, and lower levels of serum C3 and/or C4 in ~30% patients with lupus nephritis (203, 204). Binding of such autoantibodies to C3b interfere with the interaction between C3b and regulatory molecules such as factor H or CR1 and thus exacerbates the activation and consumption of serum C3. Increased C3b autoantibodies have been observed to be more specific but less sensitive than anti-C1q for lupus nephritis flares (205).

C3 and C4 nephritic factors bind to neoepitopes present in the AP C3 convertase (C3bBb or C3bBbP) and the CP C3 convertase (C4b2a), respectively. They prolong the half-life of the convertases from minutes to hours and enhance the consumption of serum C3 and variably C5 as well. Both C3 and C4 nephritic factors are associated with membranoproliferative glomerulonephritis (206, 207). C3 nephritic factors are associated with acquired partial lipodystrophy (206, 207). C4 nephritic factors are associated with SLE and post-infectious acute glomerulonephritis (208, 209).

V. COMPLEMENT IN THE IDIOPATHIC INFLAMMATORY MYOPATHIES

IIM are a group of autoimmune diseases characterized by myositis-related autoantibodies, infiltration of leukocytes into muscles and/or the skin, and destruction of blood vessels and muscle fibers, which together cause chronic weakness and fatigue. While complement-mediated destruction of capillary endothelia is implicated in pediatric and adult dermatomyositis, the complex role of C4 in IIM pathology is largely unknown.

A. C4 genetic diversity and IIM

C4 GCN were quantified for 1,644 Caucasian patients with IIM plus 3,526 matched healthy controls using real-time PCR or Southern blot analyses, as summarized in Figure 9 (79). C4T GCN (C4T<4) and C4A deficiency were correlated with increased risk of IIM with odds ratios of 2.58 (2.28–2.91, $p=5.0\times10^{-53}$) for C4T; and 2.82 (2.48–3.21, $p=7.0\times10^{-57}$) for C4A. Contingency analyses showed that amongst patients with C4A deficiency, the presence of HLA-DRB1*03 (DR3) became insignificant as a risk factor in IIM except for inclusion body myositis (IBM), in which 98.2% of patients had HLA-DR3 with an OR of 11.02 (1.44–84.4). This study revealed that C4A deficiency is a relevant risk factor in both juvenile-onset and adult-onset dermatomyositis, that both C4A deficiency and HLA-DRB1*03 contribute interactively to the risk of polymyositis, and that HLA-DRB1*03 is singularly important in IBM (79).

Further analyses revealed that low GCNs of total C4, i.e., C4T=2 [OR=2.26 (1.74–2.95)] and C4T=2+3 [OR=2.62 (2.32–2.95)], had similar magnitude of effects on the genetic risk

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of IIM. In the case of C4A deficiency, 4.2% and 38.6% of subjects with IIM had 0 and 1 copies of C4A, respectively, with odds ratios of 2.49 (1.76–3.54; $p=3.6\times10^{-7}$) for *C4A*=0 and 2.82 (2.48–3.21; $p=2.9\times10^{-57}$) for *C4A*=0+1. Of note, this finding is analogous to an autosomal dominant phenotype in Mendelian genetics and stands in stark contrast with results in SLE, in which the risk associated with C4A deficiency exhibits gene dosage effects (OR= 5.27 for C4A=0, OR=1.61 for C4A=1).

B. Serum complement levels and their activation products in IIM

Phenotypic studies support a pathological relationship between circulating complement protein levels and IIM. Our work has shown that in addition to differences in circulating complement protein levels between healthy subjects and patients with IIM, there are also differences between subgroups of IIM patients, especially when comparing those with or without specific myositis-specific (MSA) and myositis-associated (MAA) antibodies, as shown in Figure 10 (210). These data suggest that MSA and MAA may modulate complement protein levels and activity, possibly through formation of immune complexes that in turn activate and consume complement.

Additional work has supported the relationship between autoimmune myopathy and complement, including JDM. Our group took advantage of complement biology in which complement C4 byproducts, such as C4d, are deposited on cell surfaces (including on erythrocytes) upon activation and cleavage of C4. This deposition of the byproduct C4d (erythrocyte-C4d or E-C4d) creates record of complement activation (76, 174). Figure 11 shows mean fluorescence intensity, a measure of antigen density on target cells, of E-C4d in patients with JDM versus controls (left upper panel) and compares E-C4d levels based on C4A and C4B deficiency (bottom panels). E-C3d levels are also shown, though patients with JDM do not demonstrate any appreciable differences in C3d deposition than controls. The results demonstrate that E-C4d levels are higher in JDM compared with controls, likely reflecting chronic complement activation and inflammation. However, higher E-C4d levels were also seen in patients with C4A deficiency and in patients with higher GCN of C4B. This remarkable relationship suggests that activated C4B may be the primary isotype of C4 involved in complement activation and deposition on red blood cells, which can lead to complement-mediated cellular or tissue destruction. Alternatively, C4A may be less efficient at or protect against this process. In either case, these findings offer a hint of the mechanisms connecting complement genetics and autoimmune diseases such as JDM (77–79).

VI. COMPLEMENT IN OTHER AUTOIMMUNE DISEASES

A. Rheumatoid arthritis

Autoimmune arthritis is one of the most common rheumatological disorders and includes diseases such as rheumatoid arthritis (RA) in adults and juvenile idiopathic arthritis (JIA) in children. Autoimmune arthritides are thought to be largely T cell-mediated diseases, but complement has been implicated in both the initiation and ongoing pathogenesis of RA (211–216), psoriatic arthritis (214, 217), and JIA (218–220).

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A study published very recently by Banda *et al* examined the role of complement in early RA. This work showed that gene expression of complement components and receptors such as *C2*, *FCN1*, *FCN3*, *CFB*, *CFP*, *C3AR1*, *C5AR1*, and *CR1* positively correlated with disease activity as measured by the DAS28-ESR (disease activity score in 28 joints for erythrocyte sedimentation rate) (215). Conversely, expression of other complement genes such as *Colec12*, *C5*, *C6*, *MASP-1*, *CFH*, and *MCP* was inversely correlated with disease activity. Synovial samples underwent immunohistochemical staining that showed local alterations in complement and complement-activating and inhibitory proteins. The authors interpreted these results as evidence that an imbalance between complement activation and regulatory activity contributes to early pathogenesis in RA.

Most studies have examined serum complement levels, not genetic diversity in RA. A notable exception is a study in which C4 gene copy number was elucidated for 160 patients with RA and both healthy control subjects (n=51) and patients with rheumatological diseases besides RA (221). Unlike SLE, there was no increased risk of RA associated with C4A deficiency; in fact, C4B deficiency was most strongly associated with RA, especially in patients with autoantibodies such as rheumatoid factor (RF) or anti-citrullinated protein antibodies (ACPA).

Other groups have examined how single nucleotide polymorphisms (SNP) such as those identified in genome wide association studies impact disease activity and complement activation in RA (222–226). One group specifically interrogated the SNP rs17611 in complement C5 and found a dose-dependent effect on circulating C5 levels and activation. Variable susceptibility to cleavage by proteases such as elastase was hypothesized to underlie differences in C5 activation and disease activity observed in association with the SNP (227).This association with C5 is perplexing, as C5a receptor blockade does *not* reduce markers of joint inflammation in RA (228) and especially because C5 knock out mice have been shown to be resistant to collagen-induced antibody-mediated arthritis (229). Another group found that the SNP rs292001 in C1q was associated with reduced circulating C1q levels, though no functional assays were performed (225). In a study of Brazilian patients with RA and their healthy family members, alleles associated with lower production and relative deficiency of mannose-binding lectin (MBL) were associated with increased risk of disease (230), though other studies have not corroborated that finding and have instead showed that MBL deficiency is associated with increased disease severity in RA (231, 232). Mechanistic studies to understand the underlying pathophysiology connecting SNP of MBL with disease in RA are notably lacking.

B. Juvenile idiopathic arthritis

Juvenile idiopathic arthritis (JIA) is one of the most common pediatric autoimmune disorders and therefore is of high clinical significance. Much like our work in lupus and JDM, our group has investigated the relationship between complement C4 GCN and disease in patients with JIA versus controls (233). We interrogated total C4, C4A, C4B, C4L, and C4S gene copy number in 103 patients with oligoarticular JIA and over 3,500 race-matched controls. In contrast to the phenomenon observed in adult RA, described above, we did not detect significant differences in *C4B* GCN in patients with JIA (221).

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While there were no differences in the patterns of total C4, C4B, or long C4 genes, we observed significantly different distributions of C4A and C4S GCN. More than two-thirds of patients with oligoarticular JIA had two copies of C4A (68.9%), compared to 51.3% in controls; nearly half (46.6%) of patients with oligoarticular JIA had zero copies of short C4 genes (controls: 32.3%). Further clinical studies are necessary to understand the physiologic implications of such variations and whether GCN variations in oligoarticular JIA are secondary to HLA-class II gene polymorphisms (221).

