Additional analysis of the APPLE (Atherosclerosis Prevention in Paediatric Lupus Erythematosus) trial identifies novel determinants of patient heterogeneity and a distinct lipid metabolomic signature associated with atherosclerosis progression

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Abstract

Background: Juvenile-onset systemic lupus erythematosus (JSLE) is associated with chronic inflammation and increased risk of atherosclerosis. The APPLE trial was a randomised, placebo-controlled trial of atorvastatin for atherosclerosis progression in JSLE, using carotid intima-media thickness (CIMT) measurements as primary outcome.

Methods: Unsupervised clustering analysis was used to stratify JSLE patients by their baseline CIMT and identify patterns of CIMT progression over 36 months. An additional in-depth metabolomic analysis was performed to identify lipidomic signatures predictive of CIMT progression. Correlation and univariate regression analyses explored associations between patient and disease characteristics and serum biomarkers. Machine learning techniques and ROC analyses were used to identify and validate a serum metabolomic signature of high CIMT progression.

Findings: Baseline CIMT measurements stratified JSLE patients into three groups with distinct CIMT progression trajectories irrespective of the treatment allocation. Two distinct CIMT progression rates (high vs. low), characterised by higher total and low-density lipoprotein (LDL) cholesterol levels (P=0.001 and P=0.002, respectively) were found in the placebo group, while patients treated with atorvastatin had three distinct CIMT trajectories (high, intermediate and low progression), not associated with any relevant biomarkers. A robust metabolomic signature predictive of high CIMT progression in the placebo arm was identified (AUC = 80.7%).

Interpretation: This complementary analysis of the APPLE trial provides new evidence for the significant heterogeneity of subclinical atherosclerosis in JSLE and its distinct progression trajectories irrespective of treatment allocation. Clinical trial patient stratification using the newly identified metabolomic signature predictive of increased natural atherosclerosis progression rate may improve results. Despite being effective in lowering serum lipids, atorvastatin did not prevent the CIMT progression in many at risk JSLE patients, highlighting the need for personalised therapies to address various molecular mechanism driving atherosclerosis in JSLE.

Research in context

Evidence before this study

Juvenile-onset systemic lupus erythematosus (JSLE) is associated with an increased risk of cardiovascular disease (CVD) in young people, leading to significant CVD-related morbidity and mortality compared to healthy individuals of the same age. Despite this, there are limited comorbidity-tailored treatment recommendations or research directed towards stratifying and managing JSLE patients based on their CVD risk. We searched PubMed, Web of Science, and Google Scholar for research articles published between Jan 1, 1990, and November 30, 2022, using the following search terms: "cardiovascular disease", "cardiovascular risk", "cardiovascular risk factors", "cardiovascular risk management", "vascular scans", "carotid intima-media thickness - CIMT", "statins", "lipids", "cholesterol", "lipoproteins" and "(juvenile-onset) systemic lupus erythematosus". We also searched for articles published in top-rated rheumatology and cardiology journals in the same time period. Published abstracts were excluded. The earliest referenced article was published in 2001. We found that CIMT is a validated measure of atherosclerosis, which was shown to predict CVD-related events from childhood into middle-age and improve the performance of traditional CVD-risk classification methods. Several studies have found a significant increase in CIMT in children and young people with JSLE. The Atherosclerosis Prevention in Paediatric Lupus Erythematosus (APPLE) trial was a randomized (1:1), double-blind, placebo-controlled study of atorvastatin for subclinical atherosclerosis prevention in young people with JSLE (mean age 15 years). The APPLE trial, which is the only interventional trial addressing subclinical atherosclerosis in JSLE, did not meet its primary endpoint which was significant reduction in CIMT progression over 36 months in the statin arm compared to placebo. A secondary analysis suggested that post-pubertal JSLE patients with increased high sensitivity CRP levels were more likely to respond to statins. Growing evidence suggests that molecular stratification of CVD-risk in JSLE patients would benefit future clinical trials for targeted interventions to be successful.

Added value of this study

This study is a re-analysis of the APPLE trial data which presents the first CVD-risk stratification in JSLE based on comprehensive CIMT measurements and shows that JSLE patients had considerable heterogeneity at the start of the trial. This analysis also independently explored CIMT progression in both the placebo and atorvastatin arms of the

study and stratified patients based on how they progressed during the study (high vs. low progression). Treatment with statins made a significant difference in the CIMT progression observed in the statin vs. placebo arm only for the patients stratified in the low progression group. In addition, the serum lipid metabolomic profile of JSLE patients recruited to the APPLE trial was investigated at baseline, leading to the identification of a predictive signature of increased CIMT progression over 36 months in the placebo arm. However, this predictive lipid metabolomic signature was not observed in the patients treated with statins, despite evidence of high CIMT progression in a subgroup. Overall, these data suggested that statins were not able to prevent high subclinical atherosclerosis progression in a specific subgroup of JSLE patients, likely due to factors independent from lipid dysregulation.

Implications of all the available evidence

This in-depth additional analysis of the APPLE trial confirms the heterogeneity of CVD-risk in JSLE patients at baseline and over 36 months as well as patient and disease-related factors associated with this heterogeneity. The newly identified lipid metabolomic signature at baseline could allow for the precision stratification of children and young people with JSLE based on their CVD-risk. However, despite this, lowering serum lipids with atorvastatin was not effective in preventing subclinical atherosclerosis progression in a subset of at-risk JSLE patients. The evidence from this study is important for future research for tailored strategies to address CVD-risk in JSLE and supports the use of omics analyses for biomarker stratification to inform patient selection for clinical trials. This study also provides evidence for the need to explore other mechanisms related to atherosclerosis progression in JSLE independent of lipid dysregulation. These findings are particularly relevant for a disease like JSLE, associated with chronic inflammation likely to significantly contribute to increased CVD-risk and which has no cure, in addition to affecting young people who have to live with the disease for longer. Future research is required for personalised treatment strategies based on risk stratification as well as novel atherosclerosis mechanism identification to enable the future success of clinical trials.

