Risk factors in **E**arly **M**ultiple **S**clerosis

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I. Hypotheses

Multiple sclerosis (MS) is a progressive disabling disease of the central nervous system (CNS) which is associated with substantial personal, financial and societal costs. There now are 15 approved disease modifying therapies (DMTs) available, and data supports that early intervention is associated with best outcomes. The ultimate cause of MS remains unknown, but increased risk is associated with a host of genetic and environmental factors. First degree relatives (FDRs) of MS patients have a 10-20 fold increased risk of being diagnosed with MS themselves. But diagnosis, still, is delayed for many, in part due to lack of a specific biomarker of disease activity, and also due to vagueness of early symptoms in many. More recent studies, have clearly outlined a prodrome in individuals ultimately diagnosed with MS, prior to overt clinical symptoms typical of MS. Thus, it would be extremely helpful if we could develop a risk stratification algorithm that outlined those with both high and low risk of developing MS as early as possible in the disease process. The central hypothesis of this protocol is that it is possible, using FDRs of patients with MS and assessing a variety of both known and unknown risk factors for MS, to define a risk algorithm for earliest signs of development of MS. In addition, while starting with a relatively limited cross-sectional analysis, we hypothesize that development of a larger, longitudinal study will allow us to improve sensitivity by simple accrual of larger numbers of participants over time with the endpoint of early signs of MS on MRI scans, but also improve sensitivity by adding additional markers of MS risk as they become available.

II. Specific Aims, among asymptomatic first degree relatives (FDRs), aged 18-30, of multiple sclerosis (MS) patients:

1: Determine the prevalence of brain MRI lesions disseminated in space (DIS), consistent with MS

2: Gather data on potential risk factors or early signs related to MS development, including markers for: genes, immunological function, environmental factors, neuroaxonal damage, Vitamin D levels, lipid metabolism, activity levels, mood abnormalities, and cognitive function. From this, develop a risk factor score, incorporating all relevant potential markers of increased risk of DIS.

3: To use this pilot, cross-sectional study as a base for development of a long-term, longitudinal, multi-center study to determine genetic and environmental risks for pre-symptomatic MS

4: To create and maintain a biobank of specimens for future analysis as other potential biomarkers become available.

III. Background and Significance

MS is a progressive, inflammatory and degenerative condition of the CNS with mean clinical onset at age 30[1]. MS is the most common CNS inflammatory disorder, with a prevalence of about 1/500 women and 1/1000 men in Colorado. It is a major cause of disability in young adults, and financial and psychosocial costs to the patient and society are great[2]. Diagnosis remains challenging, as early symptoms may be vague and transient, and there is no highly sensitive and specific biomarker or diagnostic test[3]. But there are now 15 disease modifying therapies (DMTs) available, all of which are most effective in those who are younger and relatively newly diagnosed[4]. Delays in treatment are associated with greater accumulation of clinical symptoms and magnetic resonance imaging (MRI) scan abnormalities[5-11]. Thus, there is a substantial need to develop early detection techniques for MS, so as to treat as early as possible.

But what is early? While clinical symptoms are the first overt indication of MS, evidence is rapidly expanding that there is a prodrome[12], or pre-clinical phase, prior to first symptoms and then diagnosis of MS. A clinical prodrome may be defined as the time between the onset of decline in a baseline level of functioning until formal diagnostic criteria are met. In the Nurses Health Studies I and II, the 341 women diagnosed with MS after entry into the study had lower levels of physical activity four to six years prior to MS diagnosis compared to age-matched control women[13]. This prodromal period may be associated with

increased health care use and costs, as seen in a recent Canadian study of the five years preceding the diagnosis of MS[14]. In an Argentinian case-control study[15], diminished cognitive performance in school was reported in individuals who developed a first attack consistent with MS, so-called clinically isolated syndrome (CIS), up to 10 years after leaving school. MRI scans also detect many asymptomatic lesions in MS, especially early in the course[16,17]. Similarly, individuals destined to develop MS have early signs of anxiety and depression several years prior to diagnosis of MS[18]. The majority of patients with CIS have already developed MRI evidence consistent with demyelination at some prior, indeterminate time point, i.e. the first scans typically show multiple areas of abnormality that have not been associated with any clinical symptoms. In CIS clinical trials, the average number of asymptomatic lesions on first brain MRI scan after a first clinical attack has exceeded 17[5-11]. In addition, a small percentage of people undergo MRI scans for reasons seemingly unrelated to MS, yet the scans reveal abnormalities highly consistent with MS. This is referred to as radiologically isolated syndrome (RIS)[19-21], and there is now a clinical trial underway to determine if very early treatment is beneficial in these patients (NCT02739542). Both CIS and RIS are predictive of new clinical disease activity consistent with MS. In a study of RIS, over a quarter of participants had cognitive impairment similar to that seen in MS[21]. RIS, CIS and early onset RRMS are all associated with diminished brain volumes compared to age matched controls[22-24], suggesting neurodegeneration occurs very early, even in the prodromal phase.