C. Type I diabetes mellitus

Type I diabetes mellitus (T1DM) is an autoimmune disease of the young in which the immune system attacks and destroys β islet cells in the pancreas that are responsible for producing insulin. While the direct damage to the pancreas is thought to be mediated by cytotoxic T cells, increased deposition of complement C4 has been observed in T1DM (234, 235). Genetic analyses have implicated various haplotypes of the major histocompatibility complex (MHC) on chromosome 6 as risk factors for T1DM (236–238), but several complement genes (including *C2*, *C4A*, and *C4B*) are also located nearby between the human leukocyte antigen (HLA) class I and class II regions. Due to extensive linkage dysequilibrium, it remains relevant to critically analyze which genetic variants confer the greatest risk and if multiple variants contribute to increased risk or disease progression from onset to complications of T1D.

As a part of The Environmental Determinants of Diabetes in the Young (TEDDY) study, 15 known SNPs within complement genes were studied for possible associations with the development of autoantibodies associated with T1DM, including those against insulin, GAD65, or IA2 and/or association with the onset of clinical T1DM disease (239). Only one SNP, rs2230199, remained significant after correction; this variant was strongly associated with the development of T1DM in patients who carried two copies of HLA-DRB*04:01 (DR4/4), with a hazard ratio of 3.2 (239). While these results offer somewhat underwhelming evidence of complement's role in T1DM, it is important to note that the group did not study gene copy number variation, which others have shown to correlate with T1DM risk or progression to full-blown disease after partial remission (235). Specifically, Kingery *et al* showed that higher C4A GCN and plasma levels of C4A protein were associated with greater preservation of insulin production (as measured by C peptide levels) 1 month after diagnosis (235). Conversely, lower C4B GCN and protein concentration were protective at 9 months after diagnosis. This apprarent dichotomy between C4A and C4B is consistent with effects we have observed in other autoimmune diseases, such as JDM, pediatric lupus (170), and recurrent pregnancy loss in antiphospholipid syndrome (187).

VII. CONCLUDING REMARKS

Although extremely rare in prevalence, complete deficiencies of C1q, r, or s and C4 have provided strong evidence for the role of early components of the classical complement activation pathway in the pathogenesis of systemic autoimmune disease such as SLE. Acquired deficiency of C1q by autoantibodies and genetic deficiency of the C4A isotype secondary to gene copy number variation of C4 genes are frequent complement

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abnormalities that can be present in 30–50% of SLE patients, particularly those of European ancestry. While likely often missed clinically, complement deficiency should be included in the differential diagnosis of patients presenting with recurrent infections and/or signs of SLE-like autoimmune disease, especially when similar problems exist in other family members and when patients present with symptoms at a young age.

We are gaining an increasing understanding of complement genetics and the role of complement polymorphisms and deficiency in various diseases. While some complement components are essential and lead to high penetrance immune disorders, the effects of deficiencies of other components are more subtle. Complement biology continues to teach us much about how the immune system functions and offers an avenue to further explore the interface of antimicrobial immunity, autoimmunity, and immunodeficiency.

This review has summarized what is known regarding complement C1q, r, and s; C2; C3; and C4 deficiencies and their role in autoimmune disease. C1 and C4 deficiencies in particular present with severe infections and highly penetrant autoimmune disorders. In contrast, disease severity in C2 deficiency is generally lower, and C3 deficiency may present without autoimmune symptoms at all. The body of evidence strongly suggests, however, that complement is involved in the pathogenesis of various autoimmune conditions.

Complement C4 in particular is genetically extraordinary with inter-individual gene copy number variations, gene size dichotomy, and polymorphic protein isotypes with distinct biochemical properties. It is that diversity that allows for much of the flexibility and efficacy of the classical complement pathway. Associations with complement components including C4 may vary between disorders, however, which to us suggests a nuanced model of complement's role in autoimmunity: one in which the *balance* of various complement components and other immune factors determines disease phenotype. For example, our work has shown that there is a clear C4A gene dosage effect on the risk of disease susceptibility in SLE and other conditions such as inflammatory myositis. In contrast, C4B shows no such strong associations for most diseases. An exception to this rule is rheumatoid arthritis, in which low C4B GCN increases the odds of disease. Low gene copy number or deficiency of C4A remains a common risk factor for SLE across different racial groups despite divergent HLA haplotypes. In SLE and patients with antiphospholipid antibodies, high copy numbers of C4B are associated with greater incidence of cardiovascular complications including a history of hypertension, higher incidence of thrombosis, and recurrent pregnancy loss in female patients. Additional evidence implicates complement C4A in autoimmune arthritis and type I diabetes mellitus, but the determination of whether associations suggest a protective or risk role for various complement components is incomplete.

Additional subtleties exist regarding the downstream effects of complement deficiencies in autoimmune disorders. Patients with SLE and myositis both present with type I interferon stimulated gene expression signatures in peripheral blood samples and local tissues. However, this phenomenon is less conspicuous among patients with C4A deficiency or with HLA-DR3 haplotypes. The mechanistic relevance of C4A deficiency and HLA-DR3 in type I interferon induced gene expression requires further inquiry, but these findings suggest that

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complement signaling may contribute to autoimmunity in a way that is distinct from type I interferon pathways.

Despite ongoing advancements in our understanding of complement biology, there remain many puzzles to solve. Examples include the relative contribution of various complement deficiencies or polymorphisms and HLA haplotype in various autoimmune disorders, the relevance of the long versus short C4 genes, as well as the mechanisms by which complement contributes to autoimmune phenotypes. Furthermore, technical and scientific challenges remain on the path to understanding the pathophysiology of complement in autoimmune disease. Recent advances in whole-genome or whole-exome next-generation sequencing offer alternative methodologies to impute copy number variations for some innate immunity genes. However, validation is essential in genes with copy number variation and can be cumbersome, time-consuming, and expensive.

Finally, recent utilization of bone marrow transplantation and hematopoietic stem cell transfer has enabled complementation with intact complement C1q in C1q-deficient patients. Additional prospects for future gene therapy offer encouraging and innovative approaches to treat subjects with complement deficiency. Naturally, there remain many hurdles to overcome before this idea can become a reality, including graft versus host disease and viral reactivation under immunosuppressive conditions. Nevertheless, our increasing understanding of complement biology and its implications in immunity and autoimmunity is opening the door to more rational therapeutic design and offers hope for patients with a variety of immune-mediated conditions, from immunodeficiency to autoimmune disease.

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Complement and Autoimmunity: Highlights

- While complement's role in host defense against infection is well known, this article will focus on the role that various complement components play in autoimmune diseases.
- The over-arching purpose of this review is to provide a comprehensive summary of the association of complement with various autoimmune diseases for the clinician and researcher alike. Importantly, putative mechanisms by which complement contributes to autoimmunity will be discussed.
- To facilitate clinicians' ability to address possible complement deficiencies, the clinical history and physical exam features seen in patients with complement deficiencies will be reviewed. In addition, a suggested laboratory work-up in cases of suspected complement deficiency will be provided.
- The complex role of complement in systemic lupus erythematous, including a point-by-point discussion of each complement component and known clinical cases of their deficiencies, will be reviewed. Particular attention will be paid to complement C1 and C4, which are most strongly associated with autoimmune disease.
- Complement C1q in particular is strongly associated with lupus-like autoimmune diseases. Treatment options for C1 deficiency will be discussed.
- Complement C4 is also strongly associated with systemic lupus erythematous, as well as other autoimmune disorders.
- C4 is a unique molecule. Not only does it boast multiple layers of genetic polymorphisms, but there also exists a wide range of gene copy number that contributes an additional dimension of complexity to C4's role in autoimmune disease. Our group has studied complement extensively, and what is known regarding the genetics of complement C4 in autoimmune disease is briefly summarized.
- Complement's role in autoimmune disease is best studied in systemic lupus erythematous, but this review will also briefly address what is known regarding the role of complement in other autoimmune disorders such as the idiopathic inflammatory myopathies, autoimmune arthritis, and type I diabetes mellitus.

