Appendix A-4

National Longitudinal Study of Adolescent to Adult Health (Add Health)

Ancillary Study Proposal Form

I. Basic Study Information

1. **Full study title:** The role of persistent infections and inflammation in cognition.

2. **Principal investigator(s) (name, address, phone and fax numbers, e-mail address):**

Allison E. Aiello
[redacted]

Yang Claire Yang
[redacted]

3. **Collaborator(s) (name, address, phone and fax numbers, e-mail address):**

[redacted]

4. **Brief abstract describing the study (200 words maximum):**

Advances in the biological sciences have identified key processes involved in the immune system that may play a role in cognitive decline and Alzheimer's Disease (AD). These studies have suggested that infectious and inflammatory processes may be a trigger for cognitive decline and AD. However, these bench science infectious markers have yet to be explored in relation to cognition in studies of young adult to mid life age individuals. We propose to address this research gap by leveraging stored biological samples on individuals who participated in Add Health and test them for infections and inflammation implicated in cognitive decline. We will also assess whether measures of socioeconomic position (SEP) are associated with infection and inflammation and whether these biomarkers mediate observed associations between SEP and cognition. The resulting infectious phenotype data—when linked with rich, longitudinal data on the social environment, stressors, and physical and cognitive health—will provide an unprecedented resource to the scientific community for testing hypotheses about bio-psychosocial pathways to cognition in the US population.

5. Proposed start and end dates:

Sept 2017 - August 2022

6. Estimated cost:

$80 per sample for all four assays

Cytomegalovirus (CMV), Herpes Simplex Virus Type 1 (HSV-1), *Helicobacter pylori* (*H. pylori*), and Interleukin-6 (IL-6)

7. Proposed funding source and planned date of submission to funding agency:

NIH - R01 for submission on February 5, 2017

8. Does this study involve the support or collaboration of a for-profit corporation, or do you intend to patent any process or product of the analysis (see Section G above)?
____Yes
_x__No

II. Use of Previously-Collected Geocodes

1. Do you propose to use previously-collected respondent geocode data?
____Yes
_x__No (skip to Section III)

2. What types of geocode data do you propose to use? Mark all that apply.

Geocode	Wave I	Wave II	Wave III	Wave IV
State				
County				

Census tract				
Block group				
Latitude and longitude				

III. Use of Previously-Collected Biospecimens

1. Do you propose to use archived biospecimens?
   _x__Yes
   ____No (skip to Section IV)

2. Please indicate in the table below the type and amount of biospecimen needed and the number of respondents for whom biospecimens are requested.

Type of Biospecimen	Amount Needed	Number of Respondents
Wave III urine*		
Wave III DNA*		
Wave IV urine		
Wave IV DNA		
Wave IV blood spots	A total of 16 hole punches (2 spots)– 4 H. pylori, 2 for CMV, 2 for HSV, 8 for IL-6	5,000
Wave IV saliva		

*Not all biospecimens were collected on all participants in Wave III

3. What are the respondent selection criteria?

A randomly selected 5,000 participants with DBS samples in Wave IV; met Add Health inclusion criteria and agreed to have DBS stored

4. What assay(s) will be performed by the ancillary study?

Prior to analyses, we will elute the samples from the bloodspot following standard laboratory protocols (Gruner et al. 2015). Briefly, using a lab hole puncher, we will punch out the required number of spots for each marker, and elute in phosphate-buffered saline containing 0.05% Tween 20 and 0.08% sodium azide over night (Gruner et al. 2015). For each infection and inflammatory assay, we will use previously published solid phase enzyme-linked immunosorbent assays (ELISAs) (Dickerson et al., 2003; Lewensohn-Fuchs, 2003; Dowd et al., 2011; Waterboer et al., 2012; Miller and McDade, 2012) to assess the presence and number of

