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14. ABSTRACT Cognitive dysfunction is increasingly recognized in multiple sclerosis (MS), and the role of cortical gray matter lesions as possible causative factors for cognitive dysfunction, and possibly as a main disease initiating factor has recently been proposed, partially by our team's observations in MS neuropathology studies. Our main hypothesis is that cortical lesions determine the formation of white matter lesions along tracts directly originating from them, and that the extent of cortical dysfunction will be directly proportional to the location and overall load of cortical lesions. In order to detect cortical lesions more efficiently, our team has developed a customized pulse sequence we termed GM-DIR, with increased sensitivity for cortical lesion detection. Utilizing standard DIR, our custom-made GM-DIR and 60-direction DTI studies, we have been able to determine in an early pilot study that connections between lesions do exist. During the study period, we have processed data on 25 early MS patients (less than 5 years of disease activity) and compared that with 24 controls, and concluded that white matter lesions do in deed appear to be connected with cortical lesions. Our first abstract related to these novel observations has been submitted to the upcoming ACTRIMS/ECTRIMS meeting. Furthermore, we started collecting and analyzing neuropsychiatric outcome measures using the MACFIMS battery. Our first results are presented herein this report. Correlative analysis of MRI and MACFIMS data has been initiated and is ongoing in our laboratory. Enrollment has been steady; we don't anticipate any problems meeting our target during the study duration. We believe that our observations will meaningfully contribute not only to our understanding of cognitive dysfunction in early MS, but also to key disease initiating events, which may pave the way to the development of more specific disease modifying therapies.					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	14
Reportable Outcomes.....	14
Conclusion.....	16
References.....	N/A
Appendices.....	N/A

Introduction:

Multiple sclerosis (MS) is an immune mediated demyelinating disease affecting young adults during their most productive years. While several treatments have become available during the last two decades to address the most common form of MS (relapsing-remitting MS), the pathogenesis still remains unknown. Cognitive dysfunction is increasingly recognized in MS, and the role of cortical gray matter lesions as possible causative factors for cognitive dysfunction, and possibly as a main disease initiating factor has recently been proposed, partially by our team's observations directly in MS neuropathology studies. Our main hypothesis is that cortical lesions determine the formation of white matter lesions along tracts directly originating from them, and that the extent of cortical dysfunction will be directly proportional to the location and overall load of cortical lesions. In order to detect cortical lesions more efficiently, our team has developed a customized pulse sequence we termed GM-DIR, with increased sensitivity for cortical lesion detection. Utilizing standard DIR, our custom-made GM-DIR and 60-direction DTI studies, we have been able to determine in an early pilot study that connections between lesions do exist. During the study period, we have processed data on 25 early MS patients (less than 5 years of disease activity) and compared that with 24 controls, and concluded that white matter lesions do in deed appear to be connected with cortical lesions. These patients included both previously enrolled patients during the locally funded pilot period, as well as patients enrolled under the DOD sponsored stage of the study. Our first abstract related to these novel observations has been submitted to the upcoming ACTRIMS/ECTRIMS meeting. Furthermore, we started collecting and analyzing neuropsychiatric outcome measures using the MACFIMS battery. Our first results are presented herein in this report. Correlative analysis of MRI and MACFIMS data has been initiated and is ongoing in our laboratory. Enrollment has been increasing and is steady; we don't anticipate any problems meeting our target during the study duration. We believe that our observations will meaningfully contribute not only to our understanding of cognitive dysfunction in early MS, but also potentially to key disease initiating events, which may pave the way to the development of more specific disease modifying therapies.

BODY

During the first year of the study, we have made excellent progress in recruiting patients, further optimizing our scanning protocol, established an intranet-based database management tool for MACFIMS data entry and analysis, and submitted our first conference abstract, so overall we consider this a very successful year. While our study start date is listed as May 1, 2013, actual patient enrollment in the context of the study only started in September. This delay was partially due to the local IRB modifications (final approval of local IRB protocol reflecting DOD as the funding source for the project; approval for study subject and control enrollment granted on July 10, 2013), DOD's HRPO approval was granted on July 11, 2013 (A-17879 HRPO Approval Memorandum; IRB Number 13-003115, Proposal Log Number MS120041, Award Number W81XWH-13-1-0098), and Mayo Clinic's Central Research Office was notified by DOD officers of the final approval and funding of our project, with Award Letter sent to Mayo Clinic's Research Central Mailbox on July 15, 2013 at 6:44 AM; it was in this note that we were informed that the official study start date was May 1, 2013. Our study initiation visit commenced in August, and included all our collaborators: Dr. Port from the Department of Radiology, Drs. Tillema and Lucchinetti from neurology, Dr. Mandrekar from biostatistics; Dr. Rowe from Psychology; radiology and psychology schedulers, Mayo MS Center leadership, Neurology research office representative, senior MRI technician, local statistician team including Mr. Stephen Weigand and Patrick Fitz-Gibbon. Following training of neuropsychiatry personnel in MACFIMS, purchasing the required MACFIMS study forms, enrollment started in September 2013.