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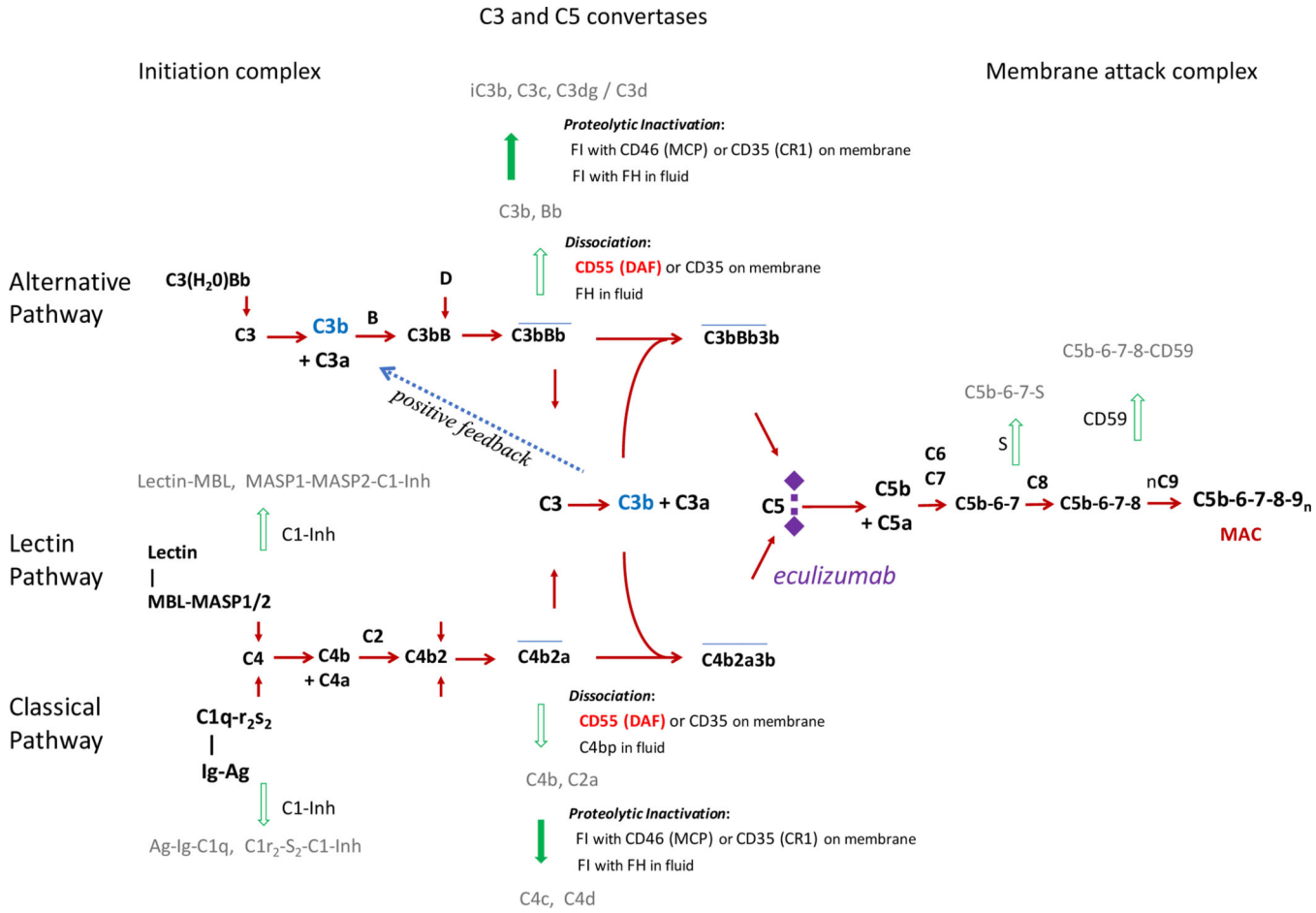

Figure 1. Activation and regulation of the human complement system.

There are 3 known arms of the complement cascade, including the classical, alternative, and lectin pathways (10). The ligands and sequence of activation are shown for each pathway. Note that all three pathways converge to cleave complement C3, which in turn allows for the generation of C5 convertases and formation of the membrane attack complex (MAC) composed of C5b, C6, C7, C8, and C9 multimers. This complex serves to perforate the outer membrane of invading microbes or aberrant cells necessitating clearance and can result in lysis or sub-lytic permeabilization of the target. Incorporated in the system are tight regulations that prevent or abort inadvertent activations on self or host cell membrane by dissociation of multimolecular complexes and proteolytic degradation of anchor proteins such as C3b and C4b. Eculizumab is a drug or biologic that blocks activation of complement C5. Activation of zymogens and progression of pathways are shown in red and regulatory steps in green. A positive feedback loop (shown in blue) leading to auto-amplification is common for all three activation pathways.

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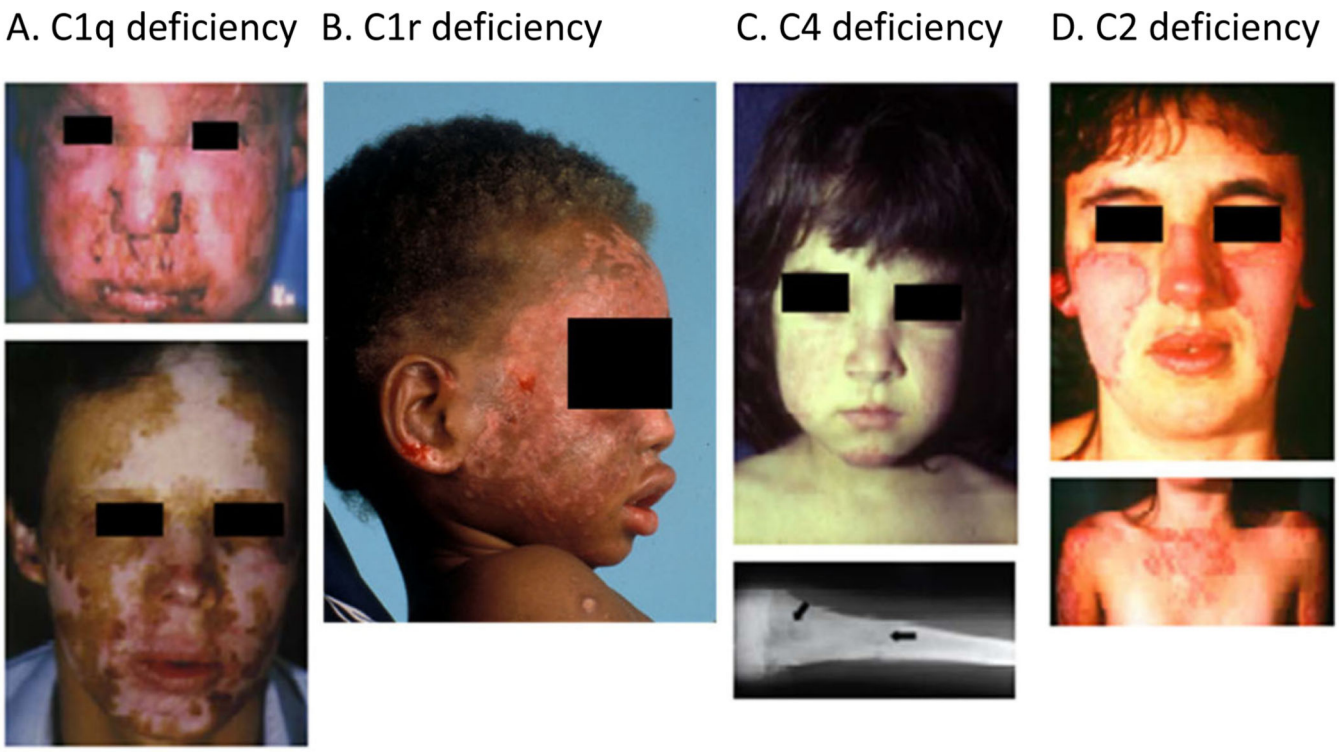

Figure 2. Systemic lupus erythematous and homozygous deficiency of early components of the classical pathway of complement activation.

Severe cutaneous lesions are common clinical presentations in SLE patients with a complete complement deficiency. (A) A homozygous C1q-deficient male child with cutaneous infection (upper panel), and with discoid lupus erythematosus and scarring lesions on face when he was 22 years old (lower panel). (B) A male child with discoid lupus at 16 months of age with homozygous C1r-deficiency. (C) Complete C4-deficiency in a girl at age 3 with a butterfly rash and cheilitis. (D) A homozygous C2-deficient young woman with acute cutaneous lupus erythematosus. The upper panel shows the butterfly rash, and the lower panel shows photosensitive lesions on sun exposed area. (Source of photographs: (116, 249).)

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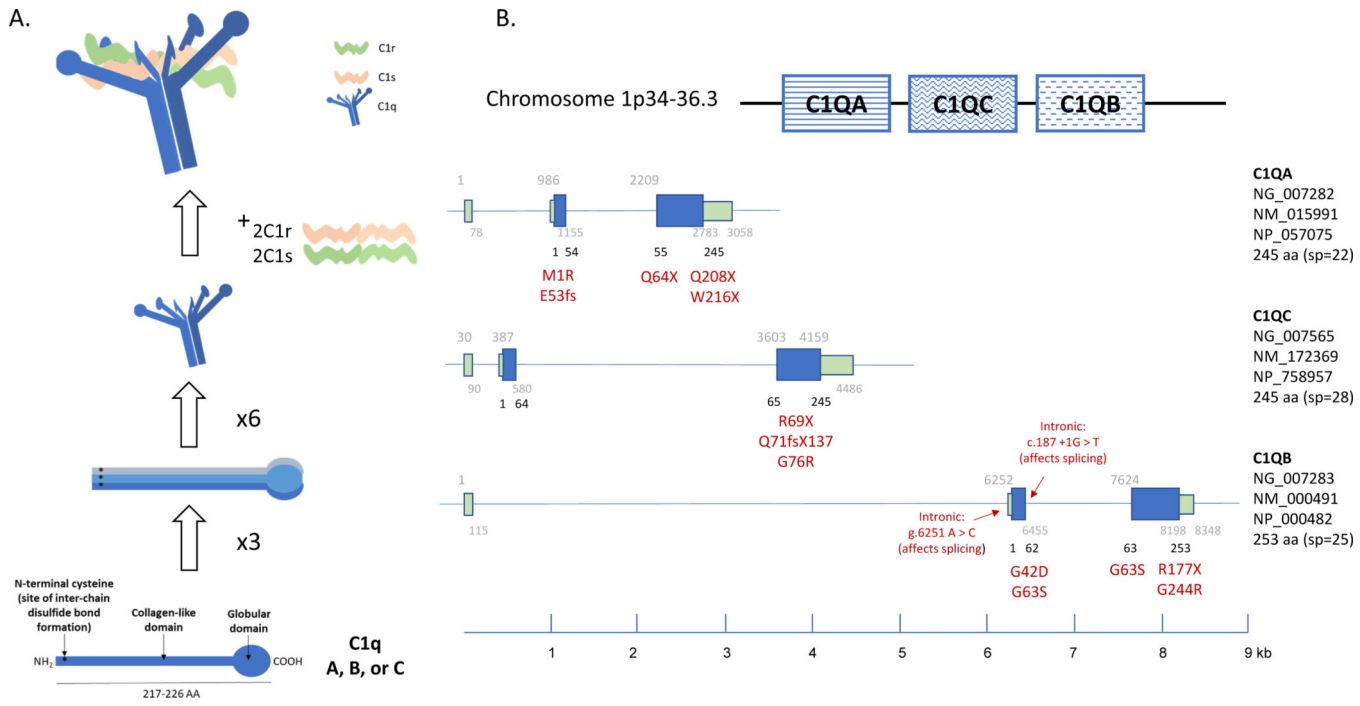

Figure 3. C1 complex: structure and genetics.

