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# Elevated serum complement levels and higher gene copy number of complement *C4B* are associated with hypertension and effective response to statin therapy in childhood-onset systemic lupus erythematosus (SLE)

Evan Mulvihill,[1,2] Stacy Ardoin,[2,3] Susan D Thompson,[4] Bi Zhou,[5] Gakit Richard Yu,[1] Emily King,[1] Nora Singer,[6] D M Levy,[7] Hermine Brunner,[8] Yee Ling Wu,[9] Haikady N Nagaraja,[10] Laura Eve Schanberg,[11] Chack-Yung Yu,[2,3,12] on behalf of the APPLE Investigators

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For numbered affiliations see end of article.

**Correspondence to**
Dr Chack-Yung Yu; Chack-Yung.Yu@nationwidechildrens.org

## ABSTRACT

**Objective** Systemic lupus erythematosus (SLE) features high frequency of cardiovascular disease (CVD) and fluctuating complement levels. The clinical trial Atherosclerosis Prevention in Pediatric Lupus Erythematosus (APPLE) aimed to evaluate whether atorvastatin treatment reduced the progression of atherosclerosis in 221 patients with childhood-onset SLE (cSLE), using carotid intima media thickness (CIMT) as surrogates. We leveraged APPLE biorepository and trial data to investigate the relationship between *complement* and CVD in cSLE.

**Methods** Gene copy numbers (GCNs) for total *C4*, *C4A* and *C4B* were measured by TaqMan-based real-time PCR and Southern blotting, and analysed with laboratory and clinical parameters through Student's t-test and $\chi^2$ analyses. Effects of total *C4*, *C4A* and *C4B* GCNs on the response to placebo or atorvastatin treatment and progression of CIMT were examined by regression analyses.

**Results** At baseline, C4 protein levels strongly correlated with GCNs of total *C4* ($p=1.8\times10^{-6}$). Each copy of *C4* gene increased mean serum C4 by 3.28 mg/dL. Compared with those without hypertension (N=142), individuals with hypertension demonstrated significantly elevated serum levels for C4 and C3 at baseline and serially (C4: $P=5.0\times10^{-25}$; C3: $P=5.84\times10^{-20}$). Individuals with ≥2 *C4B* genes had 2.5 times the odds of having hypertension (p=0.016) and higher diastolic blood pressure (p=0.015) compared with those with *C4B* deficiency. At the study end, subjects with ≥2 *C4B* and atorvastatin treatment had significantly slower increase in CIMT compared with those treated with placebo (p=0.018).

**Conclusions** cSLE with hypertension had elevated serum levels of C4 and C3 and higher GCN of *C4B*; cSLE with ≥2 *C4B* genes would benefit from statins therapy to prevent atherosclerosis.

## INTRODUCTION

Individuals with systemic lupus erythematosus (SLE) are at high risk for experiencing a major adverse cardiovascular event in their lifetimes. Their risk for myocardial infarction is 9–50 times higher than that of the general population.[1] Consequently, cardiovascular disease (CVD) is thought to be responsible for one-third of all deaths in patients with SLE.[1] This subclinical process begins early in children, whose disease course is generally characterised by higher disease activity and longer duration than those with the adult-onset form of SLE.[2] The relationship between systemic inflammation and atherosclerosis has been demonstrated in the general population[3] and is at least partially mediated by the complement system. Increased serum concentrations of the C3 complement protein, for example, are proposed risk factors for human myocardial infarction.[4 5] Under physiological conditions, the complement system protects a host by promoting immune complex (IC) solubilisation and clearance.[6–8] In a patient with active SLE, however, excessive ICs are produced, some of which are proatherogenic.[9] Inherited or homozygous deficiencies in any of the early components in the classical complement activation pathway (ie, C1q, C1s, C1r and C4) almost always result in an SLE phenotype early in life.[10–13]

Variations in gene copy number (GCN) of complement *C4* also affect the development of SLE. The human *C4* gene is located in the human leukocyte antigen class III region on chromosome 6. Each chromosome 6 contains one to five copies of *C4* genes, allowing an individual to inherit between 2 and 10 copies of total *C4* in a diploid genome.[14 15] The most common GCN for total C4 is four, and for

both *C4A* and *C4B* is two.[14] Each copy of *C4* gene encodes for one of two isotypes, acidic C4A or basic C4B. Variations at four amino acid residues between C4A and C4B, PCPVLD 1101–6 LSPVIH, result in activated C4B having a faster reaction rate but shorter half-life than activated C4A, as well as decreased binding affinity for peptide antigens such as those found on ICs.[16–18] Individuals with childhood-onset SLE (cSLE) are more likely to have fewer copies of total *C4* than those with adult SLE.[19] We hypothesise that the relative inefficiency of IC clearance but stronger reactivity by activated C4B may expose individuals with a higher *C4B* GCN to increased end-organ IC deposition and subsequent disease complications such as accelerated atherosclerosis. Furthermore, inherited variations in complement *C3* 'fast' and 'slow' allotypes (C3F and C3S, respectively) may play a role in disease. Human subjects with a C3F allotype, for example, were shown to be at higher risk for myocardial infarction and systemic inflammatory diseases such as vasculitis.[20–22]

Robust consumption of complement, leading to low serum levels of complement C4 and C3, is initiated by the presence of high concentrations of ICs. Excess complement activation has been implicated in the pathogenesis of lupus nephritis (LN), a major cause of hypertension in SLE.[23–25] The inverse relationship between SLE disease activity and serum complement levels makes C4 and C3 protein levels convenient clinical biomarkers of disease activity. This relationship becomes complicated when considering that CVD, a result of chronic inflammation in SLE, would be associated with higher serum levels of C4 and C3.