serum IgG antibody levels for each infection and levels of IL-6. This work will be conducted in [redacted] laboratory. His laboratory has a long standing record of successful immunoassay testing for numerous infection and immune markers, using state of the art instrumentation, positive and negative controls and CLIA standards. Dr. [redacted] will conduct QC procedures in his lab for each analyte using the embedded subsample of 100 Add Health respondents who provided a repeat collection of bloodspot at WIV. These intra-individual samples will allow us to calculate a measure of reliability and provide us with a QC for the lab methods. All QC testing will be conducted by laboratory personnel masked to the sample identification. Depending on the archived consent and volume of blood available, we will utilize as many IIV samples as available. These additional IIV samples will be pulled and matched to our random generated 1/3 of the sample for testing as proposed in this study (e.g. among participants with available blood spots). Indeed, Dr. [redacted] worked closely with Dr. [redacted] and Dr. [redacted] (of University of Washington Department of Medicine Dried Blood Spot Laboratory) in the application of his EBV assay and the hsCRP assay using Add Health Wave IV DBS, and has worked closely with Dr. Aiello on developing a highly sensitive CMV IgG antibody blood spot testing method that has been previously published (see reference section).

5. During what study years will the biospecimens be assayed by the ancillary study?

The first 3 to 4 years of study.

6. Can previously thawed and refrozen biospecimens be used for the assay?
   _x__Yes
   ____No (If no, provide references to supporting studies)

These are blood spot assays, and there are numerous studies showing that these analytes are all extremely robust to storage and freezing. For example, there are several large scale prospective studies, including the Womens Health and Aging Study, the Detroit Neighborhood Health Study, The Religious Orders Study, the Rush Memory and Aging Project, The Chicago Health and Aging Project, The Whitehall II Study, and the Sacramento Area Latino Study of Aging that have assayed herpesviruses (including CMV, HSV, *H. pylori,* and/or IL-6) in frozen blood samples that were collected many years ago, subsequently frozen for many years and more recently tested. Indeed, Dr. Aiello has led multiple funded studies that have included the successful testing of these infection markers in the majority of cohorts listed above (the only exception being the WHAS) (Meier et. al 2016; Weckle et al. 2015; Barnes et al. 2015; Simanek et al. 2014; Sealy-Jefferson et al. 2013; Dowd et al. 2013; Jeon et al. 2012; Roberts et al. 2010; Uddin et al. 2010; Aiello et al. 2008).

7. Provide a description of your plans for handling and storage of samples:

All bloodspot samples will be sent to [redacted] lab at Northwestern University and held in a locked laboratory space in -20 freezers.

8. Provide a description of your plans for the final disposition of samples after analyses are completed:

[redacted] will return any remaining bloodspot samples back to Add Health.

IV. Collection of New Biospecimens

1. Do you propose to collect new biospecimens from Add Health respondents?
   ____Yes
   _x__No (skip to Section V)

2. What type of biospecimen will be collected?

3. What amount of biospecimen will be collected from each respondent?

4. What are the respondent selection criteria?

5. What assay(s) will be performed by the ancillary study?

6. During what study years will the biospecimens be assayed by the ancillary study?

7. Provide a description of your plans for handling and storage of samples:

8. Provide a description of your plans for the final disposition of samples after analyses are completed:

V. Genomic Information

1. Do you propose to use genomic materials (any data from Add Health respondents' DNA)?
   ____Yes
   _x__No (skip to Section VI)

2. What specific gene(s), genotype(s), or SNPs will be investigated and by what methods of genotyping?

3. State the genetic hypothesis of interest:

4. What is/are the primary dependent variable(s)?

5. What is/are the primary independent variable(s)?

VI. Advantages for and Burden on Add Health

1. What is the advantage, both to you and Add Health, of conducting the study within the Add Health population as opposed to another population?