(A) The structure of hexameric C1q in a complex with 2 subunits each of C1r and C1s ($C1r_2s_2$) is shown in a cartoon. The structural domains of each C1q subunit are outlined in the bottom panel, including the globular and collagen-like regions. Each C1q subunit is composed of C1qA, C1qB, and C1qC. Six of these subunits then assemble into a bouquet conformation as depicted. Note the N terminal cysteine residues, which are critical for disulfide bond formation between C1qA, B, and C. (B) The configuration of C1qA, C1qB, and C1qC on chromosome 1 is shown in the top panel with each gene structure represented diagrammatically below. Known mutations and their relative location in the various introns and exons of C1qA, C1qB, and C1qC are listed (89–95). GenBank accession numbers for C1q genes, mRNA and proteins are shown.

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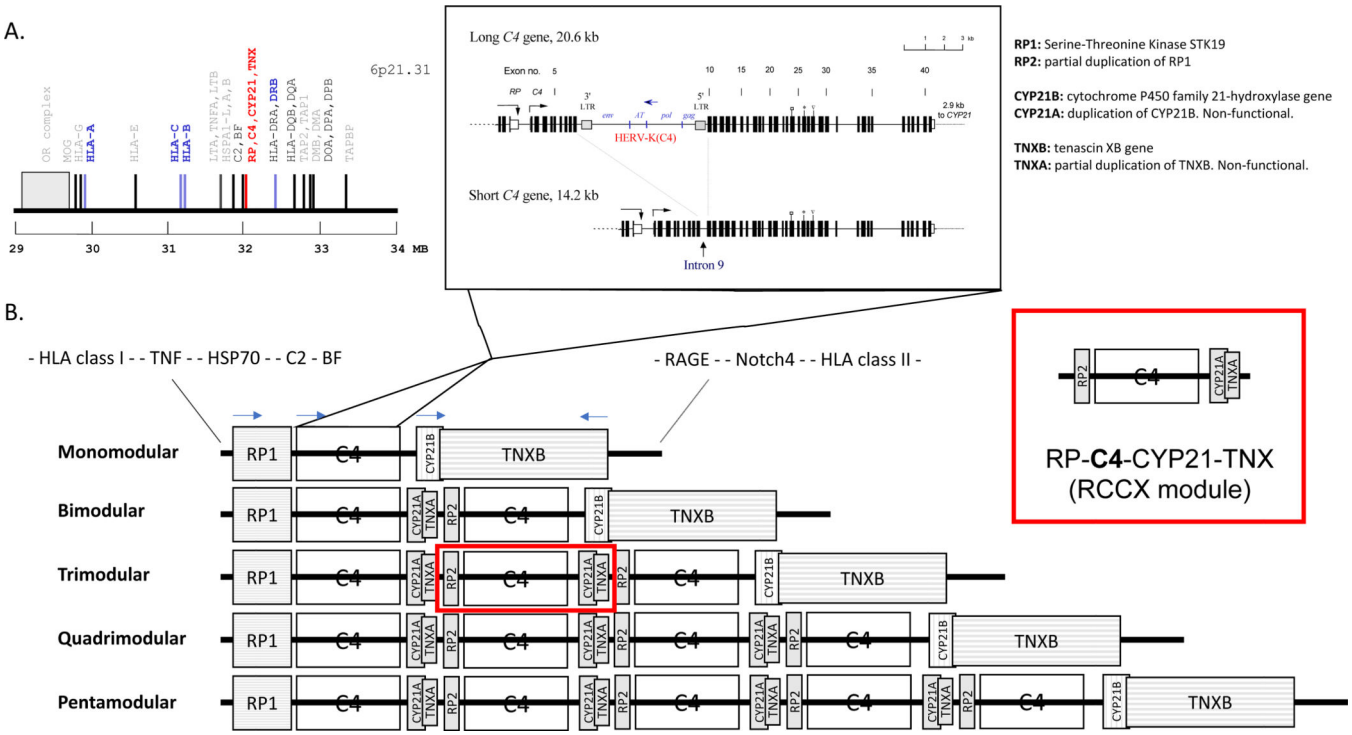

Figure 4. C4 genetics.

(A) Genetic locations for constituents of the C3 convertases for classical and alternative pathways. (B) Segmental duplications with one to five modules of the *RP-C4-CYP21-TNX* (RCCX) in haplotypes are located in the class III region of the HLA. Panel B inset: Dichotomy of human *C4* gene size with the long gene containing endogenous retrovirus HERV-K(C4) in the ninth intron and the short gene without the endogenous retrovirus. Note the otherwise complex gene structure, with 41 exons and intervening introns (133, 134, 137, 138, 250).

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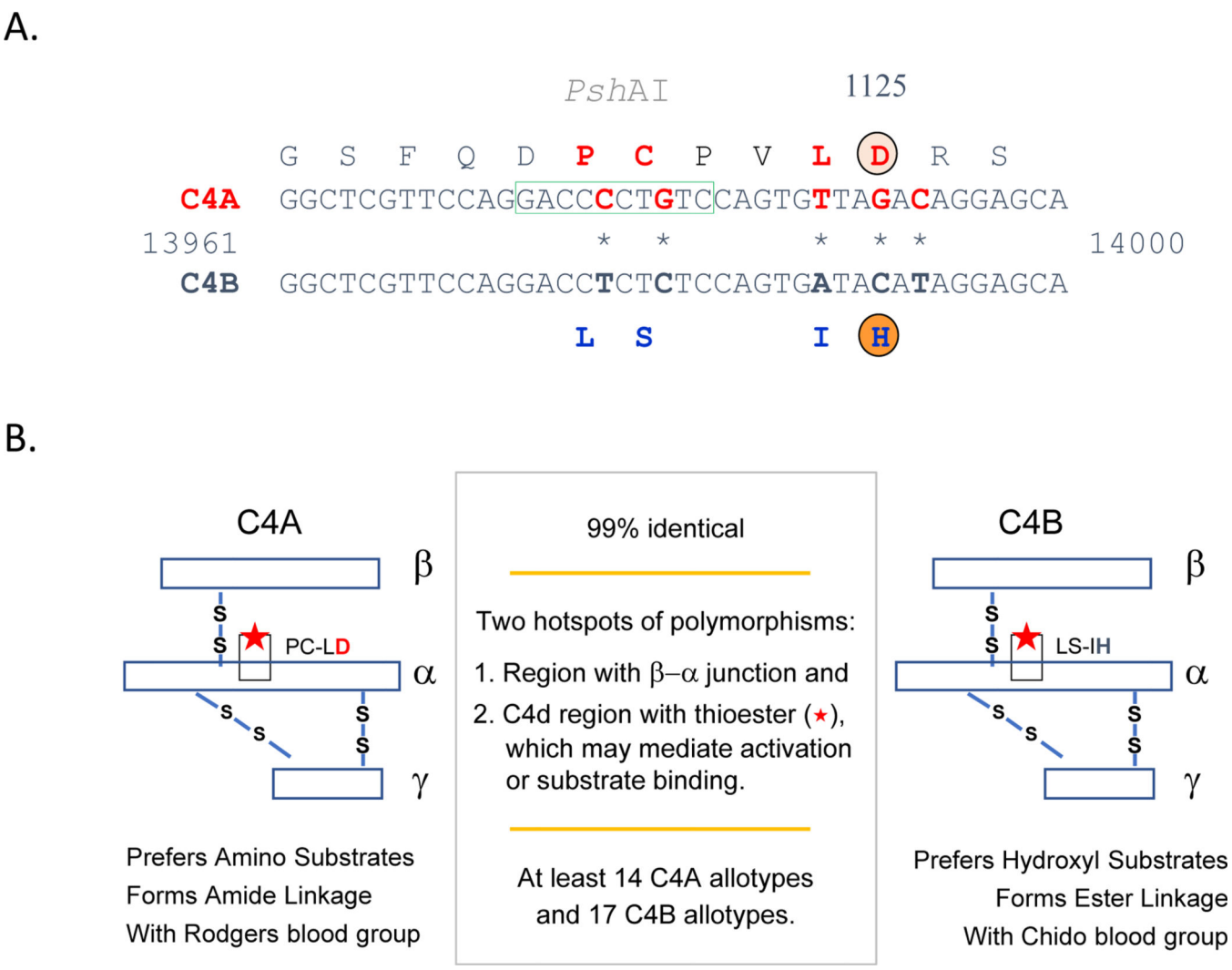

Figure 5. C4A and C4B genetic and functional differences.