Our study aims were to elucidate and clarify the dynamic relationship between CVD-related phenotypes, complement genetic profiles and response to anti-lipid therapies.

## PATIENTS AND METHODS

### Atherosclerosis Prevention in Pediatric Lupus Erythematosus trial design

The Atherosclerosis Prevention in Pediatric Lupus Erythematosus (APPLE) was a multicentre, prospective, randomised, placebo controlled clinical trial. In total, 221 children, adolescents and young adults (aged 10–21 years) with SLE, as determined by the 1997 American College of Rheumatology Revised Classification Criteria,[26] were enrolled from 21 North American centres from the Childhood Arthritis and Rheumatology Research Alliance.[27–29] Participants underwent 1:1 randomisation to receive placebo or atorvastatin (>50 kg: 10 mg/day, increasing to 20 mg/day at day 30; ≤50 kg: 10 mg/day). Participants were counselled to follow the American Heart Association Therapeutic Lifestyle Changes diet.[30] The primary endpoint of the trial was rate of progression of mean–mean common carotid intima media thickness (CIMT). A clinically significant change was defined as a 0.0045 mm/year difference in the endpoint between the atorvastatin and placebo groups at the end of the trial. This endpoint was based on previous adult studies that demonstrated a significant increase in risk for cardiovascular events for every 0.16 mm increase CIMT.[31] The authors reasoned that a child diagnosed with cSLE at 15 years of age would reach this clinically significant cut-off by the age of 50 years if they demonstrated a 0.0045 mm/year increase in CIMT. Secondary outcomes included other ultrasound CIMT measurements defined in the APPLE trial (mean–mean CIMT, mean–mean common CIMT, mean-max CIMT and mean-max common CIMT) as well as SLE disease activity, organ system damage, health-related quality of life and laboratory measures such as serum C3 and C4 concentrations, high-sensitivity C-reactive protein (CRP) and serum lipid levels. Each of these data points was collected at serial study visits over 3 years. On enrolment into the APPLE trial, physicians indicated whether participants had a past history of hypertension, which was further ascertained on multiple occasions. Automated systolic blood pressure (SBP) and diastolic blood pressure (DBP) measurements were taken under resting conditions at each visit.

### Ultrasonography of carotid arteries in the APPLE trial

Two CIMT ultrasound examinations were performed at enrolment and at the end of the trial. One CIMT examination was performed at 6, 12 and 24 months for a total of seven examinations. Standardised measurements of the bilateral common carotid arteries (CCA), carotid bifurcations and proximal internal carotid artery CIMT were obtained at each examination. Mean-max and mean–mean CIMT were calculated and recorded using measurements from each measurement site. Mean-max and mean–mean common CIMT were calculated and recorded using measurements only from the CCA.[27] A single reader with more than 20 years of experience reading CIMT research studies read all ultrasound scans for CIMT using Image Pro software (Media Cybernetics). To evaluate intra-reader reliability, a set of 68 studies were re-read. The intra-class correlation coefficient was 0.74 (95% CI: 0.61–0.83) for mean–mean common and 0.71 (0.56–0.81) for mean-max CIMT measurements.[27]

### Genotypes and phenotypes of complement C4

Biological samples were available for 200 of 221 participants. Paired genomic DNA and EDTA-plasma samples for 21 patients were not available. GCNs for total C4 (*C4T*), *C4A* and *C4B* were measured by TaqMan-based quantitative real-time PCR through five independent amplicons. Data were validated when total *C4* GCN (*C4T*) was equal to the sum of *C4A* and *C4B*.[14] GCN calls for 100 patients were further confirmed by Southern blot analyses using genomic DNA digested by *Taq*I restriction enzymes, and hybridised to genomic DNA probes corresponding to genomic fragments for (a) intergenic genomic fragments between 3′ region of *RP* and 5′ region of *C4*, (b) steroid 21-hydroxylase *CYP21A1P* and *CYP21A2* and (c) 3′ regions of extracellular matrix protein tenascin *TNXB* and *TNXA*.[32–34] Native C4 proteins from EDTA-plasma were resolved by high voltage agarose gel electrophoresis

based on gross differences in electric charges, and used to elucidate C4A and C4B protein polymorphisms by immunofixation using goat antiserum against human C4 (Diasorin, Stillwater, MN, USA).[34]

### Genotyping of complement C3: C3 fast and C3 slow

The C→G nucleotide change leading to Arg 102 Gly polymorphism for the *slow* and *fast* variants of C3 protein can be distinguished by *Hha*I restriction fragment length polymorphism.[35] Genomic DNA fragments corresponding to nucleotides 4169 and 4502 of the human *C3* gene were amplified by PCR, subjected to *Hha*I restriction digest, and resolved by agarose gel electrophoresis. The *fast* and *slow* alleles were shown as 333 bp, and 232+101 bp, restriction fragments, respectively.

