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STUDY PROTOCOLS

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Epigenetic age in African American adolescents with type 2 diabetes: A cross-sectional case–control study protocol

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Abstract

Background and Aims: Type 2 diabetes (T2D) is a disease caused by a relative insulin deficiency compared to the significant insulin requirement needed by the body to achieve glycemic control. T2D in adolescence appears to be increasing in prevalence over the past several decades, necessitating studies to understand for the onset of the disease to occur early in the lifespan. Given the high burden of disease, specifically in young African American adolescents, our study chose to focus initially on feasibility of recruitment of this population.

Methods: Data was collected at a single study center at Children's of Alabama. The protocol was completed as part of routine care or at a study visit. The study team was able to leverage the Electronic Medical Record to prescreen eligible patients to discuss the study. A variety of times of day were utilized to improve likely of success with reaching potential participants. Inclusion criteria for patients with T2D was focused on the adolescent population (ages 12–18 years), with no history of an obesity syndrome. DNA methylation age will be calculated using the EPIC 850K array. Statistical analysis will be done using linear regression analysis, adjusting for covariates.

Conclusions: This study's aim was to screen and enroll young African American adolescents for a study investigating epigenetic aging and T2D. Our study found that more direct contact (face-to-face- or phone call) improve success of recruitment. Leveraging the electronic medical record also helped improve success with pre-screening participants. Challenges included recruiting participants who might come from long distances to a tertiary care center. Consolidating appointments helped improve the success of reaching these participants. Other challenges included frequent address changes and changed phone numbers. Close attention to the barriers as well as the successes will aid in understanding effective strategies for this important population.

KEYWORDS

adolescents, African Americans, epigenetics, type 2 diabetes

1 | INTRODUCTION

Type 2 diabetes (T2D) is a disorder arising from insulin resistance (IR) and relative insulin deficiency in the absence of autoimmune beta-cell destruction.[1] While IR and hormonal changes of puberty leading to IR can precipitate the development of T2D, it still does not yet explain what leads to the rapid temporal progression that can occur in adolescents with T2D compared with adults with T2D. T2D in adolescents appears to be a more aggressive disease than adult-onset and has increased in severity and frequency over the last four decades.[2,3] Adolescents with T2D can lose up to 15 years of their life expectancy due to comorbidities.[4] The most common comorbidity of T2D in youth is obesity. Studies indicate that over 85% of children with T2D are either overweight or obese at diagnosis.[2]

Mounting evidence suggests that the comorbidities of T2D depend on multiple mechanisms beyond that which genetics and social/lifestyle determinants of health can help explain. Epigenetic changes of the methylation at cytosine-phosphate-guanine (CpG) dinucleotides across the genome could help explain some of the variance observed in the comorbidities that occur with the incidence of T2D, thus emerging as an essential mechanism to consider. Epigenetics is the study of heritable changes in gene expression that do not involve changes to the underlying DNA sequence.[3] Few studies have focused on adolescent populations, and among the studies conducted to date, even fewer—if any—have studied incident T2D in adolescents. Recent studies have identified several loci of interest associated with T2D in pediatrics, including loci within free fatty acid receptor-1 (*FFAR1*), upstream transcription factor-2 (*USF2*), and tumor necrosis factor-related protein-9 (*C1QTNF9*).[4] Studies have shown that the epigenome is an essential target for age-related physiological changes, as it is modifiable by the environment.[5] Thus, it is considered a significant potential contributor to the incidence of T2D in adolescents. Relatively little is known about epigenetic age in adolescents with T2D. Prior adolescent studies have also demonstrated that epigenetic age acceleration can be observed in adolescents and that epigenetic age acceleration can be observed in metabolically active tissues and blood.[6] Due to the metabolic disturbances associated with T2D, it is essential to delineate how T2D may impact the epigenetic signature in adolescents.

It is known that DNA methylation (epigenetic) age increases logarithmically in childhood before slowing to a linear pattern of acceleration in adulthood.[7] Studies suggest that accelerated epigenetic aging may start early as adolescence.[6,8] Little is known about epigenetic age acceleration in African American adolescents with T2D, as this cohort continues to be understudied.