(A) Gene sequence differences between C4A and C4B are shown with the corresponding amino acid differences noted above and below. (B) Graphical representation of C4A and C4B, including amino acid substitutions. Biochemical properties and bioreactivity for C4A and C4B are included (140, 143, 154–156). Note that despite high homology, there are dozens of different allotypes of C4A and C4B, allowing for incredible diversity.

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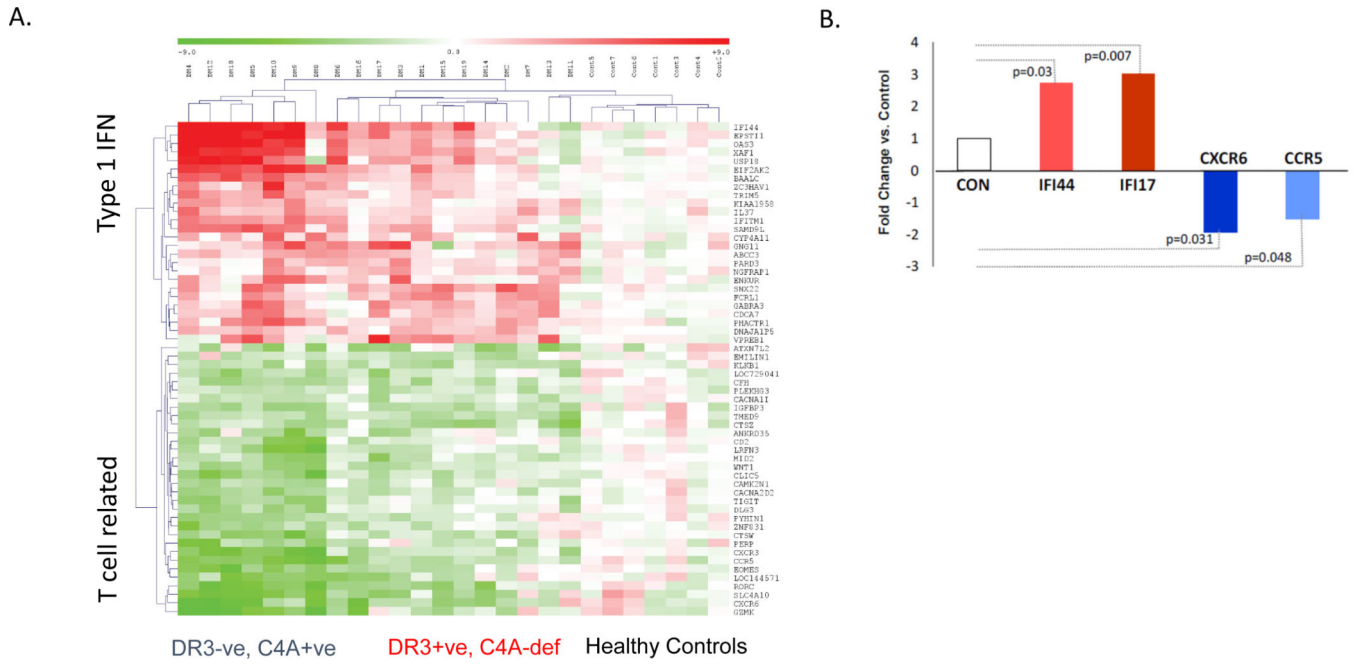

Figure 6. Interferon signature in juvenile dermatomyositis.

(A) A heat map showing a comparison of blood transcripts between patients with JDM and healthy subjects. Type 1 interferon stimulated gene expression was prominent among seven patients without HLA-DRB1*03 who were C4A proficient (DR3-ve, C4A+ve, left columns). T-cell related transcripts were markedly reduced (green), suggesting migration of T lymphocytes away from blood to tissues (possibly to muscles and skin). Those with HLA-DRB1*03 and C4A deficiency (DR3+ve, C4A-def) demonstrated moderate IFN-stimulated gene expression and the reduction of T cell specific transcripts was also less remarkable (middle columns). Healthy control results are shown in the right most columns. (B) These results were further confirmed by SYBR-green qPCR assays for IFN-stimulated gene expression (red) and reduced expression of T-cell related genes (blue) (77).

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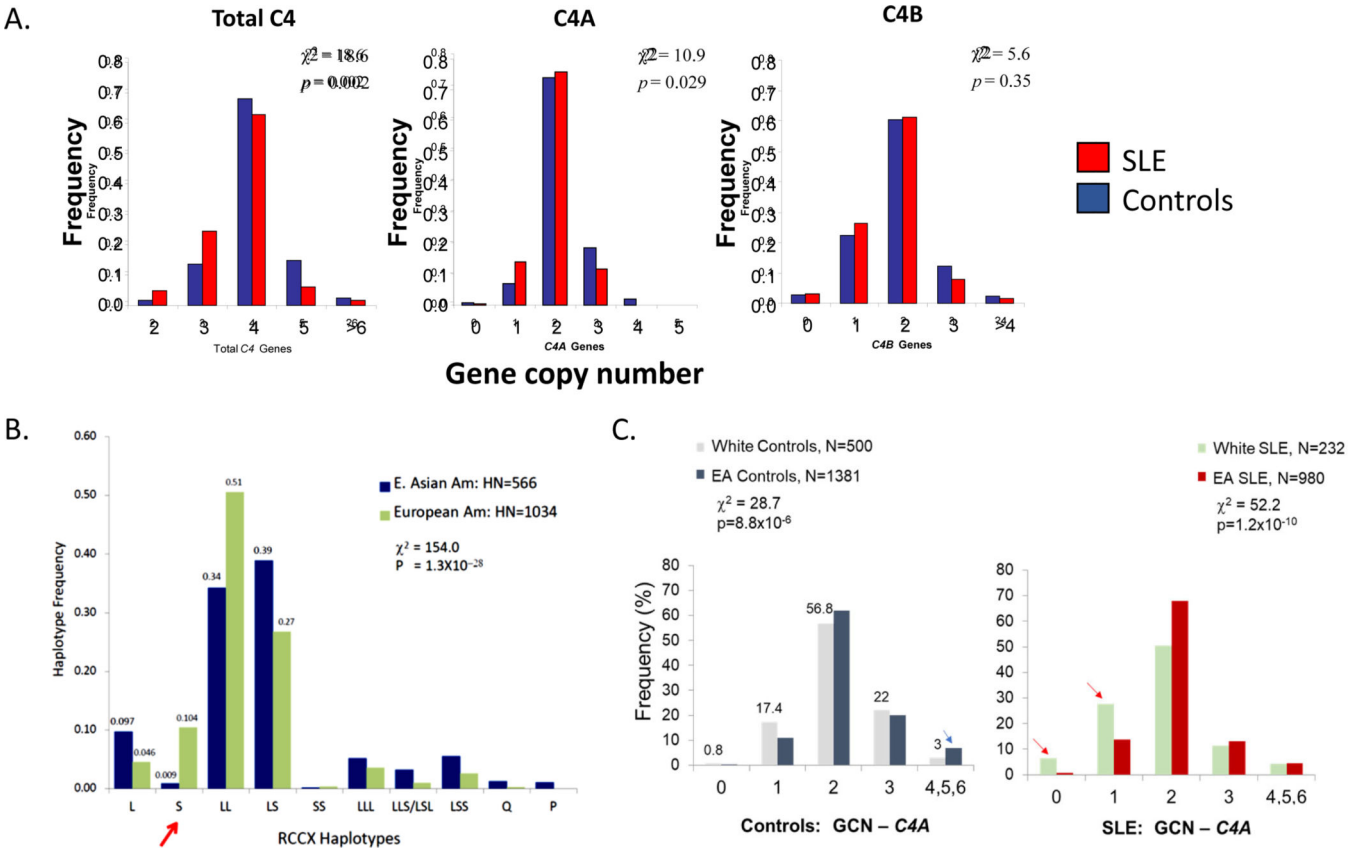

Figure 7. Copy number variation of C4 genes in healthy subjects and patients with systemic lupus erythematous.

(A) Comparisons of gene copy number (GCN) groups for total C4, C4A, and C4B between healthy American subjects and patients with systemic lupus erythematous (SLE). Both groups were of European ancestry. SLE patients show significantly higher frequencies for lower copy numbers of total C4 and C4A but not C4B. More than one-third of patients with SLE had only 0 or 1 copies of the C4A gene, compared with one-fifth in healthy White control subjects. Lower panels: (B) Comparisons of C4 GCN groups in healthy subjects of either European or East Asian (EA) descent. (C) Comparison of C4A GCN groups in SLE patients versus healthy subjects (132, 153). White subjects are of European American descent.