### Statistical analyses

We analysed baseline characteristics of APPLE participants based on dichotomised *C4* GCN groups. Specifically, participants were grouped by whether total *C4, C4A* and *C4B* GCNs were low (<4 for total *C4;* <2 for *C4A* or *C4B*) or medium to high (≥4 for total *C4;* ≥2 for *C4A* or *C4B*). Baseline variables and their associations were examined through analysis of variance (ANOVA), Student's t-test and $\chi^2$ tests. Stepwise logistic regression was used for risk factor analysis. Based on hypertensive status and treatment group, longitudinal changes in C4 and C3 protein levels and in CIMT variables were examined using repeated measures ANOVA models that included interaction effects. Significance of relationships was tested using post-hoc t-tests (Tukey). P values of ≤0.05 were considered significant. JMP V.13 (SAS Institute, Cary, NC, USA) was used for analysis.

## RESULTS

### Baseline demographics and complement profiles of the APPLE trial cohort

Baseline demographic and clinical data for 221 patients with cSLE enrolled in the APPLE trial are shown in table 1. In all, 56 (25.5%) participants were African American, 106 (48.2%) were white and 58 (26.4%) were either of mixed race or other racial background. Mean age (±SD) of participants was 15.8±2.6 years. Mean body mass index (BMI) was 24.4±5.3 kg/m$^2$. Mean SBP and DBP were 112.8±12.2 mm Hg and 66±9.6 mm Hg, respectively. In all, 73 patients (33.2%) had a history of hypertension; 104 (47.1%) had a history of kidney disorder, defined as previous or current nephrotic syndrome, nephritic syndrome or any class of LN on renal biopsy based on either the International Society of Nephrology/Renal Pathology Society[36] classification or the WHO[37] classification. At baseline, 67 participants (30.3%) were using or had previously used an anti-hypertensive medication (angiotensin receptor blocker, calcium channel blocker, beta blocker and/or ACE inhibitor). Specific indication for use of these medications was not documented.

Complement C4 and C3 genotypes were successfully determined and validated on 183 participants (82.8%).

**Table 1** Baseline demographics and complement profiles in cSLE[41] of the APPLE cohort

| | N (%) | |
|---|---|---|
| Sex | | |
| Female | 183 (83.2) | |
| Male | 37 (16.8) | |
| Race | | |
| African American | 56 (25.5) | |
| White | 106 (48.2) | |
| Multi-racial/other | 58 (26.4) | |
| Hypertension* | 73 (33.2) | |
| History of kidney disease† | 104 (47.1) | |
| Previous use of anti-hypertensive medication‡ | 67 (30.3) | |
| | **Mean±SD** | **95% CI** |
| Age (years) | 15.8±2.6 | 14.4–16.1 |
| Height (cm) | 158.6±10.7 | 157.3–160.0 |
| Weight (kg) | 62.0±17.3 | 59.7–64.3 |
| BMI (kg/m$^2$) | 24.4±5.34 | 23.7–25.1 |
| SBP, mean mm Hg ±SD | 112.8±12.2 | 111.2–114.4 |
| DBP, mean mm Hg ±SD | 66.4±9.56 | 65.1–67.7 |
| Serum C4 (mg/dL) | 15.2±7.8 | 14.1–16.2 |
| Serum C3 (mg/dL) | 100.7±29.0 | 96.8–104.7 |
| *C4* GCN | | |
| Total *C4* | 3.822±0.798 | 3.710–3.934 |
| *C4A* | 1.990±0.742 | 1.886–2.094 |
| *C4B* | 1.843±0.639 | 1.753–1.932 |
| *C3* variants | N (%) | |
| *FF+FS* | 42 (21.8) | |
| *SS* | 151 (78.2) | |

*Prior or current hypertension per baseline physician documentation.
†Prior or current nephritis, nephrotic syndrome and/or classes I–V nephritis on biopsy (regardless of classification criteria) per baseline physician documentation.
‡Prior or current use of ACE inhibitor, angiotensin receptor blocker, beta blocker and/or calcium channel blocker.
APPLE, Atherosclerosis Prevention in Pediatric Lupus Erythematosus; BMI, body mass index; *C4L*, long *C4* gene; *C4S*, short *C4* gene; DBP, diastolic blood pressure; *FF*, homozygous fast variant of *C3*; *FS*, heterozygous fast and slow variant of *C3*; GCN, gene copy number; SBP, systolic blood pressure; *SS*, homozygous slow variant of *C3*; cSLE, childhood-onset systemic lupus erythematosus.

GCNs for total *C4*, *C4A* and *C4B* varied between 2 and 6, 0 and 5, and 0 and 5, respectively. Mean total *C4* GCN was 3.82±0.80. Mean *C4A* and *C4B* GCNs were 1.99±0.74 and 1.84±0.64, respectively. Baseline mean serum C4 concentration was 15.2±7.8 mg/dL. Serum C4 protein concentrations strongly correlated with total *C4 GCN* (ANOVA P=1.8×10$^{-6}$). Based on linear regression analyses, each one-copy increase