Introduction

Juvenile-onset systemic lupus erythematosus (JSLE) accounts for approximately 15-20% of patients with SLE. JSLE is a rare disease, with ~10,000 and ~200,000 children and young people (CYP) estimated to live with the disease in the UK and the US, respectively [1,2]. JSLE is characterized by a more severe clinical phenotype compared to adults, leading to increased co-morbidity burden, including a significantly increased risk of developing cardiovascular disease (CVD). The impact of increased CVD-risk from early onset of SLE has considerable individual and societal implications. In addition, there are recognised ethnic disparities in relation to SLE incidence and prevalence rates (2-3 times higher in people of Black and Asian descent compared to White population [3]), and ethnic differences in clinical presentation and severity of JSLE [4].

Notably, JSLE patients have an estimated 100-300-fold increased CVD-related mortality compared to age-matched healthy CYP [5]. Sub-clinical atherosclerosis (chronic inflammation of the large arteries with a long asymptomatic course which is a major cause of CVD) was detected in ~32% JSLE patients [6]. A retrospective analysis of the large UK JSLE cohort (n=413) identified 12 CVD-related events, which occurred at a median age of 16 years and median disease duration of only two years [7]. However, despite strong evidence of increased CVD-risk in patients with JSLE, comorbidity-tailored treatment recommendations or research directed towards stratifying and managing JSLE patients based on CVD-risk are limited [8,9]. Notably, a growing body of evidence, including data generated by our group, support that circulating biomarkers can predict CVD-risk in healthy CYP [10,11] and CYP with JSLE [12,13].

Carotid intima-media thickness (CIMT) is a measure of atherosclerosis which can be used to predict CVD-related events from childhood into middle-age [14] and improve the performance of traditional risk factors used for CVD-risk classification [15]. Various studies have found a significant increase in CIMT in CYP with JSLE [6,16]. The Atherosclerosis Prevention in Paediatric Lupus Erythematosus (APPLE) trial was a randomized, double-blind, placebo-controlled study of atorvastatin for subclinical atherosclerosis prevention in JSLE [17]. The trial failed to meet its primary end point, which was a significant decrease in the rate of CIMT progression between atorvastatin and placebo arms, although it showed rates of CIMT progression in the placebo group comparable to those reported in CYP with familial hypercholesterolemia[18]. A secondary analysis identified that atorvastatin-treated post-pubertal patients with elevations in baseline high sensitivity C-Reactive Protein (hsCRP) had lower CIMT rates of progression [18], suggesting that JSLE patient heterogeneity contributed to the negative results in the primary analysis. Future clinical trials success may depend on correct patient stratification for targeted interventions.

We hypothesised that JSLE patients recruited to the APPLE trial could be stratified based on biomarkers with potential utility for tailored CVD-risk management strategies yielding better patient selection for clinical trials. To address this, we performed an in-depth analysis of patient, disease, and lipid metabolic factors that underpin CVD-risk heterogeneity in JSLE patients, using data and serum samples collected in the APPLE trial.

Materials and methods

APPLE cohort

Access to clinical, serological and vascular scan data, as well as matched serum samples from the JSLE cohort enrolled in the APPLE trial was facilitated by an international collaboration with the Childhood Rheumatology Research Alliance (CARRA) and APPLE trial investigators (USA). The APPLE trial was a prospective multi-centre cohort of 221 CYP with JSLE (age 10-18 at inclusion) recruited from various sites in North America and followed for 36 months [17]. Subjects were randomized 1:1 to receive either placebo (N=108) or atorvastatin (N=113). All subjects met well defined inclusion/exclusion criteria as per published protocol [17].

For these study analyses, we investigated a trial sub-cohort, consisting of 151 JSLE patients (77 atorvastatin-arm; 74 placebo-arm [**Supplementary Table 1**] with complete baseline data and matched serum samples. In addition, we investigated CIMT progression over 36 months in another sub-cohort of 121/151 JSLE patients (60 placebo-arm [**Table 1**], 61 atorvastatin-arm [**Table 2]**) who completed the APPLE trial and had complete datasets to enable the analysis. Data related to various patient and disease related features were available as collected per the APPLE trial protocol.

CIMT measurements in the APPLE cohort

The APPLE investigators provided relevant CIMT measurements collected as per trial protocol [17,18], which included assessment of the thickness of 12 vascular sites using similar ultrasound machines and a central reader [17]. The mean of the mean common CIMT (MMeanIMT) measurement was the revised primary endpoint of the APPLE trial [17]. CIMT measures were collected at different time points: baseline, 6 months, 12 months, and 36 months (end of trial). CIMT progression (ΔCIMT) was calculated by subtracting the mean of each of 12 CIMT measurements at 36 months from the corresponding 12 CIMT measurements at baseline.

Metabolomics

Measures of 250 serum biomarkers were acquired with an established NMR-spectroscopy platform (Nightingale Health, https://nightingalehealth.com/) [19]. These included both absolute concentrations

(mmol/L), ratios, and percentages (%) of lipoprotein composition of numerous metabolites (**Supplementary Table 2).**

Statistical analysis

Statistical tests were performed in R and GraphPad Prism. Data were assessed for normality and analysed with parametric or nonparametric tests, as appropriate. Chi-square test and Fisher's exact test were used for comparison between categorical variables. Details of statistical tests and parameters accounted for in the analyses are given in the figure legends. $P<0.05$ was considered statistically significant. Bonferroni correction was applied for multiple testing**.** Data analysis pipeline is detailed in **Supplementary Figure 1**.

Univariate logistic regression

The association between lipid serum biomarkers and JSLE parameters was assessed by univariate logistic regression analysis adjusted for age, sex and ethnicity. For each measurement, the odds ratio (OR) and the 95% confidence interval (CI) were determined. The p-value for each association was calculated in the logistic regression analysis. Results were visualized in a forest plot using the R package ggforestplot developed by Nightingale Health.

Sparse partial least squares discriminant analysis (sPLS-DA)

This supervised machine learning approach was operated using the mixOmics package in R[20] (for details see **Supplemantary Methods** explanation).