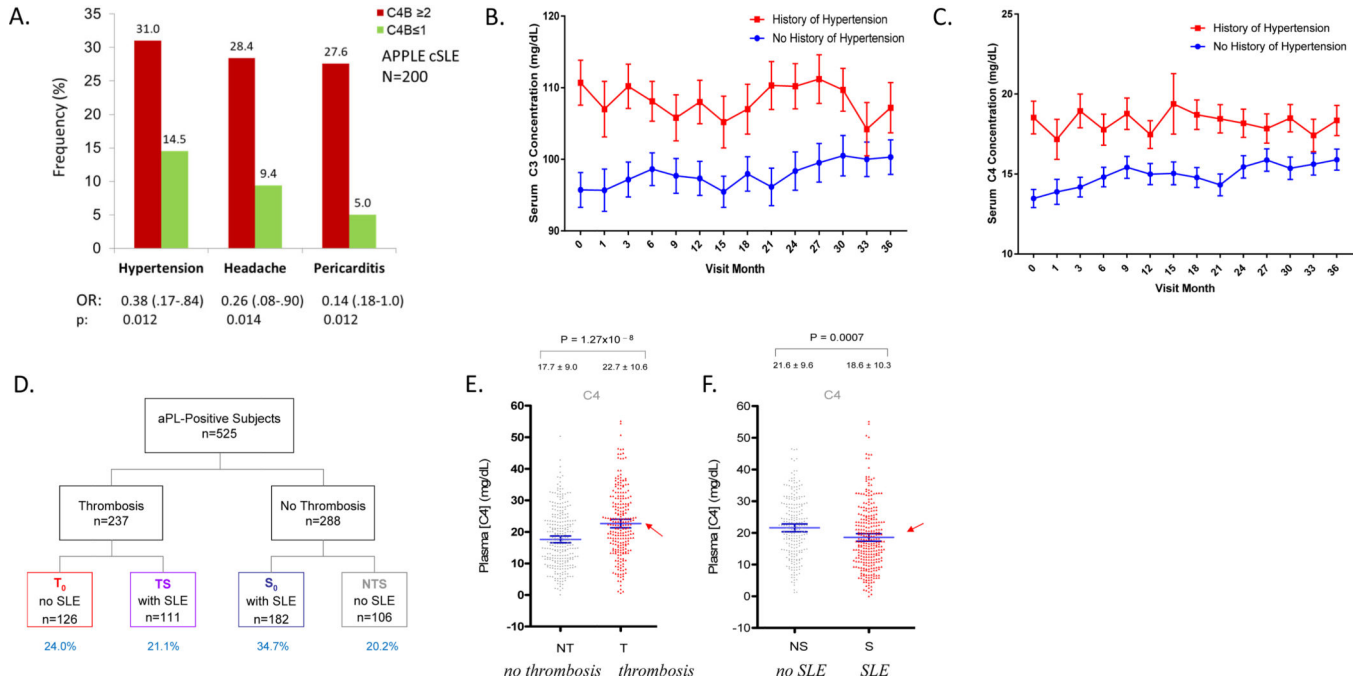

Figure 8. Complement, systemic lupus erythematous, and anti-phospholipid syndrome.

(A) Clinical differences in the frequency of reported hypertension, headache, and pericarditis between patients with more than two copies of C4B genes (red columns) and those with less than two copies of C4B genes (green columns) (170). Patients with hypertension consistently presented with higher C3 (panel B) and C4 (panel C) serum protein levels (red curves) than those without (blue curves). (D) Study design of patients with anti-phospholipid (aPL) antibodies. Subjects were divided into groups based on the presence or absence of thrombosis and systemic lupus erythematous (SLE). T_0= patients with thrombosis only. TS= patients with both thrombosis and SLE. S_0= patients with SLE only. NTS= patients with neither thrombosis nor SLE. (E) Remarkably, patients with aPL antibodies and thromboses had significantly higher serum C4 protein levels than those without thromboses. (F) As expected, patients who were diagnosed with SLE who also had aPL antibodies had lower levels of serum C4 than those who did not present with SLE (right bottom panel) (175).

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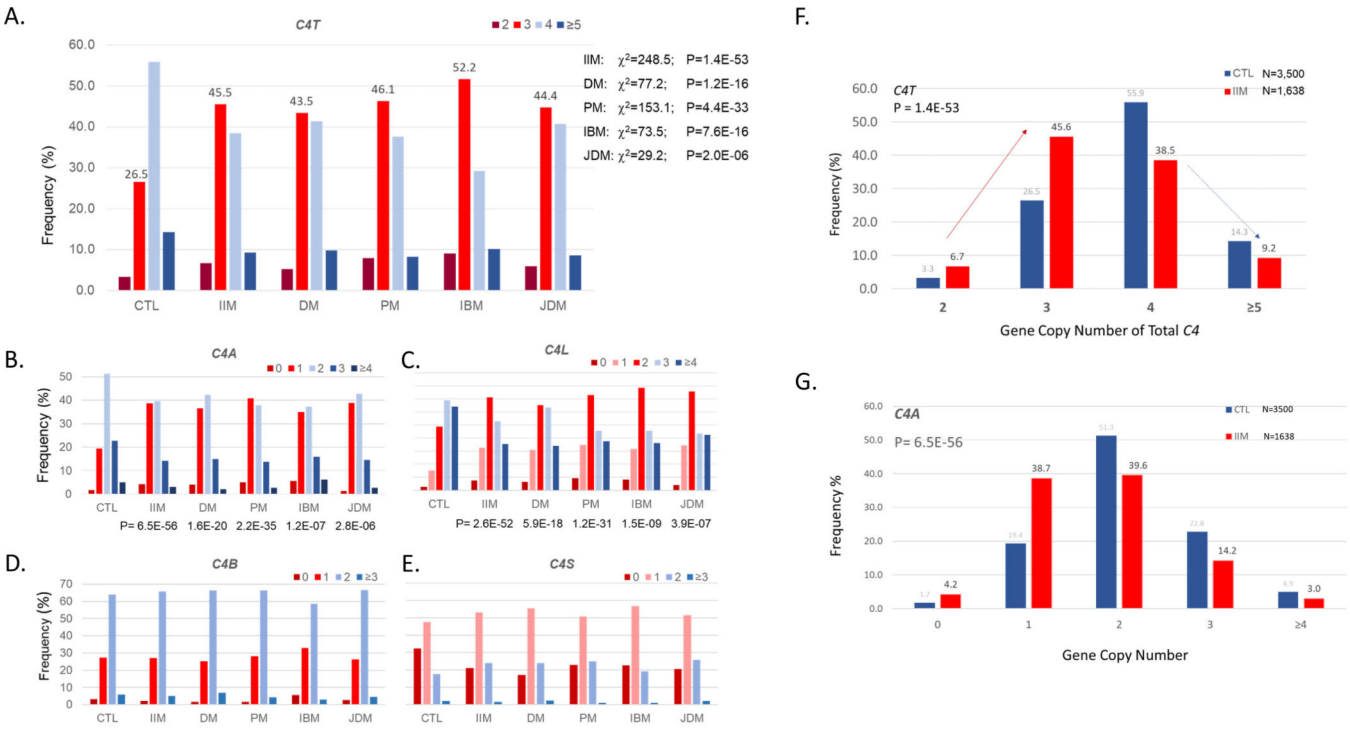

Figure 9. Complement C4 gene copy number variation in the idiopathic inflammatory myopathies.

(A through E) Gene copy number (GCN) variation of total C4 (C4T), C4A, C4 long (C4L), C4B, and C4 short (C4S) between healthy control subjects (CTL) and patients with idiopathic inflammatory myopathy (IIM) and its major subgroups: dermatomyositis (DM), polymyositis (PM), inclusion body myositis (IBM), and juvenile dermatomyositis (JDM). Note that more than half (52.3%) of patients with IIM had C4T GCN<4 (panel F); *C4A* deficiency (C4A GCN<2) was present in 42.9% of the IIM patients (panel G) (77, 79).