This is the first time any existing data and samples will be analyzed from a nationally representative, population-based study to address a major gap in social science and biodemographic research by examining whether common and persistent infections are specifically associated with cognition. Add Health is uniquely positioned with its scope of respondents and survey data as well as its plans to continue to follow participants into old age when the prevalence of Alzheimer's Disease increases significantly. The infections that we propose to test here have not only been implicated in Cognitive Decline and Alzheimer's Disease (Finch 2006; Noble et al. 2010; Schmidt et al. 2002; Yaffe et al. 2000), but also in

numerous cancers and other health outcomes (Coussens and Werb 2002; Grivennikov eta l. 2010; Hermes et al. 2006). Moreover, these infections are directly linked to stressors and provide key information on the integrity of the human immune system (Godbout et al. 2006). Finally, they are strongly patterned by socioeconomic status and race/ethnicity (Zajacova et al. 2009). Together, these infections will provide data to numerous researchers interested in asking a wide array of questions related to health and longevity. Incorporating these infection markers into the Add Health provides a unique opportunity to identify the role of infections for setting early adulthood trajectories of cognition and future cognitive decline that most aging studies designed to study AD cannot capture.

The tests we are proposing to use do not provide clinical/actionable results and would be applied to blood spot samples that were gathered almost a decade ago. Our measures are not intended to diagnose any infection in Add Health respondents, and it would be inaccurate/misleading to report a clinical finding to the participants (e.g. ELISA for signs of past infection (IgG) as we propose here versus Western blot or PCR for confirming active infection). In fact, since our infections of interest are not eradicable (with the exception of *H.pylori*), it has been recommended that researchers do not report results back to participants because the psychological stress associated with knowing that one has an asymptomatic latent herpesvirus infection is more harmful than not knowing. Indeed, the US Preventive Service Task Forces has recommended against even screening for asymptomatic genital herpesvirus infection (http://jamanetwork.com/journals/jama/fullarticle/2593575e) because the harms of reporting a positive test are more detrimental than the risks associated with asymptomatic infection (this is the recommendation for HSV-2- which has a substantially increased risk for STDs compared to HSV-1, CMV or EBV). This is because there is no way to ever clear these herpesviruses and individuals that have the infection without symptoms would be harmed by the psychological sequalae of knowing that they are infected (or falsely told that they were infected). Also, about 50-70% of middle age individuals will have signs of one or more of these infections (see below for our publications on the disease mechanisms and prevalence of these infections in multiple populations and US representative studies). If we decided to provide results and tell the participants that they were infected at some unknown point in their lifetime, we would be reporting back to the majority of individuals in Add Health that they have been infected by an untreatable infection at some point in their life (e.g. we can't actually tell them when they were infected). Moreover, the many other existing cohort studies that have collected these data, do not provide the results of these particular tests to participants because of the reasons we have outlined above. Finally, as Dr. Harris communicated to investigators on this project, EBV was tested in Add Health in Wave IV, and none of the Add Health participants were provided with the results of their seropositivity to EBV for the many reasons we mention above. For all of these reasons, we highly recommend that Add Health should decline reporting the results of these tests to participants and contend that providing results of these proposed tests may even do more harm than good to the participants.

2. If collecting new data or biospecimens, how much total respondent time (in minutes per person) will be spent on this project?

N/A

3. What types of assistance will the ancillary study require from the Add Health staff? This information will be used to estimate the amount of Add Health staff time to be spent on the project.

All assays and analyses will be conducted by the study team in collaboration with the VIM listed in this proposal, and no assistance beyond provision of access to samples will be required from the Add Health staff. Add Health staff will need to pull the blood spot cards from the archive. We propose to use dried blood spots (DBS) from a random ~40% of the Wave IV sample consented for additional blood tests (N=5,000) to assay for new markers and would therefore need Add Health staff to identify and pull these random samples- including only cards that have at least 2 blood spots available for analysis. After testing and Lab QC work is completed, results will be provided to the Add Health data manager to link the data with survey information. Add Health staff will then need to clean the data and return it to the collaborators on this proposal. Overall, we will need Add Health staff to assist with sample selection, pulling of specimens, shipping specimens to the [redacted] lab and cleaning of the testing results to merge with longitudinal Add Health data.