Damage to the neuronal structures of the CNS can also be seen early in MS. Presumed damage to neural tissue has been associated with elevated levels of the neuronal cytoskeleton, Neurofilament light (NfL), in CIS and early MS patients, and are independently associated with relapses, disability progression, and MRI markers of brain lesion volume and atrophy[25-29]. Similarly, evaluation of lipids may be important in early MS. MS is a demyelinating disorder of the CNS, and about 25% of total body cholesterol is located in the brain, most incorporated in the myelin sheaths wrapping around axons[30]. Comorbidities linked to dyslipidemia are associated with increased risk for development of MS[30] and disability progression[31-33]. After CIS, elevated total cholesterol (TC) level has been associated with Gad-enhancing lesions[34], and TC and LDL levels have been associated with development of new and newly enlarging T2 lesions on brain MRI[35]. Four years after CIS, and with use of interferon, there are rapid decreases in LDL, HDL, and TC, which then are followed by a slow return to baseline levels. Greater percent decrease in HDL at 3 months is associated with less brain atrophy over four years[36]. Thus, abnormal lipid profiles appear to be associated with damage to the CNS, even early in the course of MS. Neither NfL nor lipid profiles have been evaluated as early markers CNS damage in pre-symptomatic individuals at high risk of developing MS.

The preponderance of evidence is consistent with the concept that MS results from the imposition of one or more environmental insults in individuals with a genetic background that predisposes them to MS[1]. Epidemiological studies have identified both genetic[37-41] (over 200 genes have been identified) and environmental risk factors (exposure to Epstein-Barr virus[42], obesity[43-48], vitamin D deficiency[49-54], and smoking[55-60]) associated with the development of MS, and/or more progressive disease over time, but the triggers at onset remain unknown. Finally, asymptomatic individuals with parents or siblings with MS (first degree relatives - FDRs), have a 10-20 fold increased lifetime risk of developing MS compared to those without a relative with MS[61]. This translates to about 4-5% of girls/women and 2-3% of boys/men who might be ultimately diagnosed with MS in their lifetimes. Diagnosis in multiple family members enhances this risk further. In addition, a variety of cross-sectional MRI studies[62,63] have identified that about 10% of asymptomatic FDRs of MS patients have brain MRI lesions that could be demyelinating in nature, including fulfilling criteria for lesions disseminated in space (DIS)[63-66]. Thus, given that the median age of onset of relapsing MS is about 30 in multiple studies, we might expect that by age 30, 2-5% of FDR might have developed MS, but up to 5-10% may have developed MRI signs that place them at risk of developing MS, i.e. RIS.

In the recent Genes and Environment in Multiple Sclerosis (GEMS) study[67] of 65 female FDRs of MS patients, those with a combined genetic (based on 64 known single nucleotide polymorphisms, SNPs, in 2012) and environmental (female sex, prior history of mononucleosis and smoking) high risk score were compared to FDRs with a lower score for developing MS. Those with a high risk score showed a trend toward higher BMI and weight, but there were no differences in age, height, cigarette smoking exposure,

25-hydroxyvitamin D level, or history of infectious mononucleosis or migraine. Five (four in high risk, one in low risk groups) of the 65 women had MRI evidence of lesions disseminated in space as seen in MS. Those in the high risk group had relatively impaired quantitative vibratory sensation testing, but there were no differences in quantitative brain volume measurements or retinal nerve fiber layer (RNFL) thickness on optical coherence tomography. The overall conclusion of this cross-sectional study was that high risk individuals showed early subclinical risk signs of MS, noting the study was underpowered to detect a difference in neuroimaging outcomes between higher-risk and lower-risk individuals.