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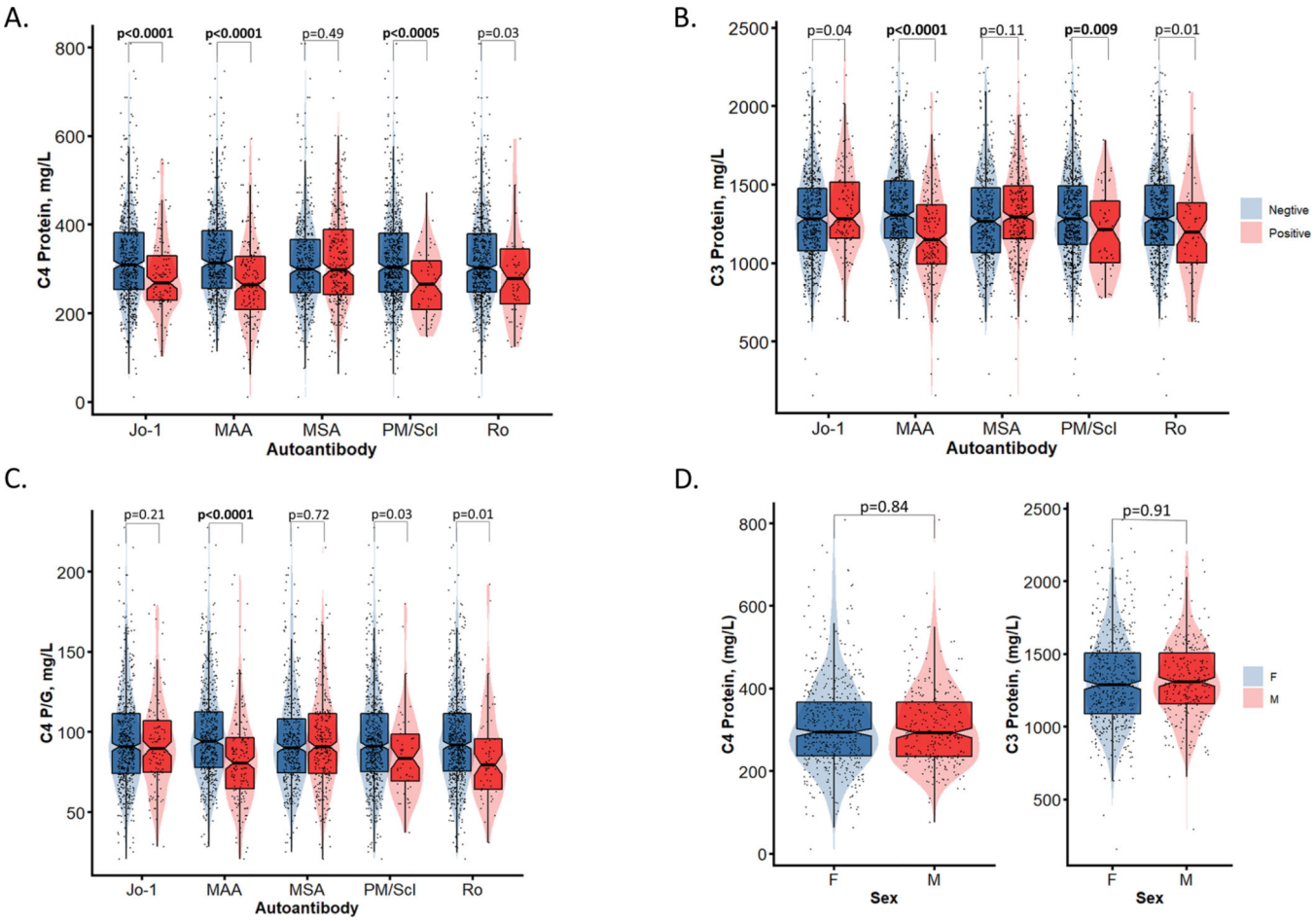

Figure 10. Comparisons of plasma protein levels among patients with idiopathic inflammatory myopathies. Plasma complement levels were determined by single radial immunodiffusion assays.

(A and B) Serum C4 and C3 protein levels were measured in patients with inflammatory idiopathic myopathies (IIM) who also had myositis-specific (MSA) and myositis-associated (MAA) antibodies. Specific MSA including Jo-1 are shown, as are the MAA PM/Scl and Ro. (C) C4 protein levels were corrected for gene copy number (GCN) variation, and C4 protein yield per copy of C4 gene was plotted for patients with MSA, MAA, Jo-1, PM-Scl, and Ro antibodies. (D) C4 (left) and C3 (right) protein levels were compared between males and females (79).

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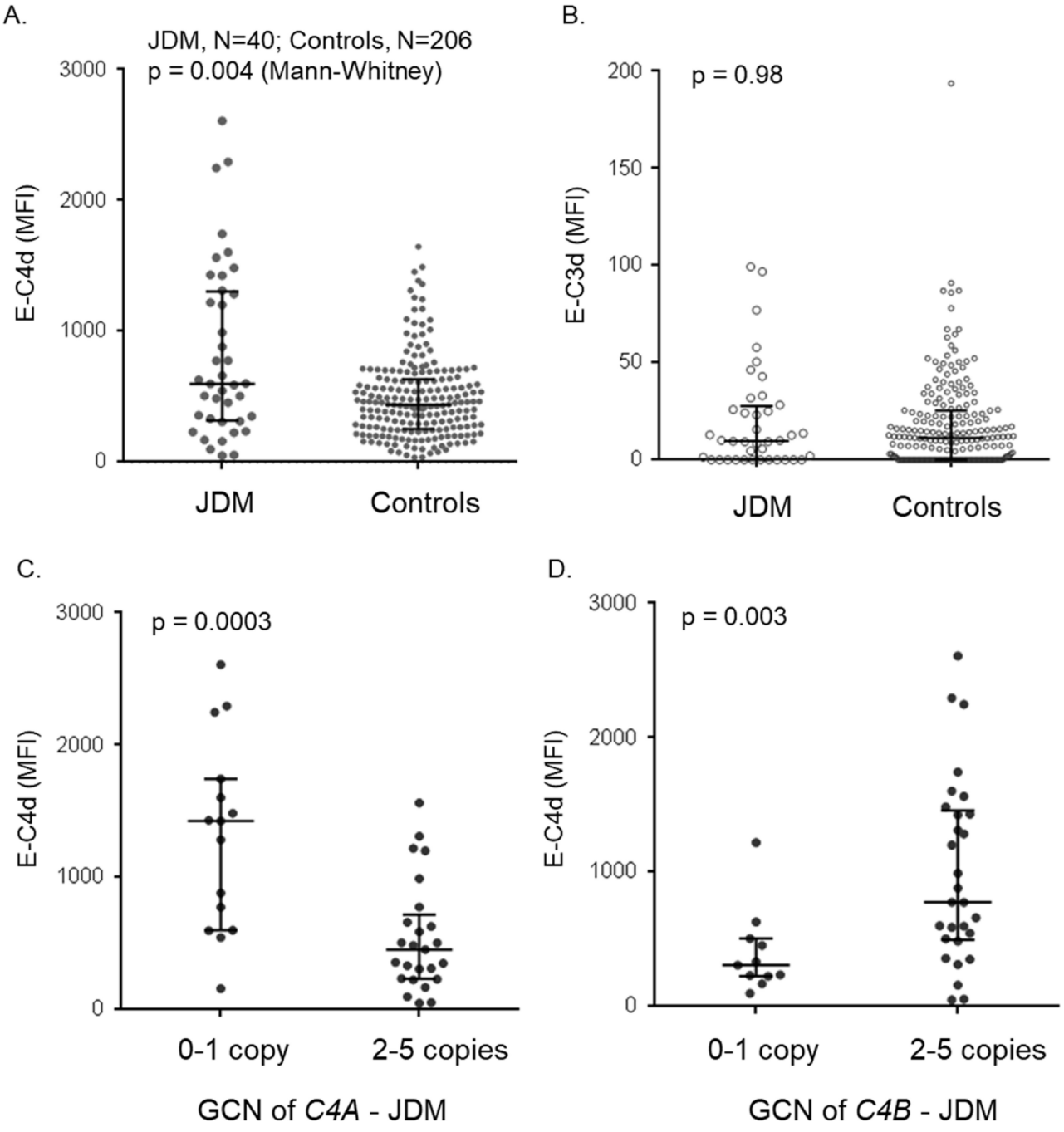

Figure 11. Erythrocyte-bound C4d (E-C4d) and E-C3d in patients with juvenile dermatomyositis and healthy controls.

(A and B) A comparison of E-C4d and E-C3d in juvenile dermatomyositis (JDM) and controls. (C) A comparison of E-C4d in C4A-deficient (C4A gene copy number <2) and C4A-proficient (C4A GCN≥2) JDM patients. (D) A comparison of E-C4d in C4B-deficient (C4B GCN <2) and C4B-proficient (C4B GCN≥2) JDM patients. The median for each group is indicated by a horizontal bar, while the shorter bars represent interquartile ranges; the p-value for Mann Whitney test is indicated.

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Table 1.

Autoimmune diseases associated with complement deficiencies

Component	Autoimmune phenotype	Sources
C1q	Severe lupus-like disease, including nephritis, discoid rash, oral ulcers and anti-Smith antibodies with an unusually negative anti-dsDNA and less frequent arthritis. Female to male ratio approximately 1:1.	(37, 87–95, 240, 241)
C1r	Glomerulonephritis, severe cutaneous lupus-like disease. May have increased interferon signaling. Female to male ratio approximately 1.5:1.	(112–114)
C1s	Glomerulonephritis, severe cutaneous lupus-like disease. May have increased interferon signaling. Female to male ratio approximately 1.5:1.	(112–115)
C2	Childhood onset but more limited lupus-like symptoms with more severe skin manifestations but milder kidney disease; may include sunsensitive skin lesions, alopecia, febrile episodes, and arthritis. Anti-DNA antibody tests are usually negative, and severe kidney disease is rare. The penetrance of C2 deficiency on SLE is only about 10%. Anti-Ro often positive. Female predominance.	(120–124)
C3	More likely to present with increased risk of severe infections. Autoimmune phenotype may be very mild or even asymptomatic. Very few known cases of autoimmune phenotype.	(125–127, 240)
C4	Early disease onset, a severe photosensitive skin rash, presence of anti-Ro/SSA, and high ANA titers are common. May also present with vasculitis, lupus nephritis. Nearly 80% of homozygous deficient patients develop SLE. Complete deficiencies are associated with a female to male ratio of approximately 1:1.	(79, 132, 146–154, 159, 162, 168, 169, 240, 242, 243)
Other components: MBL, complement factor H, membrane cofactor protein, Factor I, Factor B	More commonly present with increased infection susceptibility. May develop atypical hemolytic uremic syndrome. MBL deficiency has been seen in SLE, and anti-MBL antibodies have been detected, though their clinical significance is unclear at this time.	(66, 175, 244–246)

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Table 2.

