Complement and Cardiovascular Risk in Adolescents

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SPECIFIC AIMS

African-Americans have increased incidence of and mortality from cardiometabolic diseases, including stroke,[1-3] hypertension,[4] and type 2 diabetes.[4-6] The incidence of myocardial infarction in African-Americans may be lower than in Caucasians,[7] but mortality is increased, particularly at younger ages. These conditions are all outcomes of the metabolic syndrome. There are clear and consistent racial differences in how the metabolic syndrome presents.[4,8-10]. The cause of these racial differences and how they affect the long term outcome of the metabolic syndrome are unknown.

Recent studies have demonstrated an important role for the complement system in the development of the metabolic syndrome and its complications. This may be particularly important in relationship to racial differences in the metabolic syndrome and its outcomes since adipose tissue both produces and responds to complement. Specifically, complement component C3 is produced by adipocytes, macrophages, and endothelial cells and adipocytes have receptors for complement components, C3a and C5a.[11] Beyond this C3a-desArg, a product of C3a cleavage by carboxypeptidase, increases adipocyte free fatty acid uptake and triglyceride production. Preliminary data from our laboratory clearly shows a close correlation between plasma triglyceride levels in nonHispanic Caucasian adolescents and complement C3 levels independent of obesity. The close relationship of triglyceride levels to complement has particular importance because African-Americans have reduced plasma triglyceride levels compared to Caucasians but also have increased free fatty acid levels[12,13]. ***Thus, there is good reason to hypothesize that differences in complement activation play a significant role in racial differences in the metabolic syndrome and cardiovascular risk.***

Adult cardiometabolic diseases have their origins in childhood and adolescence. The **long-term objective** of our laboratory is to develop a full understanding of the mechanisms of cardiometabolic risk in adolescents through which therapeutic interventions may be developed. The **short-term objective** of this study is to explore adolescent racial differences in complement components C3 and C4 and their relationship to early cardiovascular risk factors. In addition, we will study racial differences in specific C3 polymorphisms and C4 gene copy number.

Our **Specific Aims** are:

1. To determine whether there are racial differences in complement C3 and C4 levels in healthy adolescents and how they relate to cardiometabolic risk factors including triglyceride and LDL levels, inflammation, and vascular function.
2. To determine whether specific C3 polymorphisms including F versus S, A>G at rs11569562 and/or rs2250656, are associated with increased cardiometabolic risk in African-Americans.
3. To determine whether C4 gene copy number variation is associated with increased cardiometabolic risk in African-American adolescents.

Both traditional and nontraditional cardiometabolic risk markers, including measures of body habitus [BMI, waist circumference, percent body fat], blood pressure, lipids [triglycerides, HDL and LDL cholesterol], endothelial function [reactive hyperemia, endothelin 1], insulin secretion and sensitivity [oral glucose tolerance test], inflammation [high sensitivity c-reactive protein (CRP), interleukin 6 (IL6)], and clotting [fibrinogen, plasminogen activator inhibitor 1 (PAI1)] will be investigated. Doing these studies in adolescents before the development of overt cardiometabolic disease or other co-morbidities and their treatments that may confound these relationships is important. ***This research will help us better understand physiological mechanisms for racial differences in cardiometabolic risk and thus allow us to develop targeted therapeutics to decrease the increased cardiometabolic risk in African-Americans.***

BACKGROUND AND SIGNIFICANCE

Problem: While cardiometabolic diseases such as coronary artery disease, atherosclerosis, hypertension, and type 2 diabetes, usually do not produce significant mortality and morbidity until adulthood, there is clear evidence that these diseases have their origins in childhood and adolescence. Studies of soldiers killed in Korea at average age of 22 years showed 77 percent had significant atherosclerosis.[14] Autopsy studies of 15-34 years who died from accidental or non-accidental trauma demonstrated raised coronary artery lesions in individuals as young as 15 years with significantly increasing prevalence over time.[15] In addition, with the rising incidence of obesity associated with poorer eating and less physical activity in children and adolescents it is important that we study these diseases early in their course if we are to prevent future cardiometabolic morbidity and mortality. A variety of techniques have been developed to assess early cardiovascular risk in this age group.[16]

The primary goals of this study are to explore the roles of complement components C3 and C4, physiology and genetics in relationship to cardiometabolic risk in Caucasian and African-American adolescents. Extensive literature exists (discussed below) indicating complement plays an important role in the development of future cardiometabolic disease.