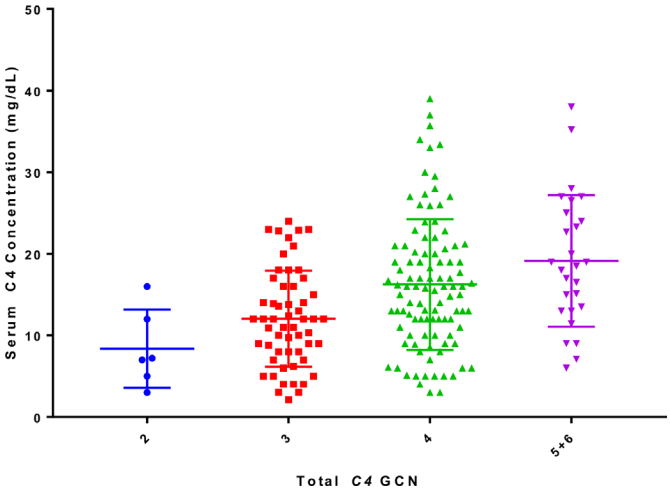


**Figure 1** Baseline serum C4 concentrations in APPLE participants based on total *C4* GCN. Mean and SD are shown. Participants with five and six copies of total *C4* were grouped together because of small sample size (analysis of variance p value: $3.0\times10^{-5}$). The linear regression formula for C4 protein concentration (mg/dL) was 2.62+3.28 x GCN of total *C4*. APPLE, Atherosclerosis Prevention in Pediatric Lupus Erythematosus; GCN, gene copy number.

in *C4* GCN resulted in an increase in mean serum C4 concentration of 3.28 mg/dL (figure 1).

Baseline mean serum C3 concentration was 100.7±29.0 mg/dL (table 1). For the C3 fast (F) and slow (S) variants, the most common genotype was SS (frequency 78.2%). Heterozygous fast and slow genotype FS, and homozygous genotype FF had a combined frequency of 21.8%. C3 F and S genotypes had no significant effect on serum C3 levels.

### Baseline laboratory values and CIMT measurements

Patients with low copy number of total *C4* genes has an earlier age of disease diagnosis than patients with medium or high copy number of total *C4* (GCN=2 or 3: 12.53±3.08 years old; GCN=4 or 5: 13.47±2.75 years old; p=0.033). Participants with medium-to-high GCN of total *C4* had a higher mean BMI than those with low total *C4* GCN (p=0.03, table 2). Compared with participants with a low *C4B* GCN, those with a medium-to-high *C4B* GCN were more likely to have history of hypertension (p=0.02), a higher DBP (p=0.02) and a past history of pericarditis (p=0.031). Lipid profiles were similar between each GCN pair (low vs medium to high) as was baseline CRP. Individuals with cSLE and a GCN of *C4A* less than 2 were more likely to have a history of myositis than those with a *C4A* GCN of 2 or more (20.5% vs 7.59%; p=0.03).

Participants with medium-to-high GCNs of total *C4* and *C4B* had significantly higher serum concentrations of serum C4 protein ($P=1.2\times10^{-5}$ for *C4T* and 0.01 for *C4B*). These patients were also slightly older than participants with low GCN of total *C4* (P=0.02) and *C4B* (P=0.06), respectively. Serum C4 concentrations and age did not differ between *C4A* GCN groups. No significant differences in distribution of race or gender were observed when comparing participants based on GCNs of complement *C4* and its isotypes.

CIMT measurements were similar in participants with low total *C4* GCN and low *C4B* GCN compared with those with medium-to-high total *C4* GCN and medium-to-high *C4B* GCN, respectively. Compared with participants with ≤2 *C4A*, those with >2 *C4A* had significantly higher mean–mean CIMT (0.463 vs 0.451 mm; P=0.04), mean–mean common CIMT (0.471 vs 0.455 mm; P=0.05), and mean-max common CIMT (0.603 vs 0.580 mm; P=0.03). Conversely, mean-max CIMT was similar between the two *C4A* groups (0.570 vs 0.590 mm; P=0.09).

No significant relationship was appreciated when comparing baseline CIMT measurements to baseline serum C3, C4 and CRP protein levels.

### Complement profiles in hypertensive and normotensive participants

Hypertensive and normotensive participants had similar mean age and racial distribution (table 3). Those with hypertension had significantly higher mean values of BMI and body weight but not height (p=0.0046). Compared with normotensive participants, the frequencies of nephrotic syndrome or nephritis syndrome, and other renal involvement were dramatically higher in hypertensive participants (table 3). We also observed significant differences in baseline serum levels of complement C4 between hypertensive and normotensive participants (18.5±8.6 vs 13.5±6.7 mg/dL; $P=4.7\times10^{-6}$). Baseline serum C3 levels were significantly higher among hypertensive participants (110.7±26.4 vs 95.7±29.0 mg/dL, P=0.0003).

Mean serum C4 and C3 levels over time in hypertensive and normotensive participants are shown in figure 2A, B. A history of hypertension was with higher serum mean C4 ($P=5.0\times10^{-25}$) and C3 levels ($P=5.8\times10^{-20}$) over time.

To investigate the genetic basis for higher baseline and serial complement levels among hypertensive participants, we compared GCNs of total *C4*, *C4A* and *C4B* in the hypertensive and normotensive groups (table 3). Hypertensive participants demonstrated significantly higher mean *C4B* GCN (2.0±0.6 vs 1.8±0.7, P=0.023), and significantly lower mean *C4A* GCN (1.8±0.7 vs 2.1±0.8, P=0.032) than normotensive participants. Hypertensive participants had a higher *C4S* GCN (P=0.0054) and a lower mean *C4L* GCN (P=0.0098). Total *C4* GCN was not significantly different between groups, nor were frequencies of fast and slow genotypes of C3.

We performed stepwise logistic regression analyses to identify independent risk factors for hypertension at baseline. As shown in part *b* of table 3, in the order of decreasing effects, nephrotic syndrome, nephritic syndrome, serum levels of complement C4, BMI and the GCN of long *C4* genes were independent risk factors for hypertension (model P: $2.6\times10^{-24}$; $R^2$: 0.51; area under curve: 0.932).