In the R01 proposal associated with this ancillary study, we budgeted 0.6 months' effort each for [redacted] and [redacted] for lab support in all five years. [redacted] will assist in the preparation of data for sampling purposes (indicators of GWAS data, archive consent), sampling of respondents, shipping samples, receiving and checking assay results, oversee codebook construction, and merging assay results with Add Health data. Angel will assist with pulling the blood spot cards from the archive once the sample is drawn, checking and interpreting lab results, working on the codebook under [redacted] and checking over documentation prepared by the investigators.

VII. Assurances

1. What new ancillary study data will be integrated into the Add Health database? Please specify the number and type(s) of variables that will become available to Add Health users. Any request to later amend this information must be communicated formally to the Add Health PI.

Infection data will be integrated into the database, including CMV, HSV, and H. pylori seropositivity and IgG antibodies. In addition, inflammation data on the IL-6 will also be added to the database. We will include values indicating batch number and kit lot number as well as a time stamp for testing each batch.

2. In what month and year should the Add Health project staff expect to receive the ancillary study data?

We expect to finish the ancillary study by the end of the third study year in June of 2020 with a possible extension of 6-9 months to account for unforeseen problems that may arise during the assay process.

3. What constructs, if any, will be used to create the ancillary study data (e.g., if a standardized scale will be used, what is the reference for that scale)?

N/A

4. If the ancillary study will collect new original questionnaire data on Add Health respondents or merge secondary data sources onto Add Health data, describe the sources of the questionnaire items and/or secondary data.

N/A

5. Provide investigator qualifications and prior involvement in Add Health, if any:

Allison Aiello, PhD has expertise in social and infectious disease epidemiology as well as cognitive health. Yang Claire Yang, PhD, has expertise in social disparities in health and the life course and chronic diseases of aging. Kathleen Mullan Harris, PhD, is the PI of the Add Health study. [redacted] PhD, has expertise in aging and immune system development.

6. Provide the name, position and contact information (address, phone and fax numbers, e-mail address) of individual who will receive, complete and submit annual progress report form:

Allison Aiello

[redacted]

VIII: Narrative Description

Please provide a brief narrative description of the proposed study. Do not exceed 5 single-spaced pages in length, excluding references (please use Arial 11-pt font). Include the following:

1. Purpose

The overall goal of this project is to assess infection and inflammation markers that have been implicated in dementia in the Add Health respondent population. Analyses of these inflammation and infection data will allow for more comprehensive assessments of how social determinants influence biological pathways to cognition across the life course, thereby identifying novel points of interventions to reduce health disparities in dementia in the future.

2. Brief background and significance

Alzheimer's disease (AD) and related dementia are chronic diseases of aging that take decades to develop and decline of cognitive function may occur many years before AD is diagnosed. There is a lack of knowledge of the age trajectory of change in cognitive functioning earlier in life in relation to the development and onset of AD and related dementia later in life. The proposed interdisciplinary research program addresses the major deficits in previous research by utilizing Add Health to assess whether socioeconomic position and infectious and inflammation related biomarkers influence cognition early in the life span. We intend to break new ground by using Add Health's innovative life course longitudinal design, available biospecimens for testing novel infectious and inflammation markers, and employing sophisticated analytic models to existing and newly proposed data that we will collect here. We will integrate measures of socioeconomic stressors, biomarkers of infection and inflammation pathways, to identify novel risk factors for cognitive decline from adolescence to midlife

3. Specific aims and hypotheses

The purpose of this study is to:

1) Examine novel (e.g. inflammation and infection) pathways by which social gradients in cognitive change and risks for cognitive decline occur in early adult and mid life. We will examine existing and newly tested biomarkers of infection and inflammation from subsamples embedded in the Add Health Wave IV when the cohort was aged 24-32 and we will follow them over time for cognitive decline using prospective survey data. We hypothesize that social adversity induces prolonged activation of physiological stress responses such that increased levels of C-reactive protein, IL-6, cytomegalovirus (CMV), herpes simplex virus (HSV-1), and *Helicobacter pylori* (*H. pylori*) immune response, in socially disadvantaged individuals may serve as pathways to cognitive impairment. Moreover, females may show greater immune response to stressors than males. And specific markers may operate with varying strength by sex at different points in life. To address this aim, existing blood spots will be tested for inflammation as measured by IL-6 and IgG immune response to infection, including CMV, HSV, and *H. pylori*. Individuals will be categorized as seropositive or negative to each infection as well as continuous IgG antibody levels for each infection.