One limitation of genetic and genomic studies is the significant representation of predominantly European ancestry populations. A second important limitation is the sample size of the studies that involve persons of color. Increasing the involvement of African Americans in clinical studies is essential for improving health equity and the generalizability of research findings. Barriers previously identified included logistical barriers, such as lack of transportation and difficulty missing work for study visits, and interpersonal barriers, such as lack of trust in the medical community and research. The required involvement of the participant's caregiver can be an additional layer of complexity in recruiting pediatric/adolescent patients.[9]

Our study will guide the recruitment of future cohorts of African American adolescents for incident diabetes and genetic/genomic studies. Findings from this study will establish the ability of our research team to recruit this unique population successfully. Given the fact that this population is understudied, successful recruitment will be necessary for other studies in the future as well.

2 | METHODS

2.1 | Study design and settings

Data for this study were collected from a single study center at the Children's of Alabama presenting from 1/2022 to 2/2023. The protocol was completed either at a study visit or a previously scheduled clinic visit that was part of routine care (see Figure 1 for described protocol). Inclusion criteria for affected participants ages 12–18 years old with T2D were body mass index (BMI) > 95th percentile for their age and gender, diagnosis of T2D based on history of hemoglobin A1c > 6.5% at diagnosis and negative pancreatic antibodies, and no history of a monogenic cause of obesity ($n = 19$). Inclusion criteria for the control participants include ages 12–18 years, hemoglobin A1c < 5.6%, BMI > 95th percentile for their age and gender, and no history of monogenic cause of obesity ($n = 43$). The IRB approved the research at the University of Alabama at Birmingham. All participants and their guardians provided informed consent and assent to participate. Laboratory data were obtained at that visit to accommodate the competing life demands of the youth and caregivers. Families were reimbursed $25 for the single visit. Participants were recruited from the general adolescent clinic, weight management clinic, and endocrine clinic at Children's of Alabama.

2.2 | Recruitment protocol

All recruitment efforts for the study were conducted by the study team at the University of Alabama at Birmingham, where adolescents were seen for care. The primary investigator trained the study team on the approach to patients and their families through recruitment.

Data regarding potential eligible adolescents (based on age, ethnicity, and medical diagnosis) were obtained from the electronic medical records mentioned above and phone contact information from the clinics mentioned above. Initial recruitment in the weight management clinic was done by a study team member reaching out to eligible participants and their families to discuss the study during their visit.

The study team began screening potential eligible control participants from the general adolescent clinic. Once the participant was found to be qualified and interested in the study, instructions