Association analysis

Pearson correlation coefficients were used to assess associations between serum biomarkers and patient and disease characteristics. Significant correlations ($p<0.01$) were visualized in circle plots using the R package circlize [21].

Results

Baseline CIMT measurements stratify patients with JSLE into three groups, each associated with distinct CIMT progression trajectories irrespective of treatment allocation

The baseline CIMT heterogeneity of JSLE patients recruited to the APPLE clinical trial were assessed in a sub-cohort of 151 patients with a mean age of 15.6 years (range 10.3–21.7 years, 85% females). A summary of baseline characteristics is depicted in **Supplementary Table 1.**

Unsupervised hierarchical clustering was used to stratify the cohort using 12 CIMT measures at baseline. Three groups were identified with relatively high (N=44), intermediate (N=64) and low (N=43) baseline

CIMT measurements (**Figure 1A**). Compared to patients in the low and intermediate CIMT groups, JSLE patients with high baseline CIMT were significantly older (P=0.021) and had longer disease duration (P=0.021) (**Supplementary Table 1**). No significant differences between various patient and disease-related parameters, including lipid serum levels were found (**Supplementary Table 1**), except for creatinine clearance estimations which were significantly higher in the intermediate compared to the low baseline CIMT groups (**Supplementary Table 1**, P=0.017).

As a validation, the baseline MMeanIMT was significantly different between the three groups (high vs intermediate, P<0.0001; high vs low, P<0.0001; intermediate vs low, P<0.0001) (**Figure 1B**), thus supporting significant CIMT heterogeneity across the JSLE cohort which was maintained across the timeframe of the study (**Figure 1C**). There were distinct CIMT trajectories over 36 months of the three patient groups which did not crossover (**Figure 1D),** irrespective of treatment allocation.

Together these data demonstrate significant CIMT heterogeneity at baseline and CIMT progression at 36 months, despite minimal differences in demographic and disease features, supporting further investigation of factors contributing to distinct CIMT progression rates in JSLE.

JSLE patients in the placebo arm of the APPLE trial stratified into two groups based on untreated CIMT progression over 36 months

To examine the untreated progression of subclinical atherosclerosis, the change in the 12 CIMT measures from baseline to 36 months (ΔCIMT) was assessed in all the patients allocated to the placebo arm of the APPLE study (N=60) **(Table 1)**. Unsupervised hierarchical clustering stratified patients into two groups based on ΔCIMT with high (N=35) and low (N=25) CIMT progression (**Figure 2A**). A significant increase in MMeanIMT, was seen in the high CIMT progression group (P<0.0001) while a significant decrease (potentially explained by the impact of individual growth on vascular measurements and variability between ultrasound probe positioning between assessments) in MMeanIMT (P=0.001) characterised the low CIMT progression group (**Figure 2B**).

There were no significant differences in age, sex, puberty stages and ethnicity between the high and low CIMT progression groups (**Table 1**). Unsurprisingly, serum total cholesterol (P=0.0004) and LDL-cholesterol (P=0.002), known to be associated with atherosclerosis development, were significantly elevated in the high compared to low CIMT progression group (**Table 1**). In addition, baseline serum total cholesterol and LDL-cholesterol levels positively correlated with the ΔCIMT progression in the placebo group (**Supplementary Figure 2A**). There were also positive correlations between CIMT progression and various biomarkers, including homocysteine, and negative correlations with the spot urine protein:creatinine ratio and complement fraction C4. Damage index (SLICC-DI) was also

positively associated with CIMT progression (**Supplementary Figure 2A**). Taken together, these findings indicate that both chronic inflammation, reflected by correlations with JSLE biomarkers and validated outcome measures, as well as altered lipid metabolism, reflected by the abnormal lipid profile and homocysteine levels, may contribute to atherosclerosis progression in JSLE.

JSLE patients treated with atorvastatin in the APPLE trial stratified into 3 groups based on CIMT progression over 36 months

CIMT progression over 36 months (ΔCIMT) was also assessed in the atorvastatin arm of the APPLE trial (N=61) (**Table 2**). Unsupervised cluster analysis of ΔCIMT measures identified three distinct groups: high (N=22), intermediate (N=24) and low (N=15) CIMT progression groups (**Figure 2C**). No significant differences were observed across the three CIMT progression groups at baseline (**Table 2**) and few correlations between CIMT and clinical measures were identified (**Supplementary Figure 2B**). Most notably there were no correlations between CIMT progression and serum lipids, likely due to treatment with atorvastatin. The correlation analysis performed (**Supplementary Figure 2B**), suggests that atorvastatin treatment disrupted the association between various biomarkers and CIMT progression observed in the placebo group. Interestingly, complement fractions C3 and C4, biomarkers of serological activity in JSLE, were inversely associated with CIMT progression similar to an independent analysis of the APPLE trial[22], indicating that disease-related factors may drive CIMT progression despite statin treatment normalising the lipid profile.

Significant changes in MMeanIMT over 36 months were observed in high (increased, P<0.0001) and low (decreased, P=0.002) CIMT progression groups, while the intermediate group (P=0.51) had almost stable MMeanIMT measurements over 36 months (**Figure 2D**). Of note, the MMeanIMT progression over 36 months was significantly different between the placebo and statin arms for the low, but not for the high CIMT progression groups identified in the two treatment arms **(Figure 2E),** suggesting that atorvastatin made a difference only for patients with low CIMT progression rate**.** Together these results identified that only a small proportion of JSLE patients allocated to the statin treatment group (24.5%, N=15) had low CIMT progression over 36 months based on the unsupervised cluster analysis, and that they benefitted from treatment with statins as they progressed significantly less than the JSLE patients in the placebo arm stratified in the low CIMT progression group (41.6%, N=25).

Finally, to confirm the effect of atorvastatin in JSLE, we found a significant reduction in serum LDL cholesterol levels at 36 months in 73.8% of patients (**Figure 2F, Supplementary Figure 4**, P<0.0001). Serum LDL cholesterol did not significantly decrease in the placebo arm (**Figure 2F, Supplementary Figure 4**, P=0.61). Thus, despite the decrease of serum LDL cholesterol levels with atorvastatin

treatment, a sizeable proportion of patients (N=22, 36.1%) continued to CIMT progression, suggesting that CIMT progression was driven by factors independent from dysregulation of lipid metabolism.