Major tasks associated with our project:

Subject recruitment: Before the start of the “official” DOD-funded patient enrollment, we were able to scan patient using our established IRB protocol that served as the basis of our pilot studies; we also had intramural funds available for scanning. With this mechanism, we were able to enroll 29 MS patients and 26 controls. All these were scanned using our proprietary protocol on a dedicated 3T GE scanner. In the context of the study, we established a rigorous screening mechanism of all charts of MS patients by screening the calendar of our MS Clinic, and the calendars of all staff neurologists involved in MS care. With this mechanism, up to the end of April 2014 we screened approximately 300 charts, and identified 86 patients potentially eligible for the study. Out of these, 58 were unable to join the study, most commonly due to time limitations: most of our patients come to see us not from local communities, but from all over the United States. The time they spend in Rochester is typically limited. Our study adds an approximately 40 minute MRI scan and a 90 minute cognitive study to their stay; with all logistics involved, essentially this comes down to an extra 3.5-4 hours, which due to flight schedules the patients aren’t always able to accommodate. The interest in our study is overall very high, and several patients asked for being considered for the study during their return visit. Realizing this limitation, we extended our pre-screening period from 2 weeks ahead to 8 weeks ahead, which has resulted in increased enrollment, as this way patients can plan ahead in terms of travel plans and length of stay in Rochester, Minnesota. Some patients did not return our screening calls; with the addition of a dedicated coordinator Ms. Jamie Sorum to our study team (made possible by Mayo Clinic’s MS Center, they fund her FTE as previously reported), we have established a much better mechanism of patient contacts, and the above problem no longer represents a major limitation. Overall, 29 patients agreed to be enrolled. Out of these, 2 could not come to their Mayo Clinic appointments due to unforeseen issues and rescheduled for later. 3 patients had additional logistical problems and could not keep their study appointments. Overall, we enrolled 24 study subjects over the 8 months of the actual active study period of the first 12 months, and our combined total numbers with the pilot period and the actual study period is as follows: **50 MS cases, and 32 controls**. We consider these numbers satisfactory in reaching our target numbers; however, we have made further optimizations to the enrollment, including the following:

- We developed flow charts to aid scheduling, enrollment and recruitment at every stage (study coordinator, MRI schedulers, MACFIMS schedulers)
- Our MS and Autoimmune Neurology fellows have been instructed in patient screening and enrollment
- Reminder cards provided about study to all MS physicians and trainees, also placed in every office where MS patients are seen
- Optimized prescreening of charts (now we screen 8 weeks ahead)
- For new patients (where chart prescreening is not available) we have implemented new mechanisms including screening all new patient charts the day after their appointment to identify potential candidates; optimized scheduling of MACFIM for these non-prescheduled patients

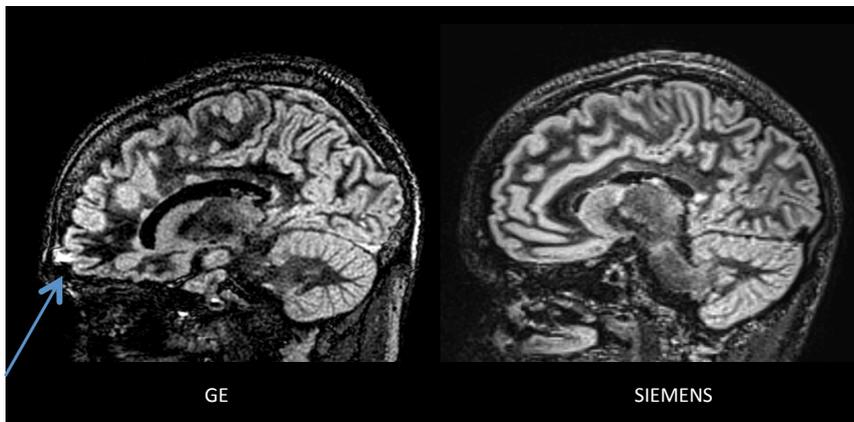
We continue to monitor our patient enrollment numbers very closely and will implement changes as needed. Our control enrollment will focus very specifically on matching patients in our database (age and gender matching) as closely as possible. Overall, we don’t anticipate difficulties in reaching our target numbers.

In the pilot stage of our projects and while the administrative issues had been clarified and the study had been set up during the first 3-4 months of our study period, we continued to scan using our preexisting IRB protocol on a 3T GE scanner.

MRI acquisition

MRI acquisition has commenced successfully during the study period. We had no safety issues whatsoever; the study doesn’t include administration of contrast dye, and our radiology colleagues have very rigorous prescreening procedures in place to make sure every patient, study or otherwise,

is safe for scanning. At the recommendation of Dr. John Port, our radiologist co-investigator on our study, we conducted a few test scans on our 32-channel Siemens Skyra 3 Tesla system and concluded that the Siemens architecture offers higher signal-to-noise ratio, which is a key issue for the types of scans we are using, we therefore decided to move our scanning protocol to that platform. Of note, there is no change to the actual scans we are acquiring, so there is no change to the protocol itself, there are no new safety or other issues; we are simply getting “higher quality” scans by making the switch to the Siemens. We did this early on in the process, which will ensure more homogeneous data collection. All of our cases where MACFIMS neurocognitive results are also available have been scanned using the Siemens platform, therefore, the MACFIMS-MRI correlational analysis will be performed using homogeneous MRI data; we won't mix data between platforms. The figure below illustrates some of the differences between the GE and Siemens scan quality.



Note that in addition to the less “noisy” appearance of these WM-DIR scans on the Siemens platform due to the higher signal-to-noise ratio, we also have fewer problems with the commonly seen artifact in the frontal lobe (arrow). We plan to maintain the Siemens platform as our main scanning platform. Our institution has purchased an additional Siemens scanner which will be installed in November 2014, therefore access will further improve to these scanners; however, due to the excellent help provided by our senior radiology technician collaborators who aid in scheduling our study patients, we have been able to access the Siemens scanner chosen for this study consistently and without problems thus far.