Type I diabetes (T1D) shares many clinical and immunological features with MS[68], including that FDRs of T1D are at high risk of developing T1D. In our institution, the long-term Diabetes Autoimmunity Study in the Young (DAISY) has sought to understand specific risk factors associated with this T1D development[69-72]. John Cambier and associates have identified loss of B cell anergy in some FDRs' of T1D patients[73]. Also here at the University of Colorado, Anschutz Campus, our colleague, Dave Wagner, and associates have identified a unique, pathogenic T cell population in T1D, an expansion of CD40 on CD4+ cells[74]. Subsequently, we have shown a similar expansion of CD40 expression in MS patients of all ages compared to controls[75]. Studies looking at CD40 expression in FDRs of T1D patients are underway. Thus, it is possible both of these immunological biomarkers, B cell anergy and T cell overexpression of CD40, may be present in FDRs of MS patients.

Innovation – The GEMS study described above is one approach to identification of early MS, but it had several limitations. It used a pre-defined risk score, including a SNP analysis of just 64 SNPs, when more recent analysis suggests over 200 may be relevant. It employed a long, detailed, and expensive brain MRI, but did not include spine or FLAIR* analysis. The cost limited participation. GEMS did not include potential immunological markers, markers of CNS damage (such as NFL or lipids), activity levels, or a detailed environmental screen. Due to privacy issues, they were unable to verify the MS diagnosis in FDRs. Finally, it was limited to 100 patients, of which only 65 (females only) were analyzed. Other studies (as yet unpublished) designed to look at risk factor algorithms for development of MS are employing larger numbers of patients, but minimal, if any, MRI analyses, no or minimal immunological or CNS damage markers, and no activity levels. Most importantly, they are using full clinical diagnosis of MS as the outcome measure, greatly limiting the number of "positive" patients, and biasing toward a much later time in the disease process. Thus, our study has great innovative value for several reasons:

a. We will define pre-symptomatic MS in 300 FDRs using stringent MRI criteria, including FLAIR* sequences[109-113], thus significantly increasing sensitivity and detection in the prodromal phase. Proband MS patients will be defined from our own population of MS patients known to fulfill formal diagnostic criteria[66] for MS.
b. We will analyze genetic, immunological, vitamin, lipid, viral, and CNS damage markers; activity levels[76]; and a mood and cognitive screen, with the goal of defining an expanded risk stratification scheme, not a pre-defined one
c. We will include a detailed environmental screen[77-84], employing approaches very similar to the DAISY study, and Dr. Norris, lead epidemiologist in DAISY, will participate as a Co-PI.
d. We will compare those FDRs with and without brain MRI abnormalities to each other, and to non-FDR controls (blood and environmental screen, not MRI)
e. We will create a biobank of blood, both plasma and PBMCs, including pre-processed serum/plasma and RNA, allowing us to add to the risk stratification model over time.

IV. Preliminary Studies

The background for the entire study is as listed above under Significance. We have extensive experience in blood and tissue banking, as Dr. Corboy has been director of the Rocky Mountain MS Center Tissue Bank, which has collected over 500 autopsied brains and spinal cords for distribution to researchers in the US and abroad. Dr. Alvarez is head of our MS Center's Biobank of CSF and blood specimens. In addition, references 73-75 outline results with T1D and MS patients with potential immunological abnormalities relevant to FDRs of MS patients. Similarly the DAISY study references speak to the capacity to do detailed environmental screening in a very similar population of young patients and the expertise of Dr. Norris. Drs.

Cambier and Wagner have already developed the lab tests described. NfL (Quanterix) and lipid studies are commercially available and we have an agreement with Quanterix to perform the studies as in references 25-29. We also have performed over 50 clinical trials in MS, and have significant experience with large recruitments, including over 450 for our Biobank and several hundred for a study looking at development of a JC Virus serology test (the STRATIFY-1, and STRATIFY-2 studies[85,86]), for which we were one of the largest recruiters internationally.