Adipose tissue, endothelium and complement: Adipose tissue over the last 20 years has been demonstrated to produce and respond to a wide variety of hormones and inflammatory markers. For the purposes of this proposal the key factor is adipose tissue production and response to complement. Complement, a key part of the immune system, plays a central role in defense against micro-organisms. Chronic complement activation, however, may play a detrimental role in the development of cardiometabolic disease.[17] Serum complement component C3 is vital to the three complement activation pathways and increased inflammation. C3 is manufactured by adipose tissue in adipocytes, macrophages and endothelial cells[11] and chylomicrons with dietary fat activate C3 production.[18,19] The increased C3 is activated to the anaphyltoxins, C3a and C3b, through the alternative pathway and C3 concentration dependent tick-over rather than through increased inflammation since the ratios of C3a/C3 are not altered in obesity.[18,20] C3a is rapidly cleaved by carboxypeptidases to remove carboxyl terminal of arginine to generate C3a-desArg.[19]

Besides producing complement adipose tissue also responds to complement. Adipocytes have C3a (C3aR) and C5 receptors (C5aR1 and C5aR2). The C5aR2 is also the only known receptor for C3a-desArg.[18] C3a-desArg, also known as acylation stimulation protein, plays an important role in lipid storage and energy metabolism. It accelerates adipocyte triglyceride metabolism and increases plasma triglyceride levels. This likely plays a role increasing insulin resistance.[18] C3 and C3aR knock-out mice are resistant to diet-induced obesity and more insulin sensitive than control mice.[18]

Endothelial cells have anaphyltoxin receptors for C3a, C5a and other complement components that increase expression of cellular adhesion molecules and pro-inflammatory cytokines. C5a and C5b-9 are associated with increased endothelial dysfunction and e-selectin secretion.[11] The complement system is thought play a dual role in the development of atherosclerosis through removal of cellular debris and amplifying inflammation.[11] A wide variety of genetic complement modifications in mice have significant effects on atherosclerosis.[21]

Complement and cardiovascular risk in humans: Cross-sectional and longitudinal studies in adults have shown consistent relationships between complement (C3, C3a, C3a-desArg and C4) and a variety of cardiometabolic diseases and risk factors. Specifically C3, C3a and C3a-desArg levels positively correlate with measures of obesity and visceral obesity in most ethnic groups and both sexes.[17,20,22-27] Beyond this several studies have demonstrated increased C3 levels in adults with the metabolic syndrome independent of obesity[20,25,27-30] and several studies have shown direct relationships with individual components of the metabolic

syndrome including insulin resistance, impaired glucose tolerance, triglycerides and lipids.[17,20,23,25-27,29,31] Patients with coronary artery disease, myocardial infarction, stroke and type 2 diabetes have been found to have increased C3 levels.[17,23,26] Beyond this, Muscari et al[32] found that increased C3 levels predicted future cardiac ischemic events in Italian men and women and Oshawa et al[25] found that change in C3 predicted change in insulin resistance measured using HOMA. Regarding C4, Nilson et al[20] found significant relationships to BMI, waist-to-hip ratio, subcutaneous and visceral adipose, blood pressure, cholesterol, and triglycerides, and a negative relationship to HDL and Oshawa et al[25] found significant relationships to BMI, triglycerides, and LDL.

In Chinese pediatric subjects, C3a-desArg levels are increased in obese subjects and correlate with cholesterol, LDL, and glucose in both sexes, and with insulin and HOMA in males. [33] Levels are increased in adolescents with the metabolic syndrome. Wei et al[34] in large population based study of 6-18 year olds found increasing levels of C3 with increasing BMI and increasing number of metabolic syndrome components within each BMI category.

Complement genetics: The C3 gene is on the short arm of chromosome 19 and several polymorphisms have been described. There are two major phenotypic variants of C3 proteins: the F-variant that travels faster and the S-variant that travels slower in an immunofixation gel based on electric charge difference of these two protein molecules. The molecular basis for such Fast to Slow (F vs S) phenotypic variation is due to a C>G DNA polymorphism that leads to glycine to arginine change at amino acid residue 102 (G102R).[35] The C3F polymorphism has been associated with myocardial infarction[36,37] and atherosclerosis[38] in several, but not all studies.[39] Phillips et al[40] found that patients with metabolic syndrome had specific polymorphisms present at the following locations rs11569562, rs2250656, rs1047286, and rs2230199. Multivariate modeling revealed that only the first two were significant. Interesting both are in the intron of the C3 gene and for both the A/A and A/G genotypes are associated with increased C3 production.[40,41] Regarding phenotype subjects with A/A or A/G alleles at rs11596562 had higher triglyceride, insulin, and c-peptide and lower HDL than subjects with two G alleles. For the rs2250656 polymorphism subjects with A/A or A/G had increased BMI, abdominal obesity, and lower HDL and insulin sensitivity compared to those with G/G.