There have been no major technical issues during the study with MRI acquisition. There was one patient whose raw DTI scans were erroneously not saved by the MRI technician, only processed FA and diffusivity information was saved. Dr. Port provided additional education to the technicians about the need to save raw DTI data for our purposes. The patient in question has graciously agreed to be scanned again upon her return appointment in June; we will also redo her MACFIMS so that it truly reflects the state she is in during MRI acquisition.

In terms of transfer and storage of data, a dedicated server is available in our laboratory. Our radiologist colleagues have standardized procedures to de-identify scans, which are implemented in our study. Secure storage of all data is password protected and approved by Mayo Clinic Information Technology standards. Consent forms and a spreadsheet identifying the study case numbers will be stored in a locked cabinet. All electronically stored imaging files are entirely de-identified and coded; the code is only available to the investigators in a password-protected file held on our own server, which guarantees full access control and robust security in every step of this process.

Neuropsychological testing

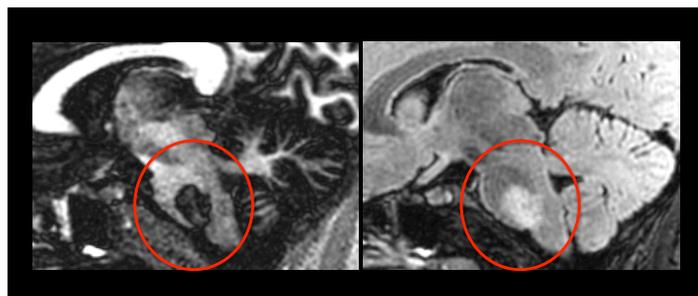
An important component of our study is the administration of the MACFIMS battery, which was specifically developed to evaluate the cognitive function of MS patients. Two trained neuropsychologists under the direct supervision of Dr. Dan Rohe, our co-investigator administers the standard MACFIMS test over approximately 90 minutes per subject will be needed for this step. We screen every patient with a standard Beck inventory for depression. The presence of depression is a

key limiting factor of cognitive performance. We have established rigorous guidelines for cases when potential suicidal ideation is identified: our neuropsychiatrist immediately notify Dr. Pirko or Dr. Tillema, who work with the neurology consultant associated with the given patient to address the situation, and/or implement emergency psychiatry consultation if needed. For quality control and initial assessment, Dr. Rohe personally reviews all MACFIMS results. Our colleagues in biostatistics developed a web-based MACFIMS score entry system, which is on our institutional intranet and requires password-protected login, only available for our study personnel. This tool collects and transfers the MACFIMS data to our study server, facilitates automated analysis. See below for our initial analysis of the currently existing MACFIMS data.

MRI data processing.

All data processing commences as outlined in our SOW document. **Briefly**, pre-processing includes data de-identification, conversion of the DICOM files into NIFTI format. Eddy current and motion correction of DTI data, brain extraction are performed using a BASH script-based pipeline, which also includes computation of probabilistic tractography (bedpost in FSL) and diffusivity measures (FA, D). All sequences are co-registered to the T1 weighted MPRAGE images and to standard MNI space. WM and GM lesion maps are generated in a semi-automated fashion by 3 investigators (IP, JT, JP) and the identified lesions are used as seeding origins for tractography. The white matter tracts from these seeding origins are then identified via probabilistic tractography. Once these tracts are identified and individually stored (from each WML and CL), we use another an automated pipeline of FSL commands to calculate which lesions are connected (both CL to WM lesion and WM to WM lesion). The output of this process is fed into an R-based statistical analysis program (WU, Vienna, Austria) developed by our statistics collaborators to further analyze this data. The data generated by our locally developed GM-DIR sequence is also analyzed by another method, where we don't use semi-automated GM lesion maps, instead we identify cortical areas connected with WM lesions by using WM lesions as seeding origins. We will consider these cortical areas as "connected cortex". Intensity analysis using a FSL script is performed on these "connected cortex" segments in comparison to the entire "non-connected" cortex. Analysis of our data strongly suggests that the vast majority of WM lesion connected GM areas ("connected GM lesions") are truly hyperintense on GM-DIR, consistent with CL-s. For additional analysis steps, please see our proposal and the SOW document.

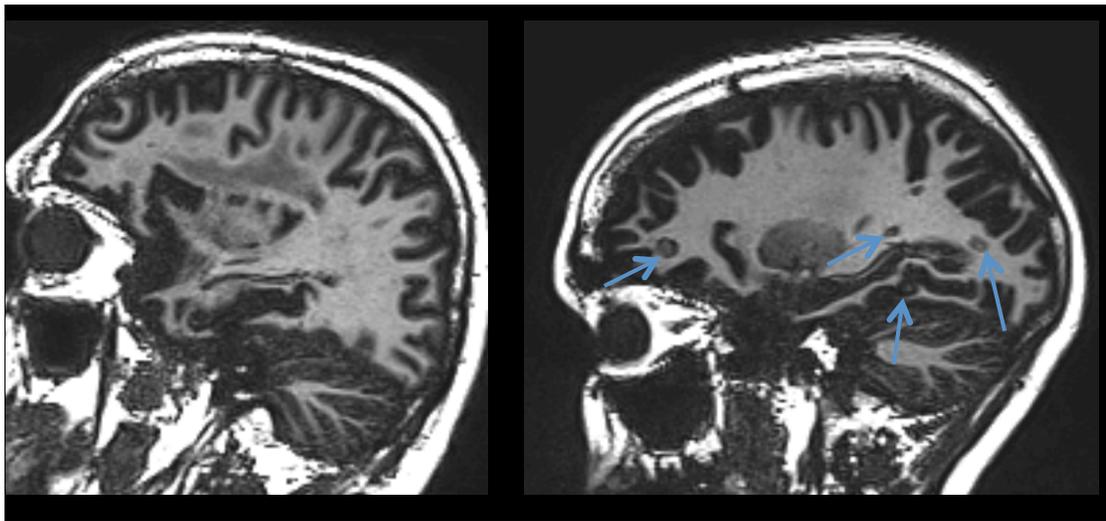
In addition to the connectivity analysis, we are also performing "qualitative analysis" of our GM-DIR scans. As discussed in the proposal, we noted that on these scans, which are otherwise used to identify cortical lesions with increased sensitivity, white matter lesions have a very peculiar appearance, and based on controls with MS unrelated white matter lesions, we hypothesize that the peculiar appearance is truly specific to MS. On GM-DIR, MS lesions appear to have a hypointense rim or ring associated with them (similar to what may be seen on SWI phase images or on FLASH-based T2* scans, especially at even higher field strength such as 7 Tesla). In addition, the central