**Table 2** A comparison of baseline laboratory and clinical data for patients with cSLE /APPLE between dichotomised GCN groups of total *C4*, *C4A* and *C4B*

| | Total *C4* GCN | | | *C4A* GCN | | | *C4B* GCN | | |
|---|---|---|---|---|---|---|---|---|---|
| | <4 (N=64) | ≥4 (N=133) | P | <2 (N=39) | ≥2 (N=158) | P | <2 (N=49) | ≥2 (N=148) | P |
| Age, years±SD | 15.0±0.3 | 15.9±0.2 | **0.02** | 15.5±0.4 | 15.7±0.2 | 0.69 | 15.0±0.4 | 15.8±0.2 | 0.063 |
| Age of SLE diagnosis, years±SD | 12.5±3.1 | 13.5±2.8 | **0.033** | 13.1±2.9 | 13.2±2.9 | 0.85 | 12.8±3.1 | 13.3±2.8 | 0.25 |
| Female, N (%) | 53 (82.8) | 110 (82.7) | 1 | 35 (89.7) | 128 (81.0) | 0.24 | 37 (75.5) | 126 (85.1) | 0.13 |
| Race, N (%) | | | | | | | | | |
| White | 17 (26.6) | 35 (26.1) | 0.81 | 7 (18.0) | 45 (28.3) | 0.33 | 12 (24.5) | 40 (26.9) | 0.73 |
| African American | 32 (50.0) | 62 (46.3) | | 22 (56.4) | 72 (45.3) | | 22 (44.9) | 72 (48.3) | |
| Other/multi | 15 (23.4) | 37 (27.6) | | 10 (25.6) | 42 (26.4) | | 15 (30.6) | 37 (24.8) | |
| BMI, kg/m$^2$±SD | 23.1±0.7 | 24.9±0.5 | **0.03** | 23.0±0.8 | 24.6±0.4 | 0.09 | 23.6±0.8 | 24.5±0.4 | 0.28 |
| Disease duration, mo ±SD | 29.8±3.5 | 29.2±2.5 | 0.89 | 28.7±4.5 | 29.5±2.3 | 0.88 | 27.4±4.0 | 30.1±2.3 | 0.56 |
| SBP, mm Hg ±SD | 111.6±1.6 | 113.8±1.1 | 0.25 | 112.7±2.0 | 113.2±1.0 | 0.83 | 111.3±1.8 | 113.7±1.0 | 0.23 |
| DBP, mm Hg ±SD | 64.2±1.2 | 67.7±0.8 | **0.01** | 65.5±1.6 | 66.8±0.8 | 0.45 | 63.7±1.4 | 67.5±0.8 | **0.02** |
| Hypertension, N (%) | 16 (25.0) | 38 (28.8) | 0.58 | 14 (35.9) | 40 (25.5) | 0.20 | 7 (14.3) | 47 (32.0) | **0.02** |
| Pericarditis N (%) | 3 (4.69) | 14 (10.5) | 0.15 | 2 (5.13) | 15 (9.49) | 0.36 | 1 (2.04) | 16 (10.8) | **0.03** |
| Myositis N (%) | 9 (14.1) | 11 (8.27%) | 0.22 | 8 (20.5) | 12 (7.59) | **0.03** | 5 (10.2) | 15 (10.1) | 0.99 |
| Serum C3, mg/dL | 102.5±3.6 | 101.9±2.5 | 0.9 | 106.3±4.6 | 101.1±2.3 | 0.31 | 100.6±4.0 | 102.6±2.3 | 0.67 |
| Serum C4, mg/dL | 11.7±0.9 | 16.8±0.7 | **$1.2\times10^{-5}$** | 13.3±1.3 | 15.6±0.6 | 0.11 | 12.6±1.1 | 16.1±0.6 | **0.01** |
| Lipoprotein (A) | 25.1 | 21.5 | 0.36 | 19 | 23.6 | 0.32 | 26.3 | 21.4 | 0.26 |
| Homocysteine | 7.72 | 7.32 | 0.4 | 7.81 | 7.36 | 0.42 | 7.51 | 7.43 | 0.87 |
| Total cholesterol | 152.1 | 157.2 | 0.4 | 159.4 | 154.6 | 0.51 | 154.4 | 155.9 | 0.82 |
| HDL | 46.3 | 46.9 | 0.77 | 47.1 | 46.6 | 0.83 | 45.5 | 47.1 | 0.44 |
| LDL | 84.1 | 87.2 | 0.53 | 88.2 | 85.7 | 0.66 | 86.9 | 85.9 | 0.85 |
| Triglycerides | 1091 | 118.4 | 0.39 | 120.3 | 114.2 | 0.63 | 110.5 | 117.0 | 0.57 |
| C-reactive protein | 2.28 | 2.78 | 0.69 | 2.28 | 2.70 | 0.78 | 2.00 | 2.82 | 0.55 |
| CIMT, mm | | | | | | | | | |
| Mean–mean CIMT | 0.46 | 0.46 | 0.21 | 0.45 | 0.46 | **0.04** | 0.47 | 0.46 | 0.23 |
| Mean–mean common CIMT | 0.46 | 0.47 | 0.37 | 0.46 | 0.47 | **0.05** | 0.47 | 0.47 | 0.38 |
| Mean-max CIMT | 0.58 | 0.59 | 0.11 | 0.57 | 0.59 | 0.09 | 0.59 | 0.58 | 0.21 |
| Mean-max common CIMT | 0.59 | 0.60 | 0.37 | 0.58 | 0.6 | **0.03** | 0.61 | 0.60 | 0.12 |

APPLE, Atherosclerosis Prevention in Pediatric Lupus Erythematosus; BMI, body mass index; CIMT, carotid intima media thickness; DBP, diastolic blood pressure; GCN, gene copy number; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure; SLE, systemic lupus erythematosus; cSLE, childhood-onset systemic lupus erythematosus.