The C4 gene is located on chromosome 6 and there is significant copy number variation. Low copy number is associated with decreased serum C4 levels[42-44] and increased risk of autoimmune disease including lupus, rheumatoid arthritis, and possibly type 1 diabetes.[45-48] Associations between C4 gene copy number variation and cardiovascular risk have not been investigated in depth but C4 gene copy number has been associated with longevity. Different forms of C4 [acidic (C4A), basic (C4B), long (C4L) and short (C4S)] also exist and there is copy number variation for each. Low C4B gene copy number has been shown to be associated with increased cardiovascular risk in several studies[49-54] and decreased longevity in Hungarian subjects[55,56] and Icelandic smokers[49] while decreased C4L gene copy number is associated with increased longevity in Germans[57].

Racial differences in cardiometabolic risk and complement: Multiple differences have been demonstrated between African Americans and Caucasians in the underlying pathophysiologic features of the metabolic syndrome. First and foremost, African-American adults and adolescents have consistently been shown to have lower triglyceride levels and higher HDL levels than Caucasians.[8,10,58] Beyond this, African-American adults have impaired endothelial function compared to Caucasian adults. Specifically, endothelin 1, a potent vasoconstrictor, is increased in African Americans adults[59] and flow mediated brachial artery vasodilation[60] is diminished. Hinderliter et al[61,62] found higher minimum FVR following vascular occlusion in African American, young adults compared to similar aged, Caucasian subjects. Endothelial dysfunction is an early predictor of both cardiovascular disease and type 2

diabetes[63]. African-American adults, also, have increased carotid artery intima-medial thickness[64]. Adolescent African-Americans with a family history of essential hypertension have increased basal and stress-stimulated endothelin 1 levels compared to similarly-selected, Caucasian adolescents[65].

In adolescents, Dr. Hoffman has demonstrated decreased endothelial function and increased insulin secretion in African-American adolescents [66]. The endothelial dysfunction occurred primarily in the obese adolescents with diminished post-occlusion shear stress-induced vasodilation [67]. In a follow up study obese African-American adolescents were found to have increased free fatty acid levels pre and post high fat meal compared to Caucasians despite having decreased triglyceride levels[13]. As discussed above there are significant interactions between complement and fat metabolism that could account for these differences.

Regarding complement, African-Americans have been shown to have increased C4 levels[68], differences in C4 gene copy number in relationship to lupus[69-72], and different C3 polymorphism frequencies[73] than Caucasians. Regarding the latter have higher C3s allele frequency compared to Caucasians (93 versus 83%).[73] Dr. Yu studied the relationships between complement and obesity in 189 African American (Black) and 331 Caucasian (White) adult subjects from central Ohio.[74,75] Between race differences in C3 and C4 levels were noted with increased BMI and C4 levels and decreased C3 levels in African-Americans. In each race group, strong correlations were seen between C3 and C4 protein concentrations, C4 protein levels and gene copy number of C4, BMI and C3 concentrations, BMI and C4 concentrations, and BMI and yield of C4 protein per copy of gene. In an earlier study of Hungarian subjects, positive correlations between serum C3 levels and total cholesterol concentration and between C3 levels and triglyceride levels were seen.[44]

Significance: There are three important areas of innovation in the proposed studies.

1. ***Our study will be the first to explore the relationships of C3 and C4 levels to cardiovascular risk factors in African-Americans.*** This is important follow-up to our current study of nonHispanic Caucasian adolescents (Data presented below) because of the demonstrated differences in cardiovascular disease between African-American and Caucasian adults and in cardiovascular risk in adolescents. These studies will help us determine potential mechanisms for these differences early in the course of the disease.
2. ***Our study will be the first to explore the relationship between C3 polymorphisms and traditional and non-traditional anthropometric, biochemical, and functional cardiometabolic risk factors in African American adolescents.*** No studies have been done examining the role of C3 polymorphisms on cardiometabolic risk in adult or adolescent African-Americans. Again because of the increased cardiovascular disease and risk and because of the different polymorphism frequency in African-Americans it is clearly important that this population be studied.
3. ***Our study will be first to explore the relationship of C4 copy number variation to cardiometabolic risk in African-American adults or adolescents.*** The effects of C4 gene copy number variation of longevity have varied among different ethnic groups. As indicated below, we have found increased C4L gene copy number is associated with impaired endothelial function in nonHispanic white adolescents. Whether this relationship is present in African-Americans in unknown.

It is important that studies be done in this age group to determine the early role of complement genetics in the development of cardiometabolic risk before the onset of overt disease or other co-morbidities and their treatment confound potential relationships. ***The results of this study will help us gain a full understanding of the role of complement in increasing cardiovascular risk in African-Americans and in potentially developing genetic methods of identifying at-risk individuals.***

PRELIMINARY STUDIES

Demographics: Thirty-four of the 75 subjects were female. Mean age was 15.0±1.7 years and mean BMI was22.0±5.8 kg/m2) were studied.