Distinct baseline serum NMR metabolomic signatures which define the high CIMT progression group in the placebo arm did not predict CIMT progression in the atorvastatin-arm

Since high CIMT progression in JSLE patients in the placebo arm was positively associated with serum LDL and total cholesterol levels (although within accepted normal ranges) (**Table 1, Supplementary Figure 2A**), a more detailed NMR metabolomic analysis was performed (250 serum lipid-based metabolites, full list in **Supplementary Table 2**) at baseline (N=60).

Forty-eight metabolites were significantly upregulated in the high compared to the low CIMT progression group in the placebo arm (**Figure 3A**). The top six significantly increased metabolites selected after stringent Bonferroni correction included total esterified cholesterol, total cholesterol, phospholipids in small LDL, cholesterol in small LDL, free cholesterol in medium LDL and total lipids in small LDL (**Figure 3A-red labels and 3B**). This suggests that JSLE patients in the high CIMT progression group had a distinct, pro-atherogenic lipid metabolomic profile, dominated by cholesterol and LDL subsets. Using the six-metabolite signature combined, receiver operator curve (ROC) analysis in multivariate logistic regression showed an area under the curve (AUC) of 80.7%, higher than the individual metabolites alone (AUC range 74.4-75.9%) (**Figure 3C**). This was also higher than the AUC for total cholesterol (AUC of 76.3%) and LDL-cholesterol (AUC of 72.5%) levels measured in the APPLE trial (**Supplementary Figure 3A**), suggesting that these six metabolites could provide a biomarker signature for predicting CIMT progression in JSLE.

To support these findings, univariate logistic regression analysis was performed on all metabolites comparing the high and low CIMT progression groups in the placebo arm, accounting for clinical and treatment features. All six selected metabolites were increased in the high CIMT progression group (**Supplementary Figure 3B**). These results were further confirmed using supervised machine learning approaches. The optimized sparse partial least squares discriminant analysis (sPLS-DA) showed separation between the two CIMT progression groups and identified similar metabolites (highlighted in red) in the first component of the model as important in driving the high versus low CIMT progression stratification (**Supplementary Figure 3C-D**). Together, the further analysis validated the six-metabolite predictive signature of CIMT progression in the placebo arm (**Figure 3A-C**).

The same NMR metabolomics analysis pipeline was applied to the atorvastatin arm of the APPLE trial. Only two metabolomic markers (the ratio of docosahexaenoic acid to total fatty acids and isoleucine) were significantly different between the high and low CIMT progression groups (**Figure 3D-E**), with poor performance under ROC analysis (**Figure 4F**). Thus, no distinct baseline metabolomic signature was found between the high and low CIMT progression groups in the atorvastatin treatment arm. As neither routine serum lipid measures (**Supplementary Figure 2B**) nor the in depth metabolomic signature correlated with CIMT progression, these results show that in atorvastatin-treated patients, baseline lipid signatures do not predict CIMT progression, and that statin treatment abrogated the predictive signature of CIMT progression found in the placebo group. This suggests that CIMT progression could be driven by factors independent from dysregulation of lipid metabolism in statin treated patients.

Discussion

The current study included a re-analysis of existing APPLE trial data to explore ways to stratify JSLE patients to improve CVD-risk assessment, in addition to serum metabolic profiling of JSLE patients at baseline for atherosclerosis progression biomarker identification.

JSLE patients recruited to the APPLE trial, despite being young, already had different degrees of subclinical atherosclerosis. This study further explored subclinical atherosclerosis heterogeneity by stratifying patients into distinct groups and by defining distinct CIMT progression rates over 36 months, irrespective of treatment allocation. The only significant predictors of baseline CIMT unsupervised patient stratification were age, disease duration and creatinine clearance, supporting previous findings that longer SLE duration is associated with increased CVD-risk [23,24]. However, the other predictors of baseline CIMT identified by the multivariable analysis of the APPLE trial[25] (minority status, higher BMI, male sex, higher lipoprotein A, proteinuria, azathioprine use, and prednisone dose) did not differ between the baseline CIMT patient groups derived from this current unsupervised cluster analysis. No patient or disease-related significant differences were identified between the high versus low CIMT progression groups in the placebo arm either, apart from the increased levels of total and LDL-cholesterol in the high progression group. Although the second analysis of APPLE trial showed that hCRP and pubertal status predicted response to atorvastatin, our unsupervised cluster analysis did not identify these markers as being different between JSLE patients stratified on CIMT at baseline or according to the rate of their progression over 36 months [18].

JSLE patients allocated to the placebo arm provided the opportunity to examine untreated CIMT progression, as a validated measure for CVD-risk [26,27], and led to the identification of two patterns of

CIMT progression and a robust serum lipid signature which defined the JSLE patients who progressed at a higher rate. Previously, lipid metabolomics was extensively used for atherosclerosis risk prediction in SLE as it provides more in-depth information that routinely measured lipids (including particle size, and components). A machine learning model (using the same metabolomic platform we employed in this study) identified a lipidomic signature which distinguished adult-onset SLE patients with vs without atherosclerosis plaques on vascular scans with a good performance (AUC=80%) [28], while a high apolipoprotein-B:A1 ratio, linked with high CD8+ T cell phenotyping and transcriptomic profile was identified as potential marker for atherogenic progression in JSLE [12]. In our study, the 6-biomarker lipid signature outperformed the LDL-cholesterol and total cholesterol (used in routine practice) in identifying JSLE patients with high rates of natural CIMT progression. This metabolomic signature provides an opportunity to explore future validation in external JSLE cohorts, which we will be pursuing.