### Effects of complement *C4* GCNs on serum complement level and response to atorvastatin treatment

APPLE participants were randomised to receive either daily atorvastatin or placebo and monitored meticulously over the 3-year trial period. Overall, there were no significant differences in serum complement levels between placebo and atorvastatin groups at baseline and at study end. Participants were then dichotomised into low versus medium-to-high groups based on total *C4* GCN and *C4B* GCN. Compared with participants in the medium-to-high groups, those with low GCNs of total *C4* or *C4B* had lower mean serum levels of complement C4 at baseline (table 2). By the end of the trial, mean serum C4 protein levels had *increased* significantly in this *low* GCN group regardless of treatment (figure 3, *panel* A). In the medium-to-high GCN group, mean serum C4 levels remained stable over time.

Table 3 Baseline demographics and complement profiles of normotensive and hypertensive APPLE participants

| | Normotensive (N=148) | Hypertensive* (N=73) | P |
|---|---|---|---|
| a. Baseline demographics | | | |
| African American | 36 (24.3%) | 21 (28.8%) | 0.67 |
| White | 74 (50.0%) | 32 (43.8%) | |
| Mixed/other | 38 (25.7%) | 20 (27.4%) | |
| Female, N (%) | 126 (85.1%) | 58 (79.5%) | 0.29 |
| SBP, mean, mm Hg ±SD | 110.4±11.3 | 117.7±12.5 | **$2.6\times10^{-5}$** |
| DBP, mean, mm Hg ±SD | 64.89±8.93 | 69.4±10.1 | **0.0008** |
| BMI kg/$m^2$ | 23.69±4.83 | 25.84±6.03 | **0.0046** |
| Body weight (kg) | 60.37±15.53 | 65.37±19.97 | **0.043** |
| Height (cm) | 159.0±9.76 | 157.90±10.69 | 0.45 |
| Nephrotic syndrome | 4 (2.74) | 33 (45.2) | **$3.9\times10^{-15}$** |
| Nephritic syndrome | 26 (17.8) | 53 (72.6) | **$1.3\times10^{-15}$** |
| Other kidney disorders | 7 (5.38) | 13 (22.0) | **0.001** |
| C4 protein, mg/dL | 13.5±6.7 | 18.5±8.6 | **$4.7\times10^{-6}$** |
| C3 protein, mg/dL | 95.7±29.0 | 110.7±26.4 | **0.0003** |
| Patient number (N) | 135 | 63 | |
| *C4T* GCN (mean±SD) | 3.844±0.836 | 3.794±0.722 | 0.68 |
| *C4A* GCN (mean±SD) | 2.067±0.755 | 1.825±0.685 | **0.032** |
| *C4B* GCN (mean±SD) | 1.778±0.665 | 2.000±0.568 | **0.023** |
| *C4L* GCN (mean±SD) | 2.889±1.182 | 2.435±1.018 | **0.0098** |
| *C4S* GCN (mean±SD) | 0.970±0.819 | 1.339±0.922 | **0.0054** |
| *C3*, FF+FS (frequency) | 0.237 | 0.175 | 0.32 |
| b. A logistic regression model of independent risk factors for hypertension in cSLE | | | |
| | $R^2$/AUC | $\chi^2$ | P |
| Overall | 0.5097/0.932† | 120.4 | **$2.6\times10^{-24}$** |
| Nephrotic syndrome | | 33.8 | **$6.1\times10^{-9}$** |
| Nephritic syndrome | | 28.5 | **$9.4\times10^{-8}$** |
| Serum C4 | | 18.1 | **$2.1\times10^{-5}$** |
| BMI | | 14.7 | **0.0001** |
| GCN of *C4L* | | 7.25 | **0.0071** |

P values <0.05 are in bold fonts.
*Prior or current hypertension per baseline physician documentation.
†An alternative model without the inclusion of serum C4 and GCN of *C4L* yielded $R^2$=0.430 and AUC, 0.905.
APPLE, Atherosclerosis Prevention in Pediatric Lupus Erythematosus; AUC, area under curve; BMI, body mass index; C4L, long C4 genes; *C4L*, long *C4* genes; DBP, diastolic blood pressure; GCN, gene copy number; R, correlation coefficient; SBP, systolic blood pressure; cSLE, childhood-onset systemic lupus erythematosus.

This would suggest that patients with low GCNs of total *C4* or *C4B* benefited from the clinical trial process to normalise serum complement levels.

As shown in figure 3, *panel* B, the medium-to-high *C4B* GCN group treated with atorvastatin demonstrated significantly slower mean–mean CIMT progression compared with participants who received placebo (P=0.018). While the mean serum C4 protein levels in the atorvastatin treatment group with *C4B*≥2 was slightly lower than that of the placebo group, the progression of changes was not significantly different between these two groups. There was also no significant difference in the rate of mean–mean CIMT progression between atorvastatin treatment and placebo groups over the 3-year trial in the low *C4B* GCN subset.