V. Research Methods

Approach - The concept in this pilot study is to perform a cross-sectional analysis of the FDRs of MS patients. FDRs were explicitly chosen as they have enhanced risk of MS, thus enriching for abnormal, pre-symptomatic MRI scans. Broadly, we will perform an abbreviated, "screening", brain MRI in 300 neurologically asymptomatic FDRs; draw blood for genetic, immunological, vitamin, lipid, viral, and CNS damage markers; and do an environmental screen, with mood and cognitive screening tests. Activity levels will be monitored with step analysis with the Godin Leisure-Time Exercise Questionnaire (GLTEQ)[87], which has been validated in both adults[88] and children[89] with MS. FDRs with abnormal brain/spine MRIs fulfilling DIS[64-66] will then be compared to age-and sex-matched FDRs *not* having MRIs fulfilling DIS, and to age- and sex-matched non-FDRs who do not carry a diagnosis of MS or have symptoms suggestive of MS (no MRI for the latter group, but all other measures taken). The long-term goal is to create a platform for a longitudinal study, likely at multiple sites, with the capacity to interrogate stored samples of DNA, RNA, plasma and PBMCs with associated environmental screening and the screening MRI scans as described above. As part of this, we also have had preliminary discussions regarding partnering with Savvysherpa for development of a long-term activity level monitoring system specifically for MS.

A. Outcome Measure(s): Individuals will be defined as having pre-symptomatic MS if they have brain/spine MRI abnormalities fulfilling DIS as defined by the Swanton[64-66] criteria. Importantly, we will also obtain sequences which allow for determination of whole brain volume measures. The following MRI sequences will be obtained:

1. Whole brain T1-weighted images acquired using 3-Dimensional fast spoiled-gradient recalled acquisitions (T1-3D-FSPGR) with 1 mm slice thickness and isotropic voxel dimensions.
2. Whole brain 3-Dimensional Double Inversion Recovery (DIR) acquisition with 1.5 mm slice thickness.
3. Whole brain T2-weighted Fluid Attenuated Inversion Recovery (FLAIR) images with 1 mm slice thickness and isotropic voxel dimensions will be combined with
4. Whole brain T2*-weighted segmented echo-planar imaging (segEPI) images with <=1 mm slice thickness and isotropic voxel dimensions to obtain FLAIR* images.

The sequences were specifically chosen to: a) screen brain with the greatest specificity (FLAIR*); b) allow for later volumetric analysis of the brain; and c) use the most sensitive technique for cortical and subcortical brain lesions (3D-DIR).

B. Description of Population to be Enrolled: The population will be a total of 300 women and men, of all races and ethnicities, between the ages of 18-30, inclusive, who have no symptoms of MS, but have at least one FDR with documented MS fulfilling McDonald 2017 criteria. Up to 1000 will be screened for inclusion, but some will be excluded due to inability to undergo a brain/spine MRI or have blood drawn, lack of interest after discussion of study requirements, or symptoms concerning for MS (the latter will be referred to a qualified neurologist for evaluation). FDRs with one or more siblings/parents with MS will be included, and more than one FDR of a proband MS patient will be allowed. A control group (based on # of FDRs with positive MRIs - we anticipate 15-30) of age- and sex-matched controls who have no known family members with MS (out to first cousins and grandparents) will have blood drawn and fill out the environmental questionnaire, but will not undergo a brain MRI. The controls will be matched 1:1:1 for those FDRs with positive MRI scans suggesting pre-symptomatic MS and those age-and sex-matched FDRs who do NOT have positive MRI scans suggesting pre-symptomatic MS. The ages were specifically chosen so as to have a good opportunity to collect an adequate number of MRI-positive, asymptomatic participants prior to the known median age of MS clinical onset (30 years), but not so young as to lower the likelihood of detecting asymptomatic brain MRI lesions.

1. FDR Inclusion Criteria
    a. Male and female
    b. All races and ethnicities
    c. Ages 18-30
    d. Must have a parent, sibling or child with MS fulfilling McDonald 2017 criteria
    e. No symptoms suggestive of MS on formal screen
    f. Ability to undergo a non-contrast MRI and venipuncture, and perform an environmental screen, mood screen and cognitive test
2. FDR Exclusion Criteria
    a. Symptoms suggestive of MS on formal screen
    b. Prior diagnosis of autoimmune disease that may be associated with CNS dysfunction and MRI lesions, e.g. Sjogren's
3. Non-FDR Inclusion Criteria
    a. Male and female
    b. All races and ethnicities
    c. Ages 18-30
    d. Must NOT have a FDR (parent, sibling, child) or second degree relative (grandparent, aunt or uncle) with MS fulfilling McDonald 2017 criteria
    e. No symptoms suggestive of MS on formal screen
    f. Ability to undergo venipuncture and perform an environmental screen.
4. Non-FDR Exclusion Criteria
    a. Symptoms suggestive of MS on formal screen
    b. Prior diagnosis of autoimmune disease that may be associated with CNS dysfunction and MRI lesions, e.g. Sjogren's