4. Design and methods

The proposed study will use existing survey data combined with novel testing of biological samples from the Add Health study. We will characterize the infectious and immunological health of Add Health respondents, a nationally representative cohort. Additionally, we will test hypotheses drawing from life history theory and the theory of inflamm-ageing in relation to cognitive decline early in life. We will assess circulating levels of *IL-6* as a new inflammation marker to enhance existing data on CRP. Add Health data will be integral in testing these hypotheses, as it is a population-based study that includes young to middle age individuals, extensive social, behavioral, and health information in addition to newly proposed infectious biomarkers to address the proposed questions here. *CRP* and *Epstein-Barr (EBV)* infection have already been tested in Add Health. This pilot study will provide a more extensive battery of inflammation and infection markers and includes several novel infections that unlike EBV have been implicated in AD as well as related conditions such as CVD and Type 2 Diabetes. This will allow us to test new hypotheses regarding the social patterning of immunological mechanisms underlying cognitive decline and potentially other related conditions. Therefore, these markers provide an unprecedented opportunity to link data and allow researchers to address a wide range of hypotheses regarding the social patterning and biological mechanisms leading to key chronic disease outcomes.

5. Data and/or biological materials requested or to be collected

We are requesting 5,000 Wave IV blood spots, which we will assay for infections (CMV, HSV, and *H.pylori*) and IL-6.

6. Sample size and justification (i.e., formal power calculation)

With 5,000 subjects who have available bloodspot samples, to our knowledge this will be the largest sample for the analysis of IL-6 and multiple chronic infections (specifically HSV-1, CMV, and *H.pylori*) in this early life age span available to date with prospectively linked cognition and health data. These data will therefore greatly improve the capacity to develop more accurate

multivariate models by including numerous covariates while maintaining statistical efficiency in regards to statistical power and studying the impact of these infections and inflammation at a much earlier period in relation to prospective disease manifestation later in life. For cross-sectional analyses using data at Wave IV only, a sample size of 5000 subjects will provide us with 90% power to detect a small change in the slope in cognitive function of 0.04, assuming a standard deviation of our independent variable (either SEP measures or infection/inflammation) ranging from 0.50 to 2.0 and a standard deviation in cognitive score of 0.50, with a two-sided alpha significance level set at 0.05. For prospective analyses, we have 1,000 subjects who currently have repeat measures of cognition and we will have 89% power to detect a small to moderate change in the slope in cognitive function (0.10), assuming a standard deviation of our independent variable (either SEP measures or infection/inflammation) ranging from 0.50 to 2.0 and a standard deviation in cognitive score of 0.50, with a two-sided alpha significance level set at 0.05. Of note, future waves of Add Health will likely include measures of cognitive function over time among the entire study population, providing an unprecedented opportunity in the future to examine the influence of the proposed infectious and inflammatory biomarkers on prospective change in cognition among the larger 5,000 person sample. Overall, we are well powered to test the impact of SES and infection on cognition.

7. Analyses

The objectives of this application are the generation and dissemination of inflammatory and infectious phenotype characteristics of Add Health for use within the broad scientific community. With the data generated under this proposal, we also plan to perform a series of analyses of the data to provide the first nationally-representative population estimates of the impact of infectious burden on cognition in early to middle age. There are several reasons that these analyses will provide a highly unique resource. First, this study will provide the first data available in a national sample of young adults that includes these biomarker measures along with prospective social and health variables to better understand the long-term consequences of these infections and inflammation on cognition over time. Second, this study will provide a rare opportunity to test these hypotheses in a sample that includes both males and females as well as diverse racial and ethnic groups in the US. Finally, these infections and inflammation markers have been shown to be associated with a wide array of diseases of aging in addition to cognition, including Alzheimer's Disease, cardiovascular disease, Type 2 Diabetes, and cancers.(Aiello et al., Adam et al., Aggrarwal et al., Barnes et al., Badawi et al., Carbone et al., Holmes, Jeon et al., Rothenbacher et al.)