Complement and body habitus: Robust rank order regression analysis with was used to assess relationships between C3 and C4 protein levels, C4 gene copy number variation to body habitus, lipids, inflammation, carbohydrate metabolism, and clotting factor. Age and sex were included in all models. The cut-off point for outliers was 3 and 100 iterations were performed. Results are presented as 95% confidence intervals.

C3, C4, C3a, C4a, and C5a levels did not differ between sexes and were not significantly related to age. C3 levels positively correlated with all measures of body mass and adiposity (Figure 1). The correlation with FMI (R2=0.41) was better than with FFMI (R2=0.22). C4 levels were not related to any measure of body habitus. For C3a, C4a and C5a the only significant relationship was between C4a and FFMI (β=9.82, CI: 1.55-18.1).

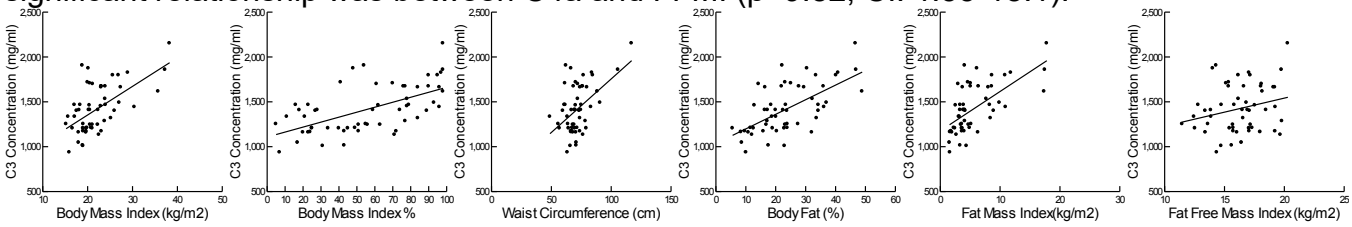

Figure 1: Relationships of C3 levels to measures of body habitus. BMI: β=35.9; CI: 23.6-48.2; BMI%: β=5.22; CI: 3.35-7.08; Waist circumference: β=15.4; CI: 10.01-20.9; Body Fat %: β=18.6; CI: 12.5-24.7; FMI: β=49.2; CI: 33.2-65.2; FFMI β=52.9; CI: 14.9-90.1.

Complement and cardiometabolic risk: C3 and C4 levels were significantly related to several cardiometabolic risk factors **(Table 1)**. Regarding vascular function, the percent fall in FVR following upper arm arterial occlusion decreased as both C3 and C4 increased. This decrease in reactive hyperemia indicates worsening endothelial function. AIx_{75} was not significantly related to either C3 or C4. Increased inflammation correlated primarily with increased C4 levels as indicated by significant relationships to white blood cell and neutrophil counts as well as IL6. White blood cell and neutrophil count also positively correlated with C3 levels. LDL and triglyceride levels positively correlated with C3 levels while HDL negatively correlated with both C3 and C4 levels. Insulin secretion increased and sensitivity during OGTT decreased as C3 levels increased. Endothelin 1 and PAI1 levels were not significantly related to either C3 or C4 levels. C3a was positively related to IL6 (β=0.003; CI: 0.001-0.006). C4a was negatively related to disposition index (β=-0.015; CI: -0.028--0.002).

Many of the cardiometabolic risk factors were related to one or more of the measures of body habitus. Adjusted values were calculated based on the body habitus measure to which they were most closely related. Reactive hyperemia, neutrophil count, LDL and insulin sensitivity were adjusted for percent body fat, HDL and triglycerides for waist circumference, and insulin secretion and PAI1 for FMI. AIx75, white blood cell count, IL6 endothelin 1 were not related to any measure of body habitus so no adjustments were done. Adjustment for CRP was not done since many of the levels were at the lower limit of detectability. After adjustment C3 and C4 levels continued to be significantly related to the reactive hyperemia response. C3 was not significantly related to any of the other adjusted variables while C4 remained positively related to adjusted neutrophil count and negatively related to HDL.