FDR participants will be enrolled from among the FDRs of MS patients seen at the Rocky Mountain Multiple Sclerosis Center at the University of Colorado in Aurora, Colorado (RRMSC at CU). Our center has approximately 3500 unique patients, of whom nearly all have asymptomatic FDRs, many in the appropriate age range. Documented MS proband patients will be made aware of the study via interactions in the office, our newsletter, and IRB-approved information given to them for their FDRs. FDRs who are then interested will initiate contact with the research group. If this does not provide adequate enrollment, we will recruit by local advertisement through known offices of neurologists in the Denver and surrounding regions. Age- and sex-matched non-FDR controls will be recruited from the friends of FDRs who are positive on the screening MRI, or will be recruited from local recruitment in the department of Neurology offices, from those non-MS conditions. FDRs and controls will be screened on the phone for basic demographic criteria (e.g. age, location where they live) and a checklist of basic MS symptoms, and to be certain they do not have previously undiagnosed MS or other autoimmune/neoplastic disorders. Those with concerns for MS at screening will be referred to a qualified neurologist for assessment.

C. Study Design and Research Methods:

1. Prescreen FDRs by phone for ability to participate and lack of MS symptoms, using a standardized script to exclude those with overt MS symptoms. Proband MS patients will have documented MS by McDonald 2017 criteria[66].
2. Qualified FDRs will be scheduled for a study visit after completing a pre-screening questionnaire by phone. If they are not excluded by pre-screen, and wish to participate, a study visit will be scheduled. A waiver of documentation of consent will be obtained over the phone so as to instruct participants to fast from midnight prior to the first study visit. This will also allow them to fill out the environmental questionnaire on-line before their visit and at their convenience. The environmental survey must be completed at least three days prior to their visit. If not, the study visit will be canceled and rescheduled once its complete.
3. A study visit will include (See Table of activities below):
    a. Read, ask questions regarding, and sign the consent.
    b. Document completion of environmental questionnaire.
    c. Obtain a measured height and weight, and compute a Body Mass Index (BMI)
    d. Perform a non-contrast brain MRI (per section V.A)

e. Perform a fasting Blood draw consisting of no more than 100 ccs of blood.
    i. Blood drawn at the primary study visit will be a maximum of 100 ccs to be processed in the following fashion. Participants will not be made aware of their blood sample results
        1. 30 ccs for immediate cell sort by David Wagner, PhD into T and B lymphocytes, for CD40 expression studies, requiring primary analysis with fresh cells, and done on all samples. Also for HLA analysis.
        2. 20 ccs processed for delayed analysis of SNPs, viral serologies (Herpes Simplex Virus 1 and 2, Cytomegalovirus, Varicella-Zoster Virus, and Epstein-Barr Virus), vitamin D levels, and neuroaxonal (NfL - Quanterix) and myelin damage (lipids), which all can be performed on stored samples.
        3. 50 ccs processed and stored for delayed analysis of plasma and PBMCs, RNA and DNA, for studies not yet determined

f. Perform the Perceived Stress Scale and the Neuro-QoL screen for anxiety and depression;
    i. Participants screening for concerns for suicidality on the mood screen will be referred to an emergency room for evaluation.
g. Perform a Symbol Digit Modality Test, a screen for cognitive function. The latter will be done in the written format.
    i. Participants will not be advised of Cognitive screen results.
h. As a measure of physical activity, we will use Godin Leisure-Time Exercise Questionnaire (GLTEQ)[87], which measures mild, moderate and strenuous physical activity during a typical seven day period. This has been validated in MS[88,89], and is scored either into three levels of activity (sedentary, moderately active, or active), or may be used as a continuous measure.
i. As a dietary assessment, we will use the Harvard 2007 Booklet FFQ[114], which is a semi-qualitative food frequency questionnaire used to assess intake of various nutrients over the past year. This has been robustly validated in a variety of populations[115-118], and nutrient intakes are computed by assigning a daily frequency weight.