Three out of six metabolites defining the CIMT progression signature in the placebo arm are lipid components of small and dense LDL particles. The association between the size of LDL particles and atherosclerosis, including their prolonged retention in plasma and enhanced ability to penetrate the arterial wall have been explored before [29-31]. Lipid lowering drugs with smaller LDL targeted reduction properties, such as rosuvastatin, may represent a better targeted treatment choice for atherosclerosis prevention [32] for patients with JSLE, highlighting the need for more precise patient stratification to address the statin response heterogeneity found in JSLE.

Although accelerated atherosclerosis has been linked to many autoimmune rheumatic diseases, the association between JSLE disease activity and CIMT progression remains controversial, with some studies finding an association [6], while others did not [33]. In our analysis, the untreated CIMT progression correlated positively with a pro-atherogenic lipid profile and presence of SLICC JSLE damage, suggesting that JSLE severity contributes to atherosclerosis, similar to previous reports [34].

One possible explanation for the APPLE trial not meeting its primary endpoint is offered by the CIMT progression stratification in the atorvastatin arm, which identified a subgroup of JSLE patients that progressed at a high rate despite atorvastatin successfully lowering their pro-atherogenic lipid profile. This indicates alternative mechanisms underpinning their atherosclerosis progression, as the high CIMT progressors receiving statin treatment were not defined at baseline by the metabolomic signature which characterised the high progressors in the placebo group. Together, these findings support the hypothesis of complementary atherosclerosis mechanisms in JSLE, very likely related to dysregulated lipid metabolism, chronic inflammation, and endothelial dysfunction, possibly modulated in distinct ways in the high vs. low CIMT progression groups. The investigation of molecular mechanisms of atherosclerosis in JSLE or that of anti-inflammatory and metabolic therapeutic benefits of atorvastatin are beyond the scope of this paper.,

As with many other CVD measures, CIMT alone is not an ideal measure for predicting CVD-risk in CYP because of challenges of standardisation across age. Factors contributing to the heterogeneity of the CIMT measures include variable ultrasound probe positioning, and potential individual heterogeneity in the context of pubertal growth during the trial, despite the use of a standardised vascular ultrasound protocol and that of a central reader in the APPLE trial. These factors, in addition to lifestyle advice provided to all patients and other unidentified factors might explain why some patients surprisingly experienced CIMT regression over time in both the low progression groups in the placebo and statin arms. However, there was no difference in the disease activity between the high or low progression groups in both arms and CIMT progression did not correlate with the disease activity.

This complementary analysis of the APPLE trial provides evidence for the limitations of restricting CVD risk factor assessment to traditional CVD variables in JSLE patients who have distinct trajectories of subclinical atherosclerosis progression. In addition, demographic, and disease characteristics, as well as routine lipid profiling did not identify JSLE patients with increased CVD-risk, and although effective in lowering serum lipids, atorvastatin did not prevent subclinical atherosclerosis progression in many at risk JSLE patients. Further research into the mechanisms driving the unique lipidomic signature predictive of CIMT progression we identified in the untreated patients, as well as investigation of other pro-inflammatory and metabolic pro-atherosclerotic mechanisms not influenced by statins may potentially support future personalised therapeutic strategies to address the increased CVD-risk in JSLE.

Funding

The APPLE study was supported by the NIH (National Institute of Arthritis and Musculoskeletal and Skin Diseases contract N01-AR-2-2265), the Edna and Fred L. Mandel Jr. Center for Hypertension and Atherosclerosis, and Pfizer, which provided atorvastatin and matching placebo.
This work was supported by a Versus Arthritis PhD Studentship (22908) and Career Development Fellowship (22856), as well as grants from the National Institute of Heath Research (NIHR) - University College London Hospital (UCLH) Biomedical Research Centre grant, BRC773/III/CC/101350 and Lupus UK and was performed within the Centre for Adolescent Rheumatology Versus Arthritis at University College London (UCL), UCLH and Great Ormond Street Hospital (GOSH) supported by grants from Versus Arthritis (21593 and 20164), Great Ormond Street Children's Charity, and the NIHR-Biomedical Research Centres at both GOSH and UCLH. The views expressed are those of the authors and not necessarily those of the National Health System (NHS), the NIHR or the UK Department of Health. Dr Lewandowski was funded by the NIAMS Intramural Program. The APPLE study was funded by the National Institutes of Health (NIAMS N01-AR-2-2265).

Acknowledgements

We would like to thank all participants and hospital sites that recruited patients for the APPLE study. We would like to thank CARRA for support for this project and review of the manuscript.

Collaborators: The following participated in this study by enrolling patients at sites or by performing study procedures at sites: Stacy Ardoin, Esi Morgan Dewitt, C Egla Rabinovich, Janet Ellis, Kelly Mieszkalski, Janet Wootton (Duke University Medical Center, Durham, North Carolina), Peter Chira, Joyce Hsu, Tzielan Lee, Christy Sandborg, Jan Perea (Stanford University School of Medicine, Palo Alto, California), Beth Gottlieb, Patricia Irigoyen, Jennifer Luftig, Shaz Siddiqi, Zhen Ni, Marilynn Orlando, Eileen Pagano (Cohen Children's Medical Center, New Hyde Park, New York), Andrew Eichenfield, Lisa Imundo, Deborah Levy, Philip Kahn, Candido Batres, Digna Cabral (Morgan Stanley Children's Hospital of New York–Presbyterian, New York, New York), Kathleen A. Haines, Yukiko Kimura, Suzanne C. Li, Jennifer Weiss, Mary Ellen Riordan, Beena Vaidya (Hackensack University Medical Center, Hackensack, New Jersey), Emily von Scheven, Michelle Mietus-Snyder (University of California at San Francisco Medical Center, San Francisco, California), Earl Silverman, Lawrence Ng (Hospital for Sick Children, Toronto, Ontario, Canada), Suzanne Bowyer, Susan Ballinger, Thomas Klausmeier, Debra Hinchman, Andrea Hudgins (Indiana University School of Medicine, Indianapolis, Indiana), Marilynn Punaro, Shirley Henry, Shuzen Zhang (Texas Scottish Rite Hospital for Children, Dallas, Texas), Nora G. Singer, Elizabeth B. Brooks, Stacy Miner, Nancy Szabo, Lisabeth Scalzi (University Hospitals/ Case Medical Center, Cleveland, Ohio), David Sherry, Libby Dorfeld, Sarajane Wilson, Jenna Tress (Children's Hospital of Philadelphia, Philadelphia, Pennsylvania), Deborah McCurdy, Tatiana Hernandez, Jyotsna Vitale (University of California Los Angeles Medical Center, Los Angeles, California), Marisa Klein-Gitelman, Angela Kress, Nicole Lowe, Falguni Patel (Children's Memorial Hospital, Chicago, Illinois), Carol Wallace, Stephanie Hamilton (Seattle Children's Hospital and Regional Medical Center, Seattle, Washington), Richard Silver, Katie Caldwell, Diane Kamen (Medical University of South Carolina, Charleston, South Carolina), Linda Wagner-Weiner, Becky Puplava, Atanas Lonchev (University of Chicago, Chicago, Illinois), Gloria Higgins, Monica Bacani (Nationwide Children's Hospital, Columbus, Ohio), Hermine Brunner, Cynthia Rutherford, Jamie Meyers-Eaton, Shannen Nelson, Alexei Grom (Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio), Larry Jung, Teresa Conway, Lacey Frank, Lori Kuss (Creighton University Medical Center, Omaha, Nebraska), Jenny Soep, Hazel Senz (University of Colorado, Aurora, Colorado), Ann Reed, Thomas Mason, Jane Jaquith, Diana E. Paepke-Tollefsrud (Mayo Clinic, Rochester, Minnesota).