Seven independent studies on associations of complement C4 gene copy number (GCN) variations with human autoimmune diseases

Studies	Parameters	Disease Cases	Healthy Controls	P	OR (95% CI)
1. European SLE: Ohio, USA; Southern blots, phenotyping **(153)**					
	N	232	500		
	Sex: F, M	216, 16	389, 111		
	C4T, GCN ± SD	3.56±.78	3.83±.69	3.56E-06	
	C4T=2, %	9.05	1.40	1.55E-06	7.00 (2.93–16.7)
	C4T=2+3, %	42.2	29.2	0.0006	1.77 (1.28–2.45)
	C4A, GCN ± SD	1.80± .90	2.09±.75	3.38E-06	
	C4A=0, %	6.47	0.80	1.70E-05	8.57 (2.81–26.1)
	C4A=0+1, %	34.1	18.2	3.67E-06	2.30 (1.63–3.31)
	C4B, GCN ± SD	1.78 ±.59	1.73±.63	0.40, ns	
	C4B=0, %	3.02	3.00	0.99, ns	
	C4B=0+1, %	22.2	28.50	0.077, ns	0.72 (0.50–1.04)
	C4L, GCN ± SD	2.64±1.16	2.95±.98	0.0002	
	C4L=0, %	5.17	0.60	9.71E-05	9.04 (2.52–32.3)
	C4L-0+1, %	14.2	6.2	0.0005	2.51 (1.50–4.21)
	C4L=0+1+2, %	45.3	32.4	0.0008	1.72 (1.25–2.37)
	C4S, GCN ± SD	0.98±.85	0.89±.79	0.17, ns	
	C4S=0, %	31.5	34.4	0.43, ns	
	C4A/C4T	0.48±.20	0.54±.17	2.40E-05	
	C4B/C4T	0.52±.20	0.46±.16	6.90E-06	
	C4L/C4T	0.72±.27	0.76±.21	0.012	
	C4S/C4T	0.30±.28	0.24±.21	0.0006	
2. East Asian SLE: Taiwan, Hong Kong, Ohio, Southern blots, real time PCR, phenotyping **(132)**					
	N	999	1347		
	Sex: F, M	920, 79	700, 674		
	C4T, GCN±SD	3.95±.97	4.14±.92	3.70E-07	
	C4T=2	2.1	1.55	0.33, ns	
	C4T=2+3	27.0	20.3	0.0002	1.45 (1.20–1.77)
	C4A, GCN± SD	2.09±.79	2.25±.82	3.40E-06	
	C4A=0, %	0.9	0.07	0.0015	12.4 (1.57–97.9)
	C4A=0+1, %	14.7	11.2		

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Studies	Parameters	Disease Cases	Healthy Controls	P	OR (95% CI)
	C4B, GCN±SD	1.85±.68	1.88±.71	0.24	
	C4B=0, %	1.14	2.28	0.038	0.50 (0.24–0.99)
	C4B=0+1, %	27.9	25.8	0.25, ns	
	C4L, GCN±SD	2.89±.79	2.93±.82	0.24, ns	
	C4L=0	0	0		
	C4L=1	0.88	0.26	0.062, NS	3.40 (0.88–13.2)
	C4L=1+2	33.2	32.7	0.82	NS
	C4S, GCN±SD	1.04±1.00	1.19±.96	0.0008	
	C4S=0	31.4	22.3	6.50E-06	1.60 (1.30–1.96)
	C4S=0+1	75.3	68.9	0.0022	1.37 (1.12–1.68)
	C4A/C4T	0.532±.151	0.543±.157	0.10	
	C4B/C4T	0.466±.151	0.450±.155	0.01	
	C4L/C4T	0.625±.338	0.615±.315	0.47	
	C4S/C4T	0.202±.202	0.230±.197	0.0009	
3. Hispanic SLE: Sao Paolo, Brazil; Real-time PCR **(168, 169)**					
	N	427	301		
	Sex: F, M	406, 21	286, 15		
	C4T (GCN ± SD)	3.87±.96	4.17±.83	<0.001	
	C4T=2, %	7.0	1.0	<0.001	
	C4T=2+3, %	29.0	13.6	<0.001	2.62 (1.77–3.87)
	C4A (GCN ± SD)	2.06 ±.86	2.24±.73	0.001	
	C4A=0, %	2.1	0.3	0.053	
	C4A=0+1, %	20.4	6.6	<0.001	3.59 (2.15–5.99)
	C4B (GCN± SD)	1.87 ±.77	1.95±.71	0.19	
	C4B=0, %	3.3	1.7	0.24	
	C4B=0+1, %	27.1	20.2	0.033	1.46 (1.03–2.08)
4. European SLE: Germany, Real-time PCR **(162)**					
	N	169	520		
	Sex: F, M	154, 15	471,49		
	C4T, GCN	na	na		
	C4T=2, %	6.9	1.9	0.0068	3.70 (1.50–9.10)
	C4T=2+3, %	43.8	31.3	na	
	C4A GCN	na	na		
	C4A=0, %	4.3	0.8	0.0077	5.33 (1.54–18.4)
	C4A=0+1	37.4	21.9	na	

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Studies	Parameters	Disease Cases	Healthy Controls	P	OR (95% CI)
	C4B=0	0	2.3	na	
	C4B=0+1	17.1	25.3	na	
5a. European SLE: London, UK, Paralog ratio test (PRT) *(242)					
	N	501	719		
	C4T, GCN ± SD	3.79±.98	3.89±.76	0.046	
	C4T=2, %	7.18	3.47		
	C4T=2+3, %	38.7	26.8		
	C4A, GCN ± SD	1.82±.93	2.08±.83	<0.001	
	C4A=0, %	5.18	1.25		
	C4A=0+1, %	38.1	22		
	C4B, GCN ± SD	1.96±.82	1.81±.72	<0.001	
	C4B=0, %	2.6	3.6		
	C4B=0+1, %	27.5	29.3		
5b. South European SLE: Barcelona, Spain; Paralog ratio test (PRT) *(242)					
	N	527	460		
	C4T, GCN ± SD	3.80 ± .92	4.13 ± 1.02	<0.001	
	C4T=2, %	6.03	4.23		
	C4T=2+3, %	36.9	23.4		
	C4A, GCN ± SD	1.82 ± .93	2.08 ± .83		
	C4A=0, %	5.18	1.25		
	C4A=0+1, %	25.0	13.1		
	C4B, GCN ± SD	1.96±.82	1.81±.72	<0.001	
	C4B=0, %	3.00	2.70		
	C4B=0+1, %	39.6	29.7		
6. Myositis: US, UK, Czech Republic, Sweden, Belgium; real time PCR, Southern blots, phenotyping (79)					
	N	1632	3276		
	Sex: F, M	1091, 541	2390, 886		
	C4T, GCN ± SD	3.50±.78	3.83±.76	1.40E-46	
	C4T=2, %	7.14	3.29	2.03E-09	2.26 (1.74–2.95)
	C4T=2+3, %	52.63	29.8	2.81E-55	2.62 (2.32–2.95)
	C4A, GCN ± SD	1.74± .88	2.10± .84	6.00E-46	
	C4A=0, %	4.24	1.74	1.90E-03	2.49 (1.76–3.54)
	C4A=0+1, %	42.87	21.01	8.70E-22	2.82 (2.48–3.21)
	C4B, GCN ± SD	1.74± .59	1.73± .62	0.45	

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Studies	Parameters	Disease Cases	Healthy Controls	P	OR (95% CI)
	C4B=0, %	2.09	3.15	0.03	0.659 (.446-.972)
	C4B=0+1, %	29.21	30.43	0.11	ns
	C4L, GCN ± SD	2.41± 1.13	2.94± 1.08	1.70E-54	
	C4L=0, %	3.67	1.38	5.09E-07	2.72 (1.85–4.02)
	C4L-0+1, %				
	C4L=0+1+2, %	51.62	33.4	2.74E-49	2.13 (1.77–2.56)
	C4S, GCN ± SD	1.06±.72	0.90±.77	3.00E-12	
	C4S=0, %	21.1	32.3	8.80E-17	0.561 (.486-.645)
	C4S=0+1	74.59	80.05	1.44E-05	0.732 (.636-.842)
7. Graves' Disease: Taiwan; real time PCR, phenotyping **(243)**					
	N	624	160		
	F, M	491, 133	128, 32		
	C4T=2+3, %	21.5	32.5	0.003	0.51 (0.34–0.78)
	C4A=0+1, %	12.7	20.6	0.01	0.58 (0.36–0.95)
	C4B=0+1, %	22.9	33.1	0.008	0.49 (0.32–0.74)

Abbreviations: GCN, gene copy number (mean); C4T, total C4; C4L, long C4; C4S, short C4; C4A, acidic C4; C4B, basic C4; F, female; M, male; SD, standard deviation

Note: Two recent publications not included in this list are Lundtoft et al. (247) and Kamitaki et al. (248) that engaged data from read-depths of next-generation sequencing and imputation of single nucleotide polymorphisms.

*Results for entry Number 5 on British and Spanish subjects have been discussed in reference (33).