4. MRIs will be read by a board-certified neuroradiologist (Dr. David Rubinstein) with extensive experience in reading brain MRIs in general, and MS MRIs in particular. All FDRs will receive a follow-up contact concerning the results of their MRI within one week. FDRs with a brain MRI fulfilling criteria for DIS will be referred to a qualified neurologist to determine if they should be treated or monitored more closely. Individuals with abnormal brain MRI scans not suggestive of or fulfilling DIS criteria, but having non-specific brain lesions (as can be seen with migraine and many other conditions), also will be referred to a qualified neurologist for evaluation as needed. Those FDRs with unexpected abnormalities (e.g. severe sinus disease, pineal cyst, pituitary abnormality, cavernous angioma, etc.) will be referred as necessary to a qualified physician.
5. FDRs with a positive brain MRI showing lesions DIS will be asked to return within four months for a repeat blood draw of no greater than 100 cc of blood, to be compared to age-and sex-matched FDRs without a positive brain MRI (who will also be asked to return within 4 months for the repeat blood draw) and age-and sex-matched non-FDRs, both in a 1:1 ratio. The former will be identified from those already in the study, and the latter will be recruited from age- and sex-matched friends of the FDRs with positive brain MRIs. The non-FDRs will be prescreened and perform all the study procedures as the FDRs on the first study visit, with the exception of the MRI.
    a. This blood draw will be for B cell anergy and cytokine studies in Dr. Cambier's lab, and repeat CD40 studies on non-FDR controls (i.e. not already done by Dr. Wagner's lab) which require fresh cells, but done only on this subset of participants. The rest of the blood will be processed and stored as described in C3ei.3 above for potential future use.
6. Data will be stored in a HIPAA-compliant database. Analysis will be done via case-cohort approach with the roughly 15-30 MRI-positive participants, controls as noted, and looking at the markers individually and together.

STUDY PROCEDURES

		Visit 1	**Visit 2** (within 4 months after V1)
FDRs	MRI positive	Vitals MRI Blood draw Environmental Questionnaire NeuroQOL SDMT Activity Questionnaire Diet questionnaire	Blood draw
	MRI negative Controls	Vitals MRI Blood draw Environmental Questionnaire NeuroQOL SDMT Activity Questionnaire Diet questionnaire	Blood draw
	MRI negative Non-Controls	Vitals MRI Blood draw Environmental Questionnaire NeuroQOL SDMT Activity Questionnaire Diet questionnaire	
NonFDR Controls		N/A	Vitals Blood draw Environmental Questionnaire NeuroQOL SDMT Activity Questionnaire Diet questionnaire

D. Description, Risks and Justification of Procedures and Data Collection Tools:

1. Blood Draws: The risks of phlebotomy are small and include temporary discomfort from the needle stick, bruising and rarely infection. The amount of blood drawn at each time point for this study is well under the established recommended maximum for adults.
2. MRI: The most common side effect of having an MRI is flashing lights in the eyes. This is caused by the magnetic waves and is not harmful. Some people also experience warmth and reddening of the skin. This usually goes away in a few minutes. This is a NON-contrast scan. In addition, there is an emotional risk of identifying abnormal MRI lesions in individuals with no symptoms concerning for MS, and an as-yet unknown risk of developing symptom concerning for, or disability related to, MS. This potential risk should be offset by the potential benefit of earliest possible intervention for a potentially disabling disorder.
3. Confidentiality: There is a potential for loss of confidentiality. A unique study number will be assigned to each subject at inclusion, immediately after informed consent has been obtained. This number will serve as the subject's identifier. Link between study number and subject's identity will be kept separately from study data on a secure server accessible only to study personnel. Data will be stored in a HIPAA-compliant database.

E. Potential Scientific Problems:

The largest potential hurdle would be inadequate numbers of FDRs with positive brain MRIs. That said, we view this cross-sectional study as a proof of concept that patients can be recruited, and that we can develop a biobank of specimens to be interrogated in the future. The long-term goal is creation of a longitudinal, multi-center study similar to the DAISY study of children at increased risk for T1D. Thus, we have no illusion that we will settle this issue with a relatively small cross-sectional study, but plan to use this study to lay the groundwork for a larger, longitudinal study, especially recruiting at other national/international sites.