Contributors

CC, ECJ, GAR and PD designed the study; SPA, LES, LL acquired the data. All authors had access to the data. GAR, CC and JP accessed and verified the data. JP and GAR analysed the data. JP, GAR, ECJ and CC wrote the manuscript. SPA, LES LL and PD reviewed the manuscript. All authors approved the final version.

Declaration of interests

The authors declared no relevant conflicts of interest.

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Figure Legends:

Figure 1: JSLE stratification (all APPLE patients with complete baseline data, N=151) by baseline CIMT (12 measures). A) Baseline CIMT measures of patients with juvenile-onset SLE were stratified using unsupervised hierarchical clustering. All 12 CIMT measures were standardised within each row by Z score and plotted as a heat map, representing the relationship to the mean of the group (red represents relatively high CIMT measures and blue represents relatively low CIMT measures). Each column represents a patient with JSLE. Three groups of patients with distinct baseline CIMT profiles were identified. B-C) Box and whisker plots show baseline and 36-month MMeanIMT measurements (APPLE primary outcome) in the identified high, intermediate and low baseline CIMT groups. Comparisons between groups were performed using Wilcoxon signed-rank test (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). D) Distinct longitudinal MMeanIMT progression from baseline to 36 months of the high, intermediate and low CIMT progression groups (Mean, 95% CI), irrespective of treatment allocation. (Only JSLE patients with completed CIMT data at 36 months were included in the panel C-D, N=121). **Legend:** CIMT- carotid intima-media thickness; MMeanIMT - Mean-Mean IMT common carotid artery measurement.

Figure 2. JSLE stratification by ΔCIMT (12 measurements) at 36 months in the placebo and atorvastatin groups. A) JSLE patients allocated to the placebo group (only the placebo arm patients with completed CIMT data at 36 months were included in the panel, N=60) were stratified based on delta (Δ) CIMT measurements unsupervised hierarchical clustering. All 12 ΔCIMT measurements were standardised within each row by Z score and plotted as a heat map, representing the relationship to the mean of the group (red represents relatively high CIMT measures and blue represents relatively low CIMT measures). Each column represents a patient with JSLE. Two groups of patients were identified with distinct CIMT progression over 36 months. B) Box and whisker plots showing comparisons between high and low CIMT progression group at baseline and 36 months**.** C) JSLE patients allocated to the atorvastatin group (N=61) were stratified based on Δ CIMT measurements using unsupervised hierarchical clustering. All 12Δ CIMT measurements were standardised within each row by Z score and

plotted as a heat map, representing the relationship to the mean of the group (red represents relatively high CIMT measures and blue represents relatively low CIMT measures). Each column represents one patient with JSLE. Two groups of patients were recognised with distinct CIMT progression over 36 months. D) Box and whisker plots showing comparison of high, intermediate and low CIMT progression group at baseline and 36 months. E) Box and whisker plots showing comparison of high/low CIMT progression group between placebo and atorvastatin arm patients. (Wilcoxon signed-rank test or t-test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$)**.** F) Clinical LDL-cholesterol trajectory of placebo and atorvastatin group over 36 months. **Legend:** CIMT- carotid intima-media thickness; MMeanIMT - Mean-Mean IMT common carotid artery measurement.

Figure 3. Baseline serum metabolomics comparisons between different CIMT progression group in placebo and atorvastatin group (a total 245 metabolites were included in the analysis after data cleaning). (A-C) Comparison between the metabolomic profiles of high (N=35) vs. low (N=25) CIMT progression groups in the placebo arm. A) Volcano plots displaying fold change of all metabolites and Log10 p values comparing high and low CIMT progression groups in the placebo arm ($p<0.01$; log2(fold change) >0.2). Top six metabolites (measured in mmol/L; significantly different after Bonferroni correction, $p<0.1$) were highlighted in red. B) Box and whisker plots showing the top six metabolite levels of the high vs low CIMT progression groups in the placebo arm. (Unpaired t-test, * $p<0.05$; ** $p<0.01$; *** $p<0.001$). C) ROC analysis for discriminating high vs low CIMT progression groups using the top 6 metabolites combine and separately, showed in AUC. D-F) Comparing metabolomic profile between high (N=22), intermediate (N=24) and low (N=15) CIMT progression patient groups in the atorvastatin arm. D) Volcano plots displaying fold change of all metabolites and Log10 p values comparing high and low CIMT progression groups in the atorvastatin arm ($p<0.05$; log2(fold change) >0.2). E) Box and whisker plots showing the top 6 metabolites (measured in mmol/L; significantly different after Bonferroni correction) from analysis comparing high (N=22) vs low (N=15) CIMT progression groups in the atorvastatin arm. (Unpaired t-test, * $p<0.05$; ** $p<0.01$; *** $p<0.001$). F) ROC analysis for discriminating high vs low CIMT progression groups using the DHA% and Isoleucine, showed in Area Under the Curve (AUC). **Legend**: AUC - area under the curve; CIMT- carotid intima-media thickness; ROC- Receiver Operator Curve; Abbreviations and full names of all metabolites are listed in the Appendix.