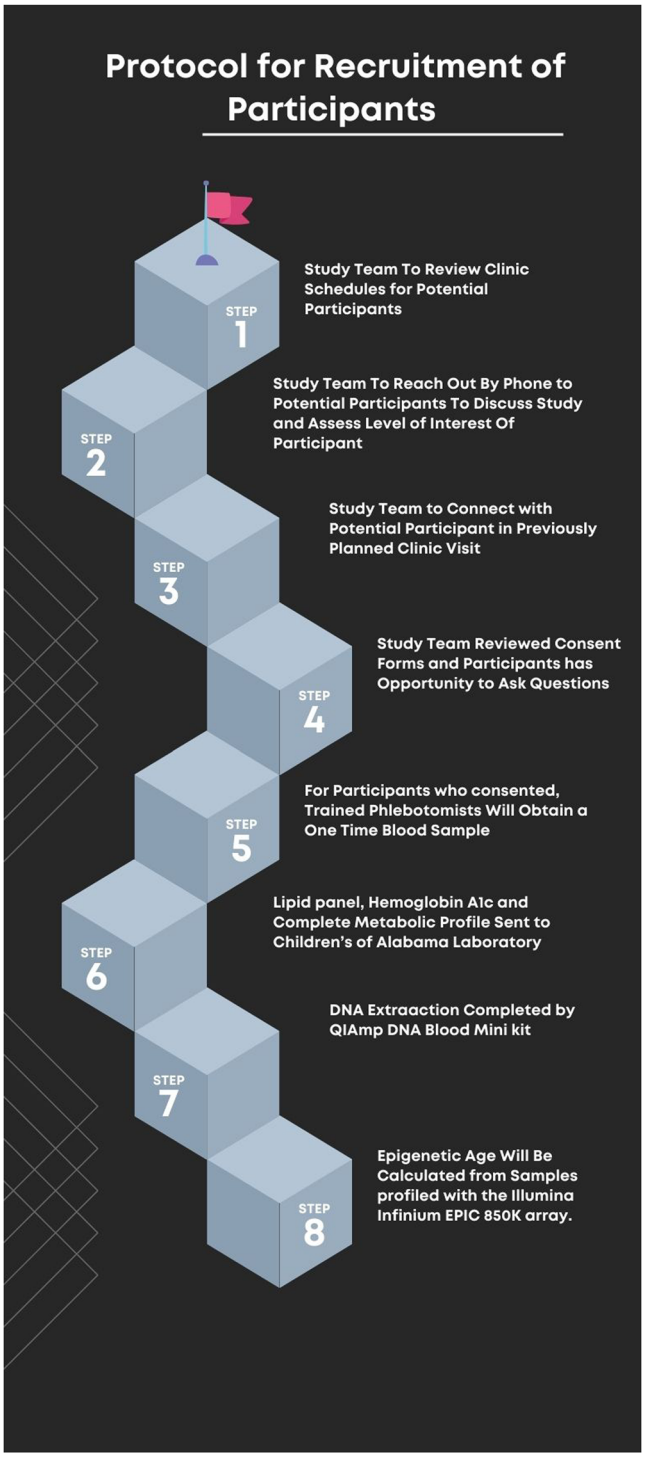

FIGURE 1 Overview of study protocol.

were given for participation. The study team then called the adolescent's family, placing them at various times of day (morning, afternoon, and evening) and on weekends to improve their chances of reaching the family. The research team met periodically to discuss recruitment efforts and brainstorm other ways to reach out to families regarding the research and its criteria. To improve recruitment, fliers were also created and circulated as well. The study recruitment lasted 13 months.

2.3 | Data collection

2.3.1 | Anthropometric data

Information including age, gender, weight z-score, height z-score, and BMI z-scores were recorded.

2.4 | Collection of whole blood sample

Appropriate sample handling and blood collection were done per clinical laboratory guidelines. All blood was collected with participants on the same day as their clinic visit, fasting for 8 h. The needle gauge was chosen based on the participant's age and size. Five milliliters were collected using an EDTA-coated tube. For the serum analytes, 5 mL were collected in a red-top vacutainer (containing no anticoagulant or preservative). Clotted blood was taken and centrifuged at 2000*g* for 15 min.[10]

2.5 | Glycemic control

Hemoglobin A1c (HbA1c), a measurement of average blood glucose during the past 2–3 months, will be used to evaluate glycemic control. These labs were completed with a DCA Vantage Analyzer and Hemoglobin A1c reagent kit.

2.6 | Cholesterol information

Lipid panels were collected to demonstrate total cholesterol, low-density lipoprotein (LDL), triglycerides, and high-density lipoprotein (HDL) levels. These assays were completed on the Abbot Architect ci8200 system. The limit of detection for total cholesterol was 6.2 mg/dL. The imprecision of the cholesterol assay is <3% of the total coefficient of variation (CV). The limitation of detection for LDL was <10 mg/dL. The interassay CV was 2%, while the intraassay was 1.1%–14%. The limit of quantitation was 5.0 mg/dL, and the detection limit for HDL was 2.5 mg/dL. The interassay CV was 0.5%–1.1%, and the intraassay CV was 1.0%–1.7%.

2.7 | DNA extraction

According to the manufacturer's protocol, genomic DNA from whole blood was isolated using a Qiagen kit (QIAmp DNA Blood Mini kit, catalog # 51104). DNA will be treated with RNase A (Qiagen, catalog # 19101) on the columns and eluted with water.

2.8 | DNA methylation

DNAm age (also referred to as epigenetic age) will be calculated from human samples profiled with the Illumina Infinium EPIC 850 K array.