F. Data Analysis Plan: Statistical Approach

a. Based on previous studies[62,63], we estimate that 5-10% of the 300 FDRs (15-30) in our pilot study will exhibit an MRI endpoint. In order to test for associations with this endpoint to obtain valuable preliminary data, we will perform a nested case control study. We will match two controls per case based on age and sex: one group will be FDRs not fulfilling DIS on brain MRI; the second will be non-FDRs who are age- and sex-matched to FDRs who do, and do not, fulfill DIS on MRI scans. The non-FDRs will perform the environmental screen and undergo venipuncture for blood analysis identical to the other two groups, but will not have a MRI scan.

b. Aim 1. Descriptive statistics

c. Aim 2. Association analyses; with individual factors and in a multiple risk factor analysis. In previous studies[37-60], HLA type, Vitamin D levels, Epstein Barr Virus exposure, smoking and early adult obesity have conferred a maximum odds ratio of 2-3 (many SNPs confer an odds ratio of 1.1-1.4) of developing MS when studied individually. In the GEMS[67] study, participants were stratified into high and low risk categories based on a deep phenotyping of 64 SNPs, and environmental screen (including sex). Comparative analyses were performed on the high and low risk groups for both neurological exam and MRI measures. In this study, we plan to use the known risk factors, and add potential biomarkers (e.g. NfL, lipid levels) to define the risk of DIS in this population. For the power analysis we made the assumption that no single marker would exhibit an odds ratio greater than 2 for increased risk of DIS, and that all markers are additive. In this early proof of concept phase, with an estimated case sample size of 30 with 60 age- and sex-matched controls, our priori power analysis suggests an expected power ranging from 20-33% for risk factors such as smoking, obesity, and vitamin D deficiency. Using frequency and odds ratio from the GEMS[67] study (OR = 2.9) we expect a power of 64% to detect an effect of HLA type risk. While this study will be statistically underpowered, and estimates will be imprecise, we expect that some results will have clinical significance. We plan to use these results and stored samples as the groundwork for larger, longitudinal, multi-site studies. These future studies will allow us to enroll samples sizes of greater than 500, necessary to reach 80-90% power. In reality, it is possible the effect some factors may depend on the levels of other factors[90]. Example hypothesis that we will test**:** *FDRs with DIS lesions are more likely to have been exposed to EBV as indicated by elevated EBV serology than FDRs of the same age and sex without DIS lesions.* By extension, we will then define a risk score combining factors that are discriminatory, e.g. Vitamin D level, smoking, obesity, elevated NfL levels, impaired B cell anergy, and HLA type may each have a roughly 2 fold increased risk, whereas those with four of these factors have a six-fold increased risk of DIS on MRI.

   Given the matched case-control design, we will use multivariable logistic regression, allowing us to adjust for confounders, to estimate and test risk factors controlled for others, and to gain greater precision. The clustering effects of matching can be incorporated using conditional logistic regression, or repeated measures methods such as GEE.

d. Aims 3 and 4 do not have statistical implications.

G. Summarize Knowledge to be Gained:

This is a highly innovative research protocol which seeks to duplicate the approach of the successful DAISY study, but in a MS population. We aim to define a set of environmental, genetic, lipid, neuroaxonal/myelin damage or immunological markers of earliest disease activity in MS. From this, we ultimately hope to develop a risk stratification algorithm for development of MS. This will allow for earliest possible intervention for this disabling disease. The purpose of Aim 4 will be to enhance the risk profile over time, as other potential, highly sensitive, precise biomarkers become available. One example is

microRNA (miRNA), which are a class of small (~21-25 nucleotides), noncoding RNAs. These are very important in posttranscriptional regulatory processes[91]. MiRNA's may directly and indirectly promote neurodegenerative action through a variety of pathways. MiR-155, known to be upregulated in MS, and silences CD47, a protein involved in phagocytic signaling processes that block macrophage activation[92]. MiRNA's may contribute to disease progression by driving differentiation of Th1 and Th17[92-95], known pathogenic T-cell subsets[96,97]. MiRNA may have potential as a tool for relapse prediction[98-100], distinguishing different subtypes of MS[101-105] (i.e. RRMS vs. PPMS vs. SPMS), and determine disease progression[104,105]. MiRNA's may have potential as therapeutic targets for controlling disease activity[98,99]. Untreated MS patients display elevated levels of miR-126 relative to healthy controls. After these patients underwent DMT treatment, levels of miR-126 decreased to similar amounts observed in controls[98]. Non-invasiveness, easy analysis, and high-stability make miRNA a desirable biomarker for disease prediction[106-108]. However, one miRNA sampled from various compartments (plasma, serum, lymphocytes) yields significantly different results, contributing to the complexity of comparing results across studies[92,107]. Additionally, RNA extraction, miRNA measurement, and analyzation do not have a standardized protocol, resulting in heterogeneous results across different studies. As novel, consistent, and standardized techniques become available, miRNA expression studies with longitudinal design at a large site with control comparators may allow for insight on miRNA's ability to predict development of MS, relapses, and even disease subtype.

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