Complement genetics: For C3 the only significant difference was lower HDL levels in subjects with at least one F allele (46.7±12.2 versus 54.1±13.1, p=0.028) which would indicate

Table 1: Relationship C3 and C4 to cardiometabolic risk factors in nonHispanic white adolescents.		
	C3 β	C4 β
Reactive Hyperemia	0.012 (0.006-0.019)	0.028 (0.013-0.043)
Adjusted	0.007 (0.001-0.014)	0.023 (0.007-0.039)
AIX_{75}	0.007 (-0.011-0.025)	0.044 (-0.002-0.089)
White Blood Cell Count	0.002 (0.0002-0.005)	0.007 (0.003-0.012)
Neutrophil Count	0.002 (0.0002-0.003)	0.007 (0.004-0.011)
Adjusted	-0.0004 (-0.001-0.002)	0.005 (0.002-0.007)
CRP	0.003 (-0.001-0.006)	0.0005 (-0.001-0.002)
IL6	0.0004 (-0.00005-0.001)	0.001 (0.0002-0.002)
LDL	0.021 (0.0004-0.041)	-0.024 (-0.074-0.027)
Adjusted	0.013 (-0.007-0.033)	-0.040 (-0.087-0.007)
HDL	-0.012 (-0.023--0.001)	-0.027 (-0.054--0.0003)
Adjusted	0.002 (-0.007-0.012)	-0.026 (-0.048--0.004)
Triglycerides	0.054 (0.025-0.083)	0.055 (-0.020-0.1)
Adjusted	0.009(-0.027-0.046)	0.057 (-0.014-0.127)
Matsuda insulin sensitivity	-0.003 (-0.004--0.001)	-0.001 (-0.006-0.004)
Adjusted	0.0003 (-0.002-0.001)	0.086 (-0.004-0.176)
Insulin Secretion	0.001 (0.0004-0.002)	0.002 (0.001-0.004)
Adjusted	-0.0001 (-0.001 0.001)	0.002 (-0.00006-0.004)
Disposition Index	-0.0004 (-0.003-0.002)	0.005 (-0.001-0.011)
Endothelin-1	-0.0002 (-0.001-0.0003)	-0.001 (-0.002-0.0004)
PAI-1	0.002 (-0.0002-0.005)	0.0003 (-0.004-0.005)
Adjusted	-0.0004 (-0.002-0.002)	-0.001 (-0.005-0.004)

increased risk in subjects with one F allele. This will be important to explore in this proposal since HDL levels differ between African-Americans and Caucasians.

FFMI decreased as C4T (β=-0.73; CI: -1.24--0.22) and C4L (β=-0.46; CI: -0.81--0.10) copy number increased. C4S, C4A, and C4B copy number were not related to any measure of body habitus. C4T gene copy number was not related to any of the cardiovascular risk factors but relationships were found for the various C4 subtypes. Interestingly, HDL was significantly related to all 4 subtypes and increased C4L copy number increased (β=3.59; CI: 1.19-6.00), decreased as C4S (β=0-4.26; CI: -7.50—1.02) and C4B (β=-5.56; CI: -10.3--0.87) increased and increased as C4A increased (β=4.33; CI: 1.00-7.66) **(Figure 2).** These relationships are particularly interesting in light of the relationship of C4 levels to HDL and suggest that C4 may play a role in determining HDL levels.

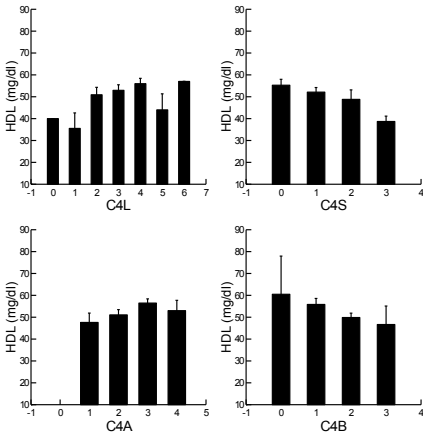

Figure 2: Relationship of HDL to C4 type copy number. C4L: β=3.59; CI: 1.19-6.00; C4S: β=0-4.26; CI: -7.50—1.02; C4A: β=4.33; CI: 1.00-7.66; C4B β=-5.56; CI: -10.3--0.87.

LDL and triglyceride levels were not associated with any of the C4 gene copy numbers. Interestingly, while increased C4L gene copy number was associated with increased HDL, it was also associated decreased insulin secretion (β=-0.24; CI: -0.44--0.05) and decreased disposition index (β=-0.61; CI: -1.14--0.08. Increased C4S copy number was associated with increased insulin secretion (β=0.29; CI: 0.06-0.52) and decreased endothelin 1 (β=-0.15; CI: -0.30--0.01). WBC (β=-0.79; CI: -1.56--0.02) and neutrophil count (β=-0.87; CI: -1.48--0.33) both decreased with increasing C4B copy number. Interestingly, the measures of inflammation, Il6 and CRP were not related to C4 copy number nor were AIx_{75} or PAI-1.

RESEARCH DESIGN AND METHODS

All three specific aims will be studied using the same subjects and research procedures.