Given that the group of interest, those with T2D, provided blood as part of routine clinical care, we chose to focus on two epigenetic clocks validated in blood. We will apply the Horvath clock, which measures DNAm age based on 353 CpGs (cytosines followed by guanines) developed using DNA methylation data from a variety of tissue specimens.[10,11] The Hannum clock is comprised of 71 CpGs based on blood specimens.

Assays will be performed at the University of Minnesota's Genomics Center (UMGC). The UMGC has extensive experience with the Illumina EPIC array, including over 850,000 methylation sites at single-nucleotide resolution selected by a consortium of methylation experts. The EPIC platform interrogates >97% of genes, focusing on the promoter and CpG-island, CpGs, gene bodies, 3′ ends, known enhancers, and differentially methylated regions (DMR)s. The EPIC arrays also carry 90% of the previous-generation HumanMethylation 450 array content to enable future replication/meta-analysis opportunities in other cohorts that may have utilized the older technology. Case and control DNA will be distributed equally within each plate being processed to avoid false associations due to lab artifacts and sample clustering. The assays entail standard Illumina DNA amplification and hybridization to the EPIC array after bisulfite conversion with EZ DNA kits (Zymo Research). The resulting intensity files will be analyzed with Illumina's GenomeStudio, which generates beta scores (i.e., the proportion of total signal from the methylation-specific probe or color channel) and "detection *p*-values" (probability that the total intensity for a given search falls within the background signal intensity).

2.9 | Epigenotype quality control (QC)

QC procedures will remove beta scores with an associated detection *p*-value greater than 0.01, samples with more than 1.5% missing data points, and any CpG probe where more than 10% of samples fail to yield adequate intensity. Next, we will eliminate any CpGs where the probe sequence maps either to a location that does not match the annotation file or to more than one locus.

2.10 | Batch effects

Background correction, within array normalization, Type I and II chemistry correction, and batch/plate/chip adjustment will be made in addition to probe and sample level QC described above. We will use quantile preprocessing procedures (normalizing all samples together) and ssNoob (normalizing one sample at a time).[11] The R package *minfi* will be used for the entire QC workflow.[12] *Minifi* provides a complete pipeline for quality control (as well as statistical testing) for CpGs and DMRs.

2.11 | Confounding by cell type in methylation analysis

Methylation variations within each blood cell type may confound epigenomic association results mirroring the statistical issues created by ancestry-related population stratification.[13,14] This is particularly important given that we will assay DNA methylation in whole blood. The natural differences in the ratio of white blood cell types could give rise to differential methylation measurements at those genes that carry cell type-specific methylation. To address this concern, we will use a method previously described.[15] which can predict underlying blood cell composition from DNA methylation patterns and include estimates of cell counts as covariates in the analysis.

2.12 | Planned data analysis

We will use linear regression to test the association of an expected approximately 850,000 CpG sites with T2D case-control status (T2D status is the predictor). Modeling the CpG as the outcome has been adopted in many epigenetic studies to enable direct adjustment for technical variables in association analysis (e.g., an estimated mixture of cell proportion, batch).[16–19] While we will let descriptive statistics (chi-square and *t*-tests) guide our covariate adjustment, our current plans include covariate adjustment for precocious puberty, glycemic control, BMI, and medication use. We will consider additional adjustments for the methylation array, plate row, and column, similarly to prior consortia reports.[18] In addition to considering single CpG sites, we will investigate DMRs using ChAMP, which uses three different algorithms (1) the bumphunter package; (2) DMRcate; and (3) the probe lasso function to identify DMRs between case–control status. Each algorithm is slightly different but considers annotated genomic features and their corresponding local probe densities to call and test the significance of DMRs between cases and controls using normalized beta values.[20–22] We plan to calculate DMRs with a methylation cut-off of 0.015 (corresponding to a 1.5% difference in beta values by T2D status).[23]

2.13 | DNA methylation age calculation

The DNA methylation age will be calculated using a publicly available code The Horvath epigenetic clock is comprised of 353 CpG probes and has been validated across multiple tissues.[7] Hannum's method is computed from methylation values of 71 CpG sites and has been validated with adult whole blood samples.[24] The deviation between epigenetic age and chronological age will be calculated based on the residuals from regressing the DNAm age on chronological age

2.14 | Multiple testing

We will adjust for multiple testing using a Bonferroni correction for an anticipated 0.05/850,000 tests setting α to 5.88×10^{-8}.