Table 1: Demographic comparison between the high CIMT progression group and low CIMT progression group in the APPLE study placebo-treated participants (N=60).

	Total Placebo	High CIMT progression group	Low CIMT progression group	P *
Number	60	35	25	-
Sex, no. (%) female	51 (85.0)	29 (82.9)	22 (88.0)	0.855
Puberty at baseline, no. (%) post puberty	38 (63.3)	21 (60.0)	17 (68.0)	0.564
Age, mean ± SD years	15.50 ± 2.48	15.46 ± 2.49	15.56 ± 2.52	0.876
Race, no. (%)				0.848
White	35 (58.33)	19 (54.29)	16 (64.0)	
Black	13 (21.67)	8 (22.86)	5 (20)	
Asian	4 (6.67)	3 (8.57)	1 (4.0)	
Other	8 (13.33)	5 (14.29)	3 (12)	
History of smoking, no. (%)	0 (0)	0 (0)	0 (0)	-
Annual household income, no. (%)				0.763
<$25,000	16 (26.67)	9 (25.71)	7 (28)	
$25,000–49,999	17 (28.33)	9 (25.71)	8 (32)	
$50,000–74,999	7 (11.67)	5 (14.29)	2 (8)	
$75,000–99,999	8 (13.33)	6 (17.14)	2 (8)	
$100,000–150,000	6 (10)	2 (5.71)	4 (16)	
>$150,000	3 (5)	2 (5.71)	1 (4)	
Body mass index, mean ± SD kg/m²	24.51 ± 6.19	24.91 ± 6.60	23.94 ± 5.66	0.555
Duration of lupus, mean ± SD months	28.05 ± 30.11	27.89 ± 34.68	28.28 ± 22.88	0.961
SLEDAI, mean ± SD	4.02 ± 3.96	4.51 ± 3.98	3.32 ± 3.90	0.253
SLICC DI, mean ± SD	0.333 ± 0.774	0.457 ± 0.886	0.160 ± 0.554	0.144
History of hypertension, no. (%)	23 (38.3)	16 (45.7)	7 (28.0)	0.262
dsDNA antibody positive, no. (%)	45 (75.0)	24 (68.6)	21 (84.0)	0.290
Creatinine clearance, mean ± SD ml/minute/m²	133.18 ± 28.66	134.59 ± 28.24	131.21 ± 29.7	0.891
C3, mean ± SD mg/dl	106.2 ± 25.24	110.50 ± 24.53	100.05 ± 25.50	0.121
C4, mean ± SD mg/dl	16.95 ± 7.72	17.85 ± 8.18	15.63 ± 6.96	0.282
Medications (past 30 days)				
Aspirin, no. (%)	43 (71.67)	24 (68.57)	19 (76)	0.735
Hydroxychloroquine, no. (%)	59 (98.33)	34 (97.14)	25 (100)	1
Multivitamin, no. (%)	42 (70)	23 (65.71)	19 (76)	0.568
Corticosteroids, no. (%)	48 (80)	29 (82.86)	19 (76)	0.743
Cyclophosphamide, no. (%)	10 (16.67)	6 (17.14)	4 (16)	1
Mycophenolate mofetil, no. (%)	11 (18.33)	8 (22.86)	3 (13.04)	0.463
Azathioprine, no. (%)	11 (18.33)	7 (20)	4 (16)	0.955
Methotrexate, no. (%)	8 (13.33)	5 (14.29)	3 (12)	1
Rituximab, no. (%)	0 (0.0)	0 (0.0)	0 (0.0)	-
NSAIDs, no. (%)	19 (31.67)	9 (25.71)	10 (40)	0.373
ACE inhibitor, no. (%)	17 (28.33)	11 (31.43)	6 (24)	0.735
hsCRP, mean ± SD mg/liter	2.88 ± 6.50	2.93 ± 6.13	2.82 ± 7.11	0.953
Homocysteine, mean ± SD µmoles/liter	7.52 ± 4.24	8.08 ± 4.97	6.76 ± 2.91	0.24
Lipid levels, mean ± SD mg/dl				
Total cholesterol	144.59 ± 31.3	156.97 ± 32.91	127.76 ± 19.12	**<0.001**
HDL cholesterol	45.92 ± 12.71	48.38 ± 13.53	42.56 ± 10.88	0.082
LDL cholesterol	74.09 ± 26.75	83.24 ± 27.98	62.00 ± 19.71	**0.002**
Triglycerides	128.12 ± 94.52	136.62 ± 115.75	116.56 ± 54.09	0.425
Lipoprotein A	12.25 ± 16.04	14.82 ± 17.61	8.76 ± 13.17	0.153

Table 1: Demographic comparison between the high CIMT progression group and low CIMT progression group in the APPLE study placebo-treated participants (N=60). *Chi-squared test or Wilcoxon signed-rank test. Tanner Stage 4-5 are classified as post-puberty. **Legend:** ACE - angiotensin-converting enzyme inhibitors; C3, C4 – complement fractions C3,C4; HDL- high-density lipoprotein; hsCRP - high sensitivity C-Reactive Protein; LDL- low-density lipoprotein; NSAIDs - non-steroidal anti-inflammatory drugs; SLEDAI – Systemic Lupus Erythematosus Disease Activity Index; SLICC DI - Systemic Lupus International Collaborating Clinics Damage Index.

Table 2: Demographic comparison between the high, intermediate and low CIMT progression group in the APPLE study atorvastatin-treated participants (N=61).