areas of the lesions show a heterogeneous pattern of hypo- and hyperintense areas. It is unclear what these areas may represent at this point, as there is no tissue available for direct comparison. Based on previous comparative studies using other sequences, we postulate that the areas of hypo intensity may overlap with the presence of macrophages in these lesions. The figure to the left illustrates this lesional

appearance. Note the GM-DIR scan on the left compared to the FLAIR scan on the right of this pontine lesion.

Because this lesional appearance may be a biomarker for MS, we are currently in the process of reviewing all our GM-DIR scans for statistical analysis and comparison in order to determine the specificity and overall significance of this finding.



To demonstrate the potential specificity of this lesional appearance to MS, we present 2 scans in the figure below: The in left image is of a patient with non-specific small vessel ischemic white matter lesions, the image on the right is of an MS case. The blue arrows indicate the

“MS-specific” lesion appearance on GM-DIR, whereas the lesions in the small vessels ischemic disease-case show uniform lesional hypointensity without dark rims and heterogeneous centers.

Statistical analysis of data

Our statistical team includes a PhD statistician Dr. Jay Mandrekar, and a master’s level statistician Stephen Weigand, along with Patrick Fitz-Gibbon, a data analyst specialist. Through their help, we have analyzed the initial data available in our project. This doesn’t yet represent the full analysis of all 50 enrolled cases and 32 controls, but provides a good insight into our data, and gives an excellent overview of the types of analyses we aim to accomplish. Analysis is ongoing, we have biweekly meetings with our statistics team, and ad hoc meetings as needed. The integration with our statistical team has been outstanding and we anticipate that through the data generated in our project, we will make truly meaningful contributions to the MS literature.

Interim MACIMS data analysis

Below we summarize the MACFIMS neurocognitive available at the time of writing this report. Overall, this includes the analysis of 17 patients, 15 female, and 2 male. See table 1 for summary of patient characteristics.

Table 1. Summary of patient characteristics.

Characteristic	Summary
Number of subjects	17
Female, n (%)	15 (88%)
Age at test date, years	
median (min,max)	42 (18, 60)
mean (s.d.)	41 (11)
Education, year s	
median (min,max)	14 (12, 18)
mean (s.d.)	15 (2)

MACFIMS includes multiple components, each are descriptors of specific cognitive functions. As detailed in the proposal, the most common way to analyze this data is by z-scores, the distribution of which is illustrated in Figure 2 and Table 2 of the currently analyzed cohort.