Subjects: One hundred Africa-American adolescents between the ages of 12 and 18 will be recruited to participate. There will be no weight or BMI criteria and we plan to recruit subjects across a wide range of BMI's. This will allow us to determine the role of C3 and C4 levels, C3 polymorphisms and C4 copy number variation in determining adolescent obesity and cardiometabolic risk. Subjects must be on no chronic medications except for contraceptives in females. Subjects will be expected to be medication free for at least 2 weeks prior to study except for contraceptives. Subjects with a history of autoimmune disease, either endocrine or connective tissue type will be excluded. Subjects with a history of hematologic or renal disease, malignancy or other chronic disease will also be excluded. Subjects with a history of asthma or attention deficit disorder will be allowed to participate as long as they meet the medication requirements.

To avoid potential racial confounders as described in the preliminary data, we plan to recruit only African-American subjects. Subjects will recruited to specifically fill 3 different weight classes (70 % lean, BMI <85%: 10% overweight BMI<95%: 20% obese BMI>95%) for both sexes. These match the reported US prevalences for overweight and obesity.[76]

Recruitment: Subjects will be recruited through advertisements in the Pediatric Clinics of Nationwide Children's Hospital, email recruitment through Nationwide Children's Hospital, and Ohio State University systems and through advertisements in local newspapers, churches, and schools. We will specifically advertise in the *Columbus Post* which is the newspaper aimed directly at the Columbus African American Community. The Columbus City School District has ~ 50,915 students, 76% of whom are minorities with most of these being African American The district uses Peachjar for electronic distribution of brochures and communications. There is a cost but it allows for distribution to be targeted to schools based on age and demographics. We will also reach out to the Whitehall City School District which has ~3,500 student, 69% minorities. We have had past success in recruiting African-Americans.[13,58,66]

Protocol:

Study Visit: The study will be performed in the Clinical Research Center (CRC) of the Wexner Medical Center at The Ohio State University. Subjects will come to the CRC at 08:00 after an overnight fast beginning at 22:00 the night before study. Upon arrival Informed Consent and Assent will be obtained. A medical history will be taken and a brief physical examination performed to assure subjects meet study criteria.

Anthropometrics: Height, weight and waist circumference will be measured. Height will be measured on a Harpendon stadiometer and weight on an electronic scale. Waist circumference (WC) will be measured to the nearest 0.1 cm at the narrowest place between the lowest rib and the iliac crest at the end of normal exhalation with a spring loaded, inelastic measuring tape.[77] Body percent fat will then be measured DEXA. CRC personnel have extensive experience performing each of the measurements.

Endothelial Function: Endothelial function will be measured using post-occlusion, shear stress-induced vasodilation after a 20 minute rest period. Venous occlusion plethysmography will be used to measure FBF and FVR before and after upper arm occlusion. This method of testing endothelial function assesses resistance vessel function. Results closely correlate with results from endothelial function assessed by intra-arterial acetylcholine infusion,[78] and correlate well with the nitrite/nitrate ratio, an index of NO synthesis.[79]

Specifically, maximal endothelium-dependent vasodilatory responses will be quantified as post-occlusion FVR (poFVR). FBF will be measured using strain gauge venous occlusion plethysmography using a Hokanson AI6 plethysmograph. Two minutes of baseline FBF will be recorded after which the upper arm cuff will be inflated to 200 mmHg pressure for 5 minutes to occlude arterial inflow. It will then be released and FBF will be measured for one minute. FVR will be calculated by dividing mean arterial blood pressure by FBF for each of four FBF

measurements after occlusion. Post-occlusion FVR will be the average of the 4 FVR values. Arterial blood pressure will be measured using automated sphygmomanometer. Mean intra-observer coefficient of variation (CV) for FBF before upper arm occlusion is 5.1% and 7.4% after upper arm occlusion.

Arterial Stiffness: Augmentation index and pulse wave velocity will be measured using the Spygmocor XCEL (Atcor Medical Inc, Itasca IL).