## DISCUSSION

Understanding that CVD is often subclinical in children with cSLE, we investigated the relationship between complement genetics and risk for CVD. Conceivably, patients with low serum complement levels at baseline would be more readily depleted during active disease. Given the linear relationship between total *C4* GCN and

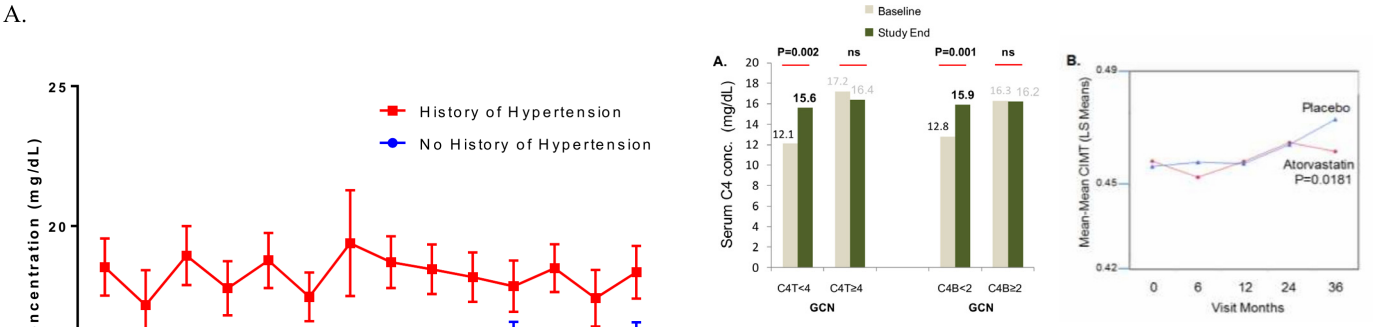


**Figure 3** Treatment effects of APPLE clinical trial. (A) improvement of mean serum C4 concentrations at the endpoint of APPLE trial among patients with low GCNs for total *C4* (<4) or *C4B* (<2), irrespective of treatment with placebo or atorvastatin, when compared with C4 concentrations at baseline. (B) Slower progression of mean–mean CIMT in cSLE treated with atorvastatin (red) than placebo (blue) among patients with GCN of *C4B*≥2. APPLE, Atherosclerosis Prevention in Pediatric Lupus Erythematosus; CIMT, carotid intima media thickness; cSLE, childhood-onset systemic lupus erythematosus; GCN, gene copy number; NS, not significant.

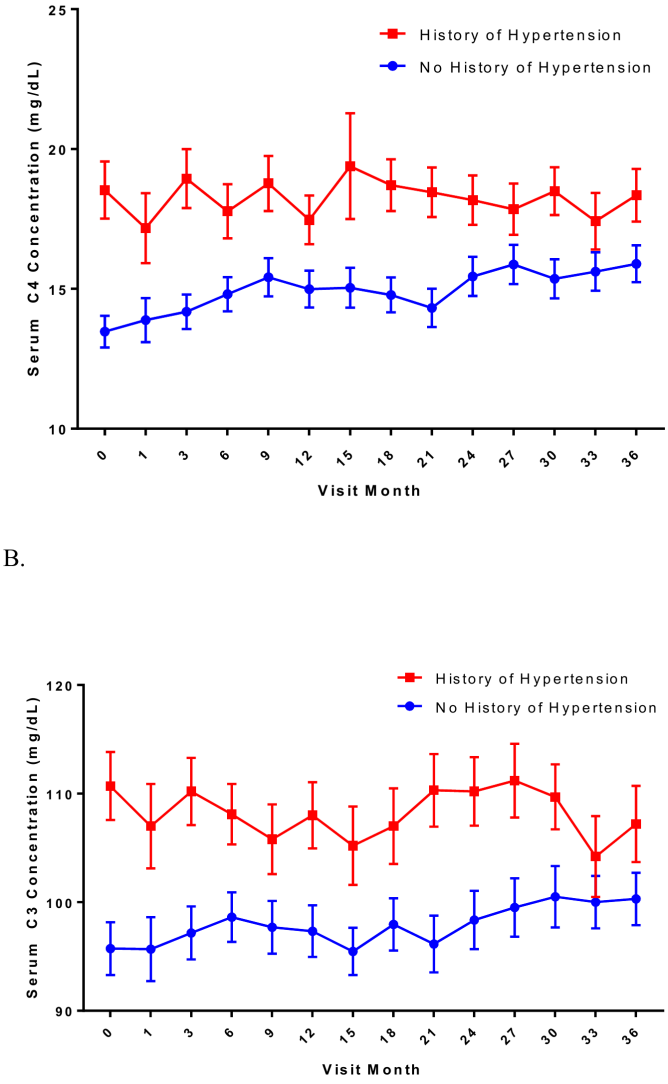


**Figure 2** Serial serum C4 (panel a) and C3 (panel B) concentrations in APPLE participants with (*red* curve) and without (*blue* curve) a history of hypertension. Mean concentrations of serum C4 or C3 with standard errors were plotted against time (visit month). APPLE, Atherosclerosis Prevention in Pediatric Lupus Erythematosus.

serum *C4* concentration demonstrated in our study, we propose that low total *C4* GCN is an inherited unmodifiable risk factor that, on early identification, may trigger more intensive clinical monitoring.