	Total	CIMT progression groups			P*
		High	Intermediate	Low	
Number	61	22	24	15	-
Sex, no. (%) female	49 (80.3)	17 (77.3)	21 (87.5)	11 (73.3)	0.503
Puberty at baseline. (%) post-puberty	35 (57.4)	13 (60.1)	13 (54.2)	9 (60.0)	0.919
Age, mean ± SD years	15.34 ± 2.72	14.87 ± 2.51	15.21 ± 2.93	16.24 ± 2.63	0.314
Race, no. (%)					0.677
White	23 (37.7)	9 (40.9)	9 (37.5)	5 (33.3)	
Black	16 (26.23)	6 (27.3)	5 (20.8)	5 (33.3)	
Asian	5 (8.2)	0 (0.0)	3 (12.5)	2 (13.3)	
Other	17 (27.87)	7 (31.8)	7 (29.2)	3 (20.0)	
History of smoking, no. (%)	0 (0)	0 (0)	0 (0)	0 (0)	-
Annual household income, no. (%)					0.167
<$25,000	17 (27.87)	4 (18.2)	12 (50.0)	1 (7.1)	
$25,000–49,999	15 (24.59)	8 (36.4)	3 (12.5)	4 (28.6)	
$50,000–74,999	6 (9.84)	3 (13.6)	3 (12.5)	0 (0.0)	
$75,000–99,999	7 (11.48)	2 (9.1)	2 (8.3)	3 (21.4)	
$100,000–150,000	7 (11.48)	2 (9.1)	2 (8.3)	3 (21.4)	
>$150,000	4 (6.56)	1 (4.5)	1 (4.2)	2 (14.3)	
Body mass index, mean ± SD kg/m²	24.17 ± 4.73	22.97 ± 4.38	24.57 ± 5.40	25.31 ± 3.91	0.298
Duration of lupus, mean ± SD months	28.26 ± 29.94	25.68 ± 20.37	28.79 ± 28.34	31.20 ± 43.37	0.858
SLEDAI, mean ± SD	5.38 ± 4.74	6.55 ± 5.83	4.38 ± 3.62	5.27 ± 4.45	0.303
SLICC DI, mean ± SD	0.393 ± 0.714	0.23 ± 0.53	0.42 ± 0.72	0.60 ± 0.91	0.295
History of hypertension, no. (%)	17 (27.9)	5 (22.7)	7 (29.2)	5 (33.3)	0.766
dsDNA antibody positive, no. (%)	51 (83.6)	18 (81.8)	19 (79.2)	14 (93.3)	0.489
Creatinine clearance, mean ± SD ml/minute/m²	147.25 ± 34.40	158.09 ± 45.41	141.95 ± 22.07	139.82 ± 29.76	0.179
C3, mean ± SD mg/dl	99.57 ± 28.05	84.28 ± 36.44	92.55 ± 41.53	96.53 ± 34.33	0.608
C4, mean ± SD mg/dl	13.87 ± 6.36	11.76 ± 5.26	14.04 ± 6.84	16.89 ± 6.25	0.058
Medications (past 30 days)					
Aspirin, no. (%)	36 (59.02)	11 (50.0)	15 (62.5)	10 (66.7)	0.543
Hydroxychloroquine, no. (%)	60 (98.36)	22 (100.0)	23 (95.8)	15 (100.0)	0.457
Multivitamin, no. (%)	44 (72.13)	17 (77.3)	16 (66.7)	11 (73.3)	0.72
Corticosteroids, no. (%)	51 (83.61)	20 (90.9)	17 (70.8)	14 (93.3)	0.093
Cyclophosphamide, no. (%)	8 (13.11)	3 (13.6)	2 (8.3)	3 (20.0)	0.574
Mycophenolate mofetil, no. (%)	15 (24.59)	5 (22.7)	6 (25.0)	4 (26.7)	0.962
Azathioprine, no. (%)	8 (13.11)	3 (13.6)	4 (16.7)	1 (6.7)	0.664
Methotrexate, no. (%)	5 (8.2)	1 (4.5)	3 (12.5)	1 (6.7)	0.598

Rituximab, no. (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	-
NSAIDs, no. (%)	20 (32.79)	7 (31.8)	7 (29.2)	6 (40.0)	0.776
ACE inhibitor, no. (%)	13 (21.31)	5 (22.7)	4 (16.7)	4 (26.7)	0.744
hsCRP, mean ± SD mg/liter	2.87 ± 9.66	2.11 ± 3.56	4.44 ± 15.00	1.48 ± 2.16	0.59
Homocysteine, mean ± SD µmoles/liter	7.17 ± 2.52	7.25 ± 2.85	6.88 ± 2.59	7.52 ± 1.95	0.731
Lipid levels, mean ± SD mg/dl					
Total cholesterol	158.48 ± 41.74	165.41 ± 43.55	157.88 ± 44.72	149.27 ± 34.22	0.519
HDL cholesterol	44.93 ± 12.68	44.00 ± 13.78	45.17 ± 12.47	45.93 ± 12.07	0.899
LDL cholesterol	92.21 ± 32.7	99.14 ± 37.65	91.12 ± 31.38	83.80 ± 26.18	0.373
Triglycerides	106.62 ± 55.85	111.09 ± 51.70	107.92 ± 63.33	98.00 ± 51.55	0.78
Lipoprotein A	27.15 ± 31.6	29.95 ± 33.27	23.33 ± 31.66	29.13 ± 30.52	0.754

Table 2: Demographic comparison between the high, intermediate and low CIMT progression group in the APPLE study atorvastatin-treated participants (N=61). *Chi-squared test, one-way ANOVA or Tukey's range test. Tanner Stage 4-5 are classified as post-puberty. **Legend**: ACE - angiotensin-converting enzyme inhibitors; C3, C4 – complement fractions C3,C4; HDL- high-density lipoprotein; hsCRP - high sensitivity C-Reactive Protein; LDL- low-density lipoprotein; NSAIDs - non-steroidal anti-inflammatory drugs; SLEDAI – Systemic Lupus Erythematosus Disease Activity Index; SLICC DI- Systemic Lupus International Collaborating Clinics Damage Index.