Blood sampling: After completion of the test of endothelial function, an intravenous catheter will be placed in one arm for initial blood sampling and for the oral glucose tolerance test. Twenty five ml of blood will be taken from each study subject for measurement of fasting plasma glucose, insulin, lipids, CRP, IL6, fibrinogen, PAI1, endothelin 1, C3, C3a, C3a-desArg, and C4 levels and for genetic analyses of complement C3 and C4. IL6 and CRP are markers of inflammation which is clearly increased in individuals at increased cardiometabolic risk.[26,34] Fibrinogen and PAI1 will be used to assess clotting risk. Both are increased in individuals with increased cardiometabolic risk and have been linked in adults to increased C3 levels.[11] Endothelin 1 is an endothelially-produced vasoconstrictor and has been shown to be biochemical marker for endothelial dysfunction.[80]

Insulin sensitivity and secretion: Subjects will be given 1.75 grams/kg of oral glucose (up to a maximum of 75 grams). Blood samples for measurement of plasma glucose and insulin levels will be drawn every 30 minutes for 120 minutes. Insulin sensitivity (IS) will be calculated using the Matsuda Index (IS = 10,000/$\sqrt{}$ [fasting glucose x fasting insulin] x [mean glucose x mean insulin])[81] while insulin secretion (SEC) will be calculated as the change in insulin divided by the change in glucose from 0 to 30 min (SEC = ΔI0–30/ΔG0–30).[82] DI will be calculated by multiplying both these values (DI = IS x SEC).[82] Disposition index is an important predictor of future type 2 diabetes.[83]

Subject Incentives: Subjects will be compensated $100 for participation in the Study Visit.

Laboratory: Genomic DNA will be prepared from peripheral blood mononuclear cells and used for assessment of C3 genetic polymorphisms, gene copy-number variations of total C4, C4A, C4B, long genes and short genes. Cell bound levels of processed activation products C4d and C3d deposited on red blood cells will be determined by flow cytometry. B-lymphoblastoid cell lines for each subject enrolled in this study will be created to facilitate future molecular biologic and genetic studies.

Complement phenotypes: Protein levels for complement C3 and C4 from EDTA-plasma will be determined by single radial immunodiffusion (RID) assays using commercial kits from the Binding Sites (UK). Fast and Slow protein variants of complement C3, acidic C4A and basic C4B allotypes of complement C4 will be elucidated by immunofixation of EDTA-plasma resolved by high voltage agarose gel electrophoresis, and scanned by densitometer to quantify the relative expression levels of polymorphic variants.[74] C3a-desArg will be assayed by enzyme-linked immunosorbent assays (ELSA) using EDTA-plasma and commercial kits (BD Bioscience). Cell-bound levels of processed complement activation products on erythrocytes, E-3d and E-C4d, will be assayed by flow cytometry using specific monoclonal antibodies against C3d and C4d, respectively (Quidel).[84]

Complement genotypes: C3 genetic variants including the DNA sequence polymorphisms for G102R for fast and slow allotypes, and SNPs for *rs11569562* and *rs2250656* that modulate *C3* plasma protein levels will be typed for all study subjects. Copy-number variations for total C4, C4A, C4B, long genes and short genes of C4 will be elucidated by Southern blot analyses of genomic DNA samples digested by PmeI, TaqI, PshAI/PvuII and resolved by gel electrophoresis.[74] Samples for subjects with ambiguous data will be subject to realtime quantitative PCR using five different amplicons and the results will be validated as the gene copy-numbers in each subject for total C4=C4A+C4B=long genes+short genes.[85]

Cardiovascular measures, glucose and insulin: IL6, CRP, endothelin 1, fibrinogen, PAI1 glucose and insulin will be measured in the CORE laboratory. Plasma lipids will be measured in the clinical laboratory of the Wexner Medical Center.

Data analysis: **Specific Aim 1:** To determine whether there are racial differences in complement C3 and C4 levels in healthy adolescents and how they relate to cardiometabolic risk factors including triglyceride and LDL levels, inflammation, and vascular function.

Statistical methods: Complement levels from African-Americans in this proposal will be compared to those from nonHispanic whites in our current study. Eligibility criteria are identical for both studies except for race. Data will be assessed for normality using the Shapiro Wilk test ($p>0.05$ indicative of normality). Non-normal data will be log normalized if possible. Unpaired t-tests will be used to compare differences in C3 and C4 levels as well as cardiometabolic risk factor results (reactive hyperemia, augmentation index, Matsuda index, insulin secretion, lipids, inflammatory markers, and PAI-1). We anticipate age and BMI differences will be minimal but should racial differences be found analysis of covariance will be done to account for any difference found.

Robust rank order regression analysis will be used to assess relationships between C3 and C4 protein levels to body habitus, lipids, inflammation, carbohydrate metabolism, and clotting factor in each race individually and in the group as a whole. Age and sex will be included in all models. The cut-off point for outliers was 3 and 100 iterations were performed. Results are presented as 95% confidence intervals.

Sample size: Based on data from our previous study with 100 nonHispanic white subjects and 100 African-American subjects we have power 0.8 to detect a 15% racial difference in C4 levels and 7.5% difference in C3 levels.