3 | DISCUSSION

This study aimed to screen and enroll young African American adolescents for a study investigating the association of T2D and epigenetic aging. Data describing recruitment for this specific patient population is often limited. Information may allow research teams to plan accordingly for appropriate contact methods to improve recruitment success. Interestingly a prior study has demonstrated that up to 10 episodes of various references may be needed to recruit this population before enrollment.[9] Our study has established some families with fewer contact hours but more direct contact, such as face-to-face contact during a prior scheduled clinic visit.

Leveraging the electronic medical record to provide demographic information so that approach to patients can be targeted was instrumental in enhancing our recruitment. Pre-screening for potential patients by reviewing target clinic schedules the week prior worked well for improved recruitment. Another implementation that worked well for recruitment was phone pre-screening to assess potential participants' interest levels. This allowed for the consolidation of appointments and targeted in-person approaches from the study team to discuss the study before consenting. Expanding to other clinics also increased our ability to recruit potential participants and widened the number of patients our study team could screen for participation.

Challenges during recruitment included difficulty with our patients traveling long distances to complete the study visit and that some patients might need to be seen in the clinic where the study team primarily works. Thinking outside the initial clinics, the study team began reaching out to the general adolescent clinic. In an attempt to consolidate appointments, our team began reaching out to families who had already scheduled appointments in a clinic before their appointments to assess their interest so that they could be fasting to coordinate any lab testing and improve the likelihood of recruitment.

Interestingly, using a variety of contact approaches, such as phone calls and in-person discussions, could also help improve the successful recruitment of African American adolescents.[25] Other challenges we encountered in recruitment included a frequent changes of address, home phone numbers, and disconnected numbers. These challenges can indicate social risks that can alter participation in a research study. Prior studies have also demonstrated similar findings.[9,26]

Limitations of this study include the fact that recruitment efforts were focused on initial contact with families who were coming for scheduled appointments, given the availability of the recruitment team. Given that this study takes place at a tertiary care center, the generalizability of the implication of the findings may be difficult. If recruitment were done in other locations, it may be challenging to apply these strategies. Given the limitation of the study sample size, this will limit our ability to consider prediabetes and insulin diabetes. We recognize that consideration to IR and prediabetes should be given and this was a limitation to this study. There may be some changes to epigenetic age acceleration that may occur in this phase. Larger studies may be needed as a future study direction.

Limitations of the analysis could include computational power and the need to recognize the complexity of the models that are generated.[27] To decrease the effect of this limitation, we will work with a statistician team who has extensive experience with this epigenetic work.

4 | CONCLUSIONS

In summary, our study demonstrates that unique targeted recruitment efforts may be needed to successfully recruit African American adolescents into a research study, particularly those with a chronic health condition, including leveraging already planned visits and increasing face-to-face contact to discuss the study may improve recruitment success for these studies. Close attention to the specifics of the recruitment efforts will aid in understanding effective strategies for targeted enrollment of African American adolescent participants for genetic and epigenetic studies.

AUTHOR CONTRIBUTIONS

Christy Foster: Conceptualization; data curation; funding acquisition; methodology; writing—original draft; writing—review & editing. **Olga Mamaeva**: Data curation; methodology; resources; writing—review & editing. **Sadeep Shrestha**: Resources; supervision; writing—review & editing. **Bertha Hidalgo**: Conceptualization; methodology; resources; supervision; writing—review & editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created at the time of this manuscript.

ETHICS STATEMENT

This study was approved by the University of Alabama at Birmingham IRB committee. Study participants and/or parents/legal guardians were individually consented for participation in the study. All the

methods included in this study are in accordance with the declaration of Helsinki.

TRANSPARENCY STATEMENT

The lead author Christy Foster affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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