Our results suggest that patients with cSLE with a medium-to-high *C4B* GCN more commonly have a history of hypertension and pericarditis. Additionally, these potentially 'high-risk' individuals who were in the treatment group of the APPLE trial demonstrated slower CIMT progression over the course of the trial. We also noted an association between low *C4A* GCN and myositis, an observation which was consistent with our earlier report on *C4A* deficiency in juvenile dermatomyositis.[38]

Importantly, we showed that APPLE participants with a history of hypertension had significantly higher serum concentrations of C4 and C3 at baseline and throughout the 36-month trial period than participants without a history of hypertension (figure 2). Elevated serum levels of complement have been described in people with CVD but, to our knowledge, this association has not previously been demonstrated in SLE or in children. This is the first study to analyse complement genetics and serial serum complement protein levels as a predictor of CVD in patients with cSLE. We leveraged data from the APPLE trial which was strengthened by its serial collection of CIMT and laboratory data in a cohort of racially and ethnically diverse children with SLE. The exclusion of patients with severe nephritis from this cohort reduced the confounding effects of kidney disease on hypertension in our analysis but also limited the generalisability of our findings to a cohort of patients with cSLE known to be highly affected by hypertension. In addition to inflammatory mechanisms, there are other factors thought to be associated with CVD in SLE such as the presence of antiphospholipid antibodies and vitamin D deficiency.[9] We were able to consider CRP which has been proposed as an independent risk factor for CVD.[39] While our study found no significant association between the levels of CRP and CIMT, further studies would be desirable to more thoroughly investigate various proposed risk factors for CVD. Among adult subjects with recurrent positivity of antiphospholipid antibodies, we observed that patients who experienced thromboses and/or recurrent pregnancy loss had significantly higher serum protein levels of complement C4 and C3 and higher GCN of *C4B* than patients who did not manifest antiphospholipid syndrome-related disorders.[40]

In view of the high CVD risk, it would be appropriate for patients with cSLE including those with hypertension, high *C4B* GCN, high serum levels of complement and a recurrent

presence of antiphospholipid antibodies to receive routine cardiovascular risk assessment, and probably prophylactic therapy to ameliorate the progress of underlying disease. It remains to be established if and to what extent anti-inflammatory therapy would give additional benefit.

Our study was limited by the lack of data on steroid exposure and SLE disease activity. Furthermore, blood pressures measured in the APPLE trial were not performed in a highly standardised fashion. Next, our study relied on CIMT as a surrogate marker for atherosclerosis. This is a widely accepted measurement but there are novel measurements of arterial stiffness such as pulse wave velocity that could be explored in future studies. The post-hoc nature of our analysis is another limitation. Prospective analysis of patients with cSLE who fall into our 'high-risk' groups using standardised and repeat blood pressure measurements would help support our findings. Our findings regarding effective response to atorvastatin in individuals with medium-to-high *C4B* GCN were also limited by the relatively short follow-up time of the trial. Longer follow-up would be necessary to verify the effect of *C4B* GCN as it pertains to attenuation of CIMT progression.

In conclusion, serial serum C4 and C3 levels are remarkably higher in patients with cSLE with a history of hypertension than in those without. Individuals with ≥2 copies of *C4B* are more likely to have a history of hypertension, and progression of atherosclerosis among these subjects was attenuated when treated with atorvastatin compared with placebo. Knowledge of a patient's complement genetic profile can be useful in risk stratification and therapy of CVD in SLE.

**Author affiliations**
[1]Abigail Wexner Research Institute, Nationwide Children's Hospital, Columbus, Ohio, USA
[2]Division of Rheumatology, Nationwide Children's Hospitatl, Columbus, OH, USA
[3]Department of Pediatrics, The Ohio State University, Columbus, Ohio, USA
[4]Center for Autoimmune Genomics and Etiology, Cincinnati Children's Hospital Medical Centre, Cincinnati, Ohio, USA
[5]Center for Microbial Pathogenesis, Abigail Wexner Research Institute, Columbus, Ohio, USA
[6]Department of Medicine, University Hospitals/Case Medical Center, Cleveland, Ohio, USA
[7]Department of Rheumatology, Hospital for Sick Children and Univeristy of Toronto, Toronto, Ontario, Canada
[8]Department of Pediatrics, University of Cincinnati, Cincinnati Children's Hospital Medical Center, PRCSG Coordinating Center, Cincinnati, Ohio, USA
[9]Department of Medical Microbiology and Immunology, Loyola University Chicago, Chicago, Illinois, USA
[10]Division of Biostatistics, College of Public Health, The Ohio State University, Columbus, Ohio, USA
[11]Department of Pediatrics, Duke University, Durham, North Carolina, USA
[12]Center for Microbial Pathogenesis, Abigail Wexner Research Institute, Nationwide Children's Hospital, Columbus, Ohio, USA

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**Contributors** SA, LES, EM and CYY conceived project. ST, SA, NS, DL, HB and LES examined and approved study protocol, provided APPLE repository genomic DNA and plasma samples, repository data and inputs on rheumatic disease studies. CYY, BZ, GRY, EHK and YLW designed and performed molecular genetics and immunology experiments. EM, SA, HNN and CYY conducted clinical, genetic and immunologic data analyses. EM, SA, HNN and CYY drafted the first version of the manuscript. All authors read and approved the final draft of the manuscript.

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**Patient consent for publication** Not required.

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**Data availability statement** Aggregate research data is presented in this manuscript. APPLE clinical trial data can be requested from the APPLE Data Committee.



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