<u>Outcomes:</u>
Change in cognition scores over time as measured by:
Memory (score ranging from 0-37)
Word Recall (score ranging from 0-15)
Digits backward (score ranging from 0-7)

<u>Independent Variables:</u>
<u>Early-life SES</u> measures will include parental education, parental household income, welfare receipt, and subjective financial wellbeing. <u>Adult SES</u> measures will include respondent education, household income, welfare receipt, household assets, and occupation. We will construct composite measures of early-life and adult SES that are comprised of SES indicators using the standardization and average approach. <u>SES Mobility</u> measures the change between early-life and adult SES. Based on the lowest quartile of each composite SES measure to indicate SES disadvantage, we construct four categories of change that include: persistent disadvantage, upward mobility, downward mobility, and never disadvantaged.

Infection and Inflammation Markers: Chronic infection markers will include EBV (already available in Add Health), CMV, HSV, and *H.pylori* IgG (both as a continuous values and using the standard kit cut offs for seropositivity or seronegativity). Inflammatory markers will include hsCRP (already available in Add Health) and IL-6

Confounders:
We will include APOE genotype variables in all analyses to control for potential confounding or modifying effects of genetic risk factors for AD. We will also conduct sensitive analysis including GWAS derived single-nucleotide polymorphism (SNP) data that has been used to create polygenic education risk scores by our collaborator [redacted] as a control covariates to address potential genetic confounding of social risk for AD. We will also include baseline individual characteristics: social demographics, e.g., age, birth cohort (5-year groups), sex, and race/ethnicity; intelligence, e.g., picture vocabulary test (PVT); psychosocial and emotional well-being, e.g., negative life events, perceived stress, depressive symptoms, and happiness; health behaviors, e.g., smoking, alcohol use, physical activities, and sleep; health conditions, including self-rated health, chronic illness, and functional disability or activities of daily living (ADLs); medications, including hypertension, diabetes, anti-inflammatory and other drug use.

Analytical Models:

We will use a multi level model to assess the influence of the SEP characteristics on cognition and the impact of infection/inflammation on cognition over time: Level-1 repeated observation model: $Y_{it} = \beta_{0i} + \beta_{1i}Age_{ti} + e_{ti}$, where Y_{ij} is the cognitive test score (e.g., global or memory) for respondent i at time t, for $i = 1,\ldots,n$ and $t = 1,\ldots,T_i$ (T_i is the number of measurements and ranges from 1 to the maximum number of waves for each study), and is modeled as a function of age (centered around cohort median), with the random within-person error of e_{ti}assumed to be normally distributed; Level-2 model: for the intercept:$\beta_{0i} = \gamma_{00} + \gamma_{01}SEP_i + \gamma_{02}Coh_i + w_{0i}$; for the linear slope: $\beta_{1i} = \gamma_{10} + \gamma_{11}SEP_i + \gamma_{12}Coh_i + w_{1i}$, where trajectory parameters, including β_{0i} and β_{1i} , depend on person-level characteristics, SEP variables and birth cohort. The level-2 model thus specifies a distinct average trajectory for SEP, adjusting for birth cohort variation. The residual random effects, w_{0i} and w_{1i} are assumed to have a multivariate normal distribution. These models have the advantage of allowing data unbalanced in time by incorporating all individuals regardless of the number of waves they contribute to the person-year dataset, thereby reducing the number of cases lost to follow-up. In case of potential correlation between attrition and cognitive outcomes, we adjust for the effects of attrition by including the status of lost to follow-up such as death and nonresponse in level-2 models.

8. Study timeline

2017 - 2022

9. Literature references

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