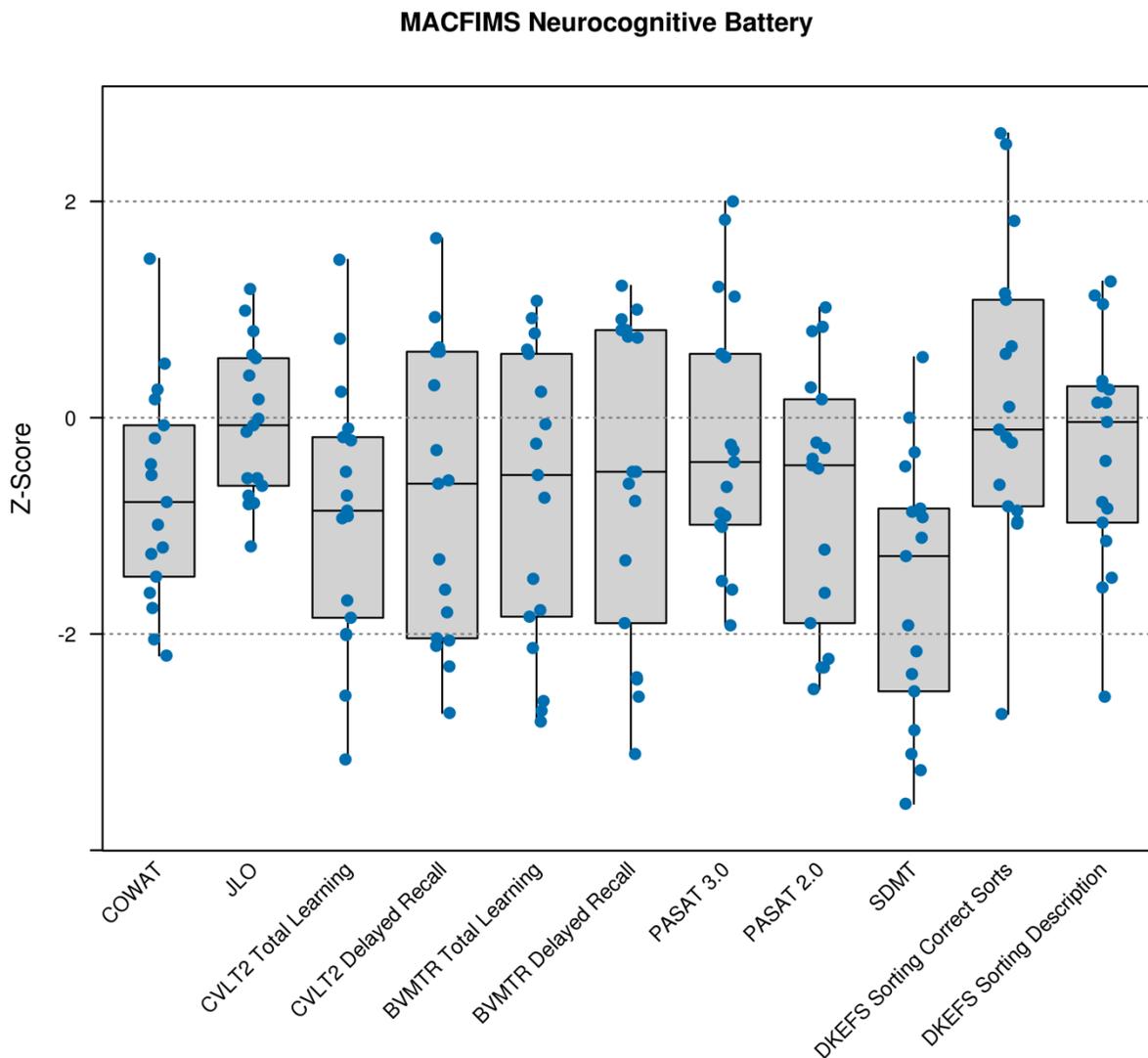


Figure 2. Distribution of MACFIMS battery z-scores.

Table 2. Summary of z-scores.

	median (min,max)	mean (s.d.)
COWAT	-0.8 (-2.2, 1.5)	-0.7 (1.0)
JLO	-0.1 (-1.2, 1.2)	-0.0 (0.7)
CVLT2 Total Learning	-0.9 (-3.2, 1.5)	-0.9 (1.2)
CVLT2 Delayed Recall	-0.6 (-2.7, 1.7)	-0.7 (1.4)
BVMTR Total Learning	-0.5 (-2.8, 1.1)	-0.7 (1.4)
BVMTR Delayed Recall	-0.5 (-3.1, 1.2)	-0.6 (1.5)
PASAT 3.0	-0.4 (-1.9, 2.0)	-0.2 (1.2)

Table 2. Summary of z-scores.

PASAT 2.0	-0.4 (-2.5, 1.0)	-0.8 (1.2)
SDMT	-1.3 (-3.6, 0.6)	-1.6 (1.2)
DKEFS Sorting Correct Sorts	-0.1 (-2.7, 2.6)	0.2 (1.4)
DKEFS Sorting Description	-0.0 (-2.6, 1.3)	-0.3 (1.1)

At this time it would be too early to draw valid conclusions from the available partial dataset, but MS related cognitive dysfunction in this early relapsing-remitting cohort appears to be most sensitively captured on CVLT2 and on SDMT out of the MACFIMS components. To further analyze this relationship, principal component analysis of the early data was also performed and is provided in Figure 3. The first five principle components explain about 90 percent of the variance in the data. Heuristically, this indicates that among these subjects there are about five distinct "dimensions" to the MACFIMS battery and as such it is a rich, multidimensional resource of clinical-MRI correlations.