Interpretation: There are two major portions of this aim. The first is to determine whether there are complement levels differences between the racial groups and the second is to determine how these affect previously demonstrated racial differences in cardiovascular risk[12]. The former will be easily determined by comparing complement levels in the two groups. The hypothesis that these racial differences are responsible for at least some of the cardiometabolic racial differences will be proven if when complement levels are included in the regression equation they retain significance but the racial differences are lost. Lastly, even if racial differences in complement are not found or do not account for cardiometabolic risk differences it will still be important for us to determine whether the significant effects of complement described above in nonHispanic whites are present in African-American adolescents.

Specific Aim 2: To determine whether specific C3 polymorphisms, F versus S, A>G at rs11569562 and/or rs2250656, are associated with increased cardiometabolic risk in African-Americans.

Statistical Methods: As indicated above, the C3F gene has been associated with increased risk of myocardial infarction in several populations[36-38] and Phillips et al[40] reported increased cardiometabolic risk in subjects with either an A/A or A/G versus subjects with a G/G genotype for both the rs11569562 and the rs2250656 sites. For rs11569562 in a European population ~ 25% of subjects had G/G and for rs2250656 ~10% had G/G. Frequencies in the African-American population are unknown. Data will be assessed for normality using the Shapiro Wilk test ($p>0.05$ indicative of normality). Non normal data will be log normalized if possible. Unpaired t-tests with unequal sample sizes will be used for comparisons for each of the measured normal variables between subjects with at least one A allele gene and those with two G alleles assuming the data is normally distributed. Mann-Whitney U tests will be used to assess non-normal data.

Sample size: Based on our preliminary data for the F versus polymorphism and the data of Philips et al[40] site we will be able to effect size variable differences of 0.47 for the F versus S polymorphism, 0.7 for rs11569562 and 1 for rs2250656 with $\alpha=0.05$ and $\beta=0.8$ within African-Americans.

Interpretation: Our hypothesis will be proven by increased BMI, waist circumference, percent body fat, blood pressure, triglycerides, LDL cholesterol, CRP, IL-6, fibrinogen, and/or PAI-1 and/or decreased HDL, insulin sensitivity, disposition index, or reactive hyperemic responses in subjects with at least one F or one A allele and/or if the subjects with at least one allele have increased worsening over 1 year than do those without. Previous studies of the effects of complement on cardiometabolic risk in adults have found differences in some risk factors but not others although there is disagreement on the areas affected.[11,18,20]

Specific Aim 3: To determine whether C4 gene copy number variation is associated with increased cardiometabolic risk in African-American adolescents.

Statistical methods: Robust rank order regression will be used to assess the relationship of C4 gene copy number for each C4 subtype and cardiometabolic risk. Age and sex will be included in all models.

Sample size: With 99 subjects we will be able to detect an $r=0.28$ with $\alpha=0.05$ and $\beta=0.80$ for each analysis without correction for multiple analyses. There is no literature available to assess whether an $r=0.28$ is a reasonable expectation but r-values below this are unlikely to indicate important mechanistic relationships

Interpretation: Our hypothesis will be proven by positive relationships between C4 copy number and BMI, waist circumference, percent body fat, blood pressure, triglycerides, LDL cholesterol, CRP, IL-6, fibrinogen, and/or PAI-1 and/or negative relationships to HDL, insulin sensitivity, disposition index or reactive hyperemic responses. The main limitation of this study is sample size. No studies have been done to identify expected effect sizes for these variables in African Americans. As indicated for rs11569562 we will be able to detect differences with an effect size of 0.7 and for rs2250656 with an effect size of 1. Smaller differences with physiological significance could be present but may not be statistically significant in this population.

Limitations: This study is limited to studying C3 and C4 genetic differences and their relationships to differences in complement and cardiometabolic risk factors and not social and lifestyle differences between the two racial groups. An extremely interesting finding would be racial differences in complement levels without significant difference in complement genetics. This would suggest that differences in lifestyle (physical activity, socioeconomic stress) may play a significant role in causing complement racial differences. Even if no racial differences are found, these studies will still generate important information regarding the relationship of complement to cardiometabolic risk in adolescents and will indicate what areas should be explored to gain a better understanding of the racial risk differences.

Future Directions: Correlational, cross-sectional studies do not prove causation so if these results demonstrate significant relationships between complement and/or complement genetics in cardiometabolic risk then future longitudinal follow-up studies will be important. It would also be important to extend these findings to additional ethnic groups. Examining the effects of socioeconomic and cultural differences on the relationships of complement to cardiometabolic risk factors may also be of value. Our results also have potential value to inform future treatment studies to prevent or reduce cardiometabolic risk. The most immediate would be using complement genetics to potentially identify subjects at high risk for future cardiometabolic disease. Furthermore, various anti-complement therapies are being developed for use in a variety of diseases.[86-88]

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