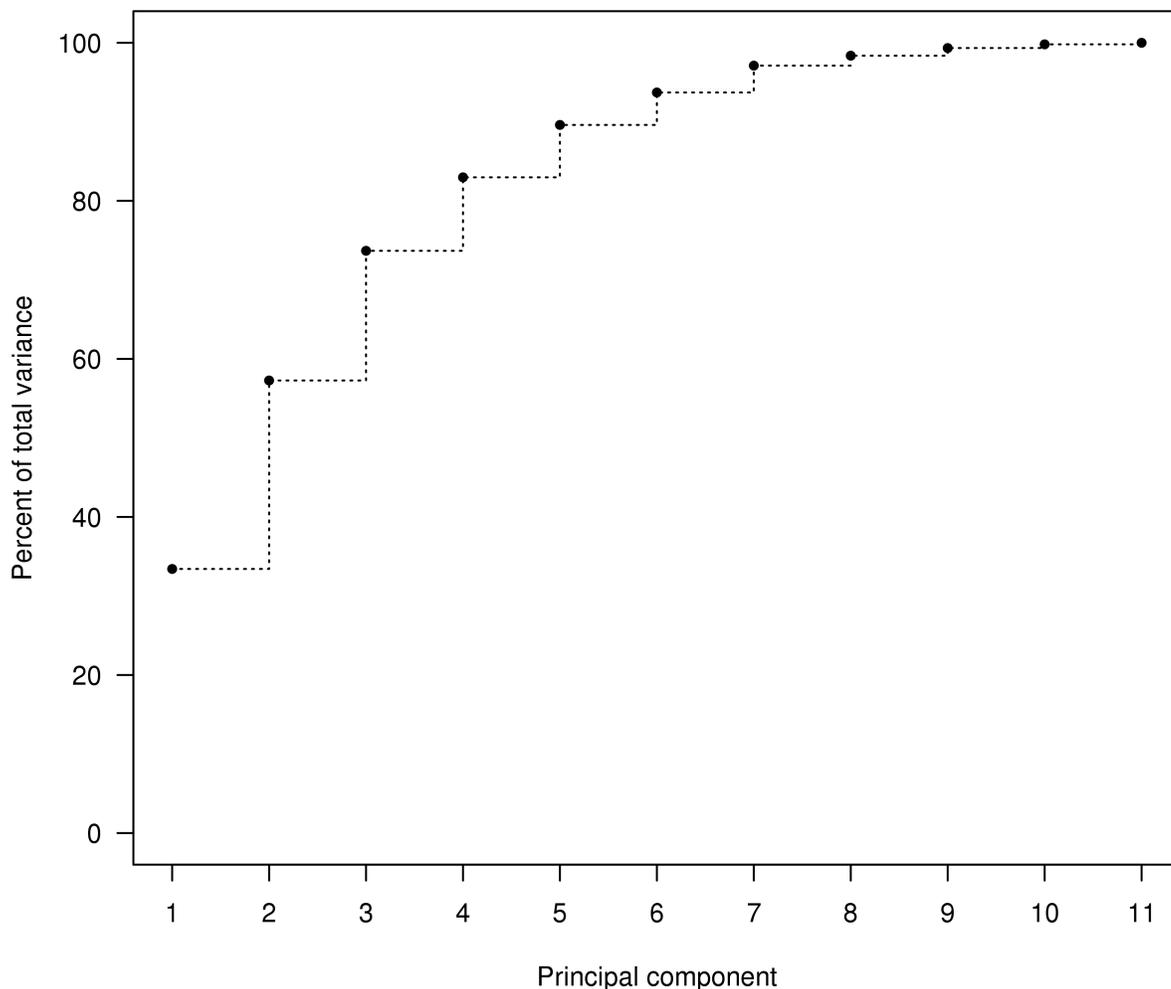


Figure 3. Cumulative proportion of variance explained by each principal component.

Statistical Analysis of MRI data

During the first funding cycle, we have submitted our first conference abstract, which is currently under review for the 2014 ECTRIMS/ACTRIMS conference to be held in Boston later this year. The abstract contains data of 25 patients and 24 controls, all of whom were scanned in the GE scanner that we used before the switch to Siemens. These patient scans were acquired prior to the inclusion of MACFIMS. The results are strongly suggestive of lesional connectivity between cortical and white matter lesions. We provide the entire abstract under the “Reportable outcomes” section. Additional interim data analysis has also been conducted, and our results are as follows.

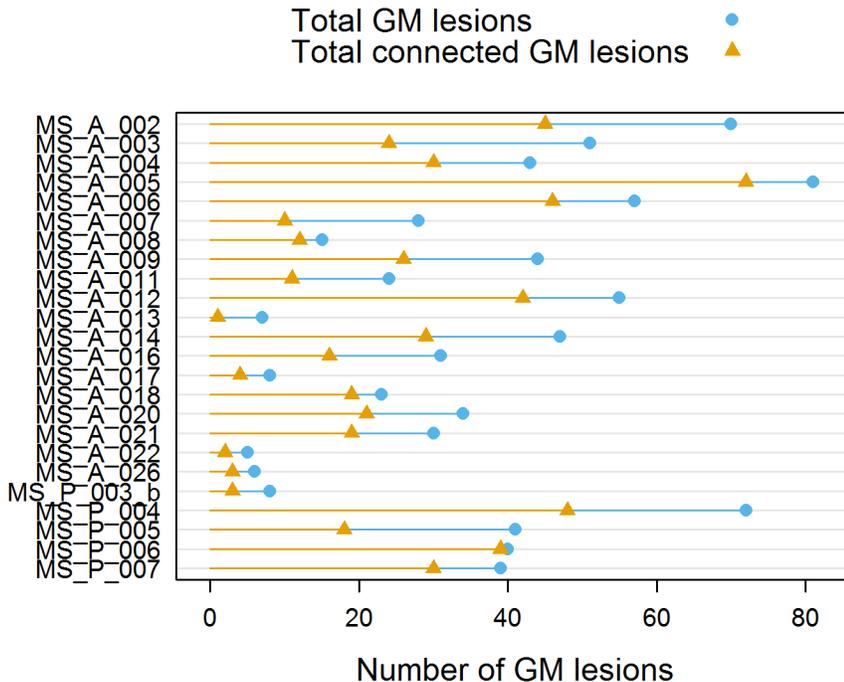


Figure 4.

Total number of cortical lesions and the number of cortical lesions (CL) connected with white matter lesions (WML) is represented in Figure 4 in our GE 3 Tesla cohort. Note that even in cases with relatively low lesion load (for example, MS_A_22 or MS_A_26) a considerable proportion of their lesions are connected.

Figures 5-6 show a different way to look at a subset of our data: each circle represents a lesion, and along the X-axis we illustrate how many other lesions that particular lesion connects to. We show WML-WML connectivity data, while WML-CL connectivity data is represented in two ways: once we are seeding from WML-s to CL-s, in the other analysis we seed from CL-s to WML-s. Lesional connectivity is again convincingly demonstrated on these figures (Figure 6).

How many WM lesions does each originating WM lesion connect with?

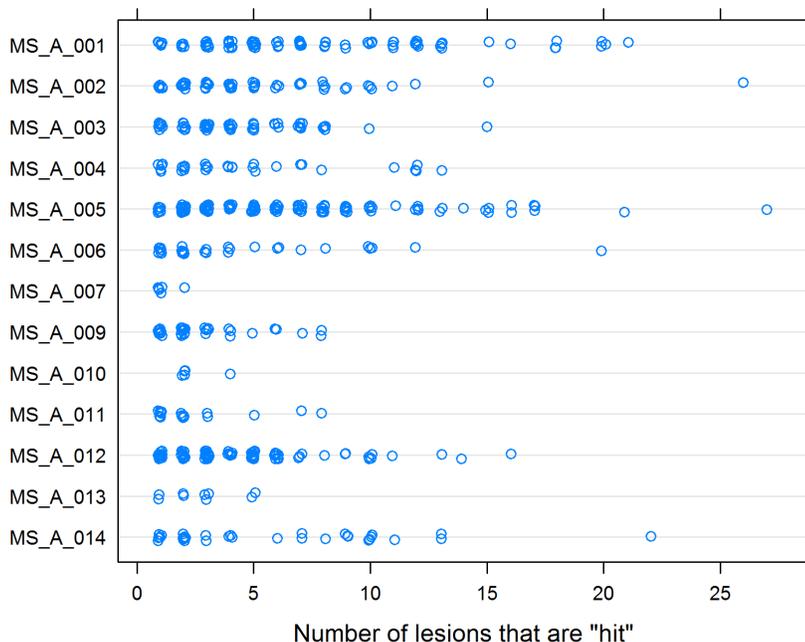
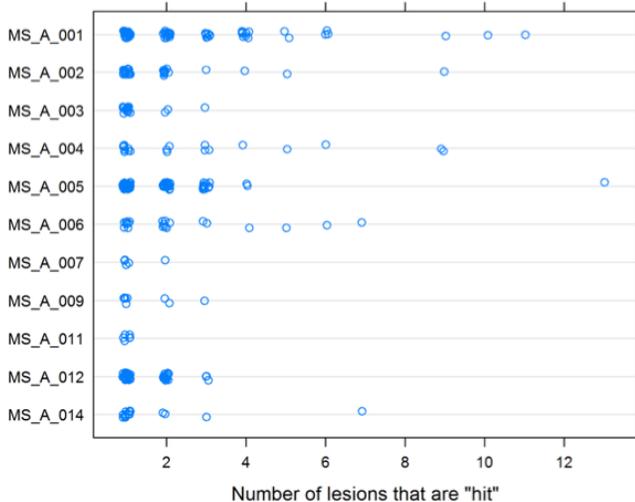


Figure 5.

How many GM lesions does each originating WM lesion connect with?



How many WM lesions does each originating GM lesion connect with?

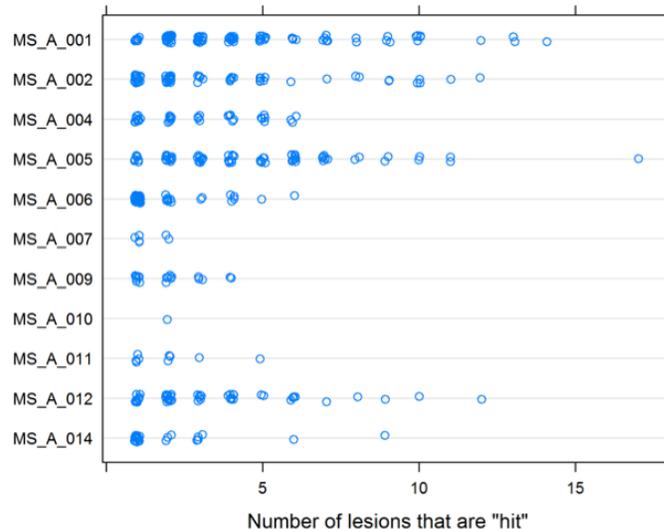


Figure 6.

The following figure shows an example of lesional connectivity using DIR images with DTI identified tracts overlaid. In this specific case, over 20 lesions are connected along the same tracts; without DTI these tracts would be difficult to “imagine”, but with the DTI overlay, the connectivity is very convincingly demonstrated.

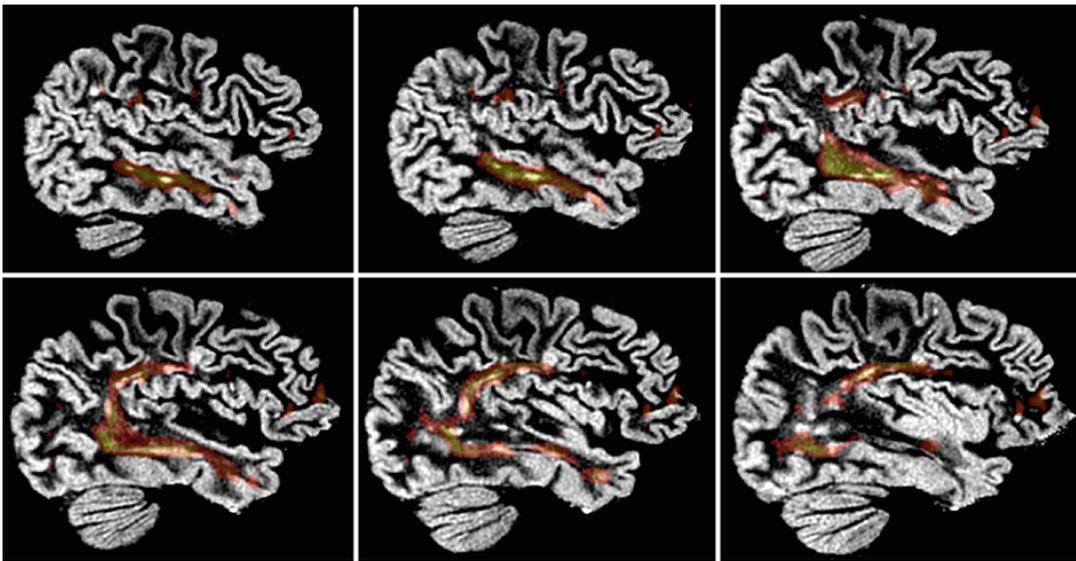


Figure 7. WML-s connected with each other along DTI identified WM tract. Originating CL is in the temporal pole (not shown).

Overall we conclude that the analyzed dataset thus far supports our hypothesis of lesional connectivity. Additional analysis, including correlational analysis is ongoing. We anticipate no difficulties in fully analyzing the datasets by the proposed study end point.

KEY RESEARCH ACCOMPLISHMENTS:

- We established a robust chart screening and patient identification system to facilitate efficient recruitment.
- We developed and successfully implemented a new way of looking at cortical lesions with a pulse sequence we termed GM-DIR.
- A novel FSL-based and BASH-scripted processing pipeline has been developed and implemented to study lesion-lesion connectivity.
- A novel web-based data entry and retrieval system has been developed for our MACFIMS data.
- Unique white matter lesional appearance may serve as a biomarker for MS, further investigations into the specificity of this finding is ongoing.
- Early analysis of our MACFIMS data highlights MS-specific z-score deviations in key MACFIMS components, and principal component analysis suggests that among the study subjects there are about five distinct "dimensions" to the MACFIMS battery and as such it is a rich, multidimensional resource of clinical-MRI correlations.
- We identified strong connectivity between white matter lesions along DTI identifiable tracts, and connections between cortical lesions with white matter lesions.
- Based on our original pathology observations, we propose that cortical lesions may be disease-initiating events that determine the formation of white matter lesions along connecting pathways. An alternative hypothesis is that through tract degeneration, the primary white matter lesions will lead to the formation of cortical lesions in a retrograde way, but this seems to be contradicted by pathology data derived from early MS. However, the two mechanisms are not mutually exclusive

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

The main reportable outcome is the submission of our first conference abstract, to be followed by the submission of our first manuscript over the next month. Our ECTRIMS/ACTRIMS abstract is pasted at the end of this section.

Another reportable outcome is the web-based MACFIMS data collection system established by Patrick Fitz-Gibbons of our statistical team, and the generation of a comprehensive database for statistical analysis

MRI Reveals Connectivity of Cortical Lesions to Deep White Matter Lesions in Multiple Sclerosis.

Jan-Mendelt Tillema, MD; John D. Port, MD, PhD; Stephen Weigand; Jay Mandrekar, PhD; Yunhong Shu, PhD; Claudia F. Lucchinetti, MD and Istvan Pirko, MD

Background: The relationship between cortical lesions (CL) and white matter lesions (WML) in MS is poorly understood. In addition to their association with disease progression, CL may also play a role in disease initiation. We hypothesized that WML-s develop along tracts directly connected with CL.

Objective: Our aim was to demonstrate this connectivity using double inversion recovery (DIR) sequences for CL detection coupled with DTI-based probabilistic tractography.

Methods: Relapsing-remitting MS patients with relative mild disease (EDSS ≤ 4) and matched controls were scanned using the same 3T MRI protocol (3D-MPRAGE, DTI (41 directions) and 3D-DIR). Freesurfer was used for segmentation of the cortex. Semi-automated outlining of WM and CL (on DIR) was performed. FSL was used for connectivity analysis. WMLs were used as individual seeds for tractography. Connectivity to the cortex for each individual tract was obtained. These connected cortical regions were combined, dividing the entire cortex into "WML connected" and "non-connected" cortex. We calculated the fraction of CL volume within the connected and non-connected cortex, quantified as the connected vs. non-connected lesion ratio (CNCLR). Automated cluster analysis was performed to include randomly selected, non-specific DIR cortical hyperintensities in patients and controls. For comparative analysis we placed topographically comparable WM seeds in each controls from matched patients via affine registration.

Results: 25 patients (median age 33) and 24 controls (median age 35) were included. Connected vs Non-connected lesion ratios were elevated in MS patients (median 2.7 [IQR 2.0-3.6]) compared to their nonspecific cortical areas (median 1.2 [IQR 1.0-1.5]; $p < 0.001$) and compared to nonspecific cortical areas in controls (median 1.0 [IQR 0.8-1.2]; $p < 0.001$). These non-specific cortical areas in MS patients had slightly higher CNCLRs than controls ($p = 0.10$).

Conclusions: CL on DIR are nearly 3 times as likely to be found in cortex that is connected to WML, providing evidence that CL and WML are topographically related. Non-specific DIR hyperintensities may be found slightly more frequently in connected cortex, suggesting that some of these non-specific regions may also represent poorly visualized CL due to sensitivity limitations. These findings are consistent with our hypothesis of CL-WML connectivity. Additional MRI and experimental pathology studies are underway to further investigate our hypothesis.

CONCLUSION: We conclude that cortical lesions are an important component of MS, and that cortical lesions are intricately connected with white matter lesions. Cortical lesions likely have a major impact on cognitive outcomes, which is to be studied further in the context of our project. Cortical demyelination as a pathological hallmark is completely specific to MS and is not seen in any other demyelinating or inflammatory diseases. While MRI never can be specific for demyelination, it is intriguing that the detected lesions truly are areas of demyelination, and the connection between these and white matter lesions implies a potential pathogenetic role for cortical lesions. Our new GM-DIR sequence also shows a very unusual appearance of white matter lesions, suggesting that this signature lesional appearance may be a viable biomarker for MS, which we are currently investigating further. We are conducting additional data processing and statistical analysis, including correlational analysis, and full analysis of our substantially higher signal-to-noise ratio Siemens MRI data, and we expect that these findings will be demonstrated even more convincingly with subsequent analyses.