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TITLE:

A Standardized Pipeline for Examining Human Cerebellar Grey Matter Morphometry Using Structural Magnetic Resonance Imaging

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cerebellum, magnetic resonance imaging, parcellation, voxel-based morphometry, grey matter, Friedreich ataxia

SUMMARY:

A standardized pipeline is presented for examining cerebellum grey matter morphometry. The pipeline combines high-resolution, state-of-the-art approaches for optimized and automated cerebellum parcellation and voxel-based registration of the cerebellum for volumetric quantification.

ABSTRACT:

Multiple lines of research provide compelling evidence for a role of the cerebellum in a wide array of cognitive and affective functions, going far beyond its historical association with motor control. Structural and functional neuroimaging studies have further refined understanding of the functional neuroanatomy of the cerebellum beyond its anatomical divisions, highlighting the need for the examination of individual cerebellar subunits in healthy variability and neurological diseases. This paper presents a standardized pipeline for examining cerebellum grey matter morphometry that combines high-resolution, state-of-the-art approaches for optimized and automated cerebellum parcellation (Automatic Cerebellum Anatomical Parcellation using U-Net Locally Constrained Optimization; ACAPULCO) and voxel-based registration of the cerebellum (Spatially Unbiased Infra-tentorial Template; SUIT) for volumetric quantification.

The pipeline has broad applicability to a range of neurological diseases and is fully automated, with manual intervention only required for quality control of the outputs. The pipeline is freely available, with substantial accompanying documentation, and can be run on Mac, Windows, and Linux operating systems. The pipeline is applied in a cohort of individuals with Friedreich ataxia (FRDA), and representative results, as well as recommendations on group-level inferential statistical analyses, are provided. This pipeline could facilitate reliability and reproducibility across the field, ultimately providing a powerful methodological approach for characterizing and tracking cerebellar structural changes in neurological diseases.

INTRODUCTION:

The cerebellum is a part of the brain historically associated with motor control[1-3] and is thought to be integrally involved in only a small set of rare diseases, such as inherited ataxias[4]. However, converging lines of research from anatomical tracing studies in nonhuman primates, as well as human lesion and neuroimaging studies, provide compelling evidence for a role of the cerebellum in a wide array of cognitive[5-7], affective[8-11], and other nonmotor functions[7,12] (see[6] for review). Furthermore, abnormalities of the cerebellum are increasingly implicated in a broad range of neurological and psychiatric disorders, including Parkinson's disease[13], Alzheimer's disease[14,15], epilepsy[16,17], schizophrenia[18], and autism spectrum disorder[19]. Therefore, it has become essential to incorporate the cerebellum into functional and structural models of human brain diseases and normative behavioral variability.

Anatomically, the cerebellum can be divided along its superior to inferior axis into three lobes: anterior, posterior, and flocculonodular. The lobes are further subdivided into 10 lobules denoted by roman numerals I–X[20 21] (**Figure 1**). The cerebellum can also be grouped into midline (vermis) and lateral (hemisphere) zones, which respectively receive inputs from the spinal cord and cerebral cortex. The anterior lobe, comprising lobules I–V, has traditionally been associated with motor processes and has reciprocal connections with cerebral motor cortices[22]. The posterior lobe, comprising lobules VI–IX, is primarily associated with nonmotor processes[11] and has reciprocal connections with the prefrontal cortex, posterior parietal, and superior temporal cerebral cortices[8,23]. Lastly, the flocculonodular lobe, comprising lobule X, has reciprocal connections with vestibular nuclei that govern eye movements and body equilibrium during stance and gait[21].

A growing body of recent work using functional neuroimaging has further refined understanding of the functional neuroanatomy of the cerebellum beyond its anatomical divisions. For example, resting-state functional magnetic resonance imaging (fMRI) techniques have been used to map the pattern of functional interactions between the cerebellum and cerebrum[24]. Additionally, using a task-based parcellation approach, King and colleagues[7] demonstrated that the cerebellum shows a rich and complex pattern of functional specialization across its breadth, evidenced by distinct functional boundaries associated with a variety of motor, affective, social, and cognitive tasks. Collectively, these studies highlight the importance of examining individual cerebellar subunits to develop complete biological characterizations of cerebellum involvement in both healthy variability and neurological diseases characterized by alterations in cerebellar structure and/or function.

The present work focuses on methods for quantifying local changes in cerebellar volume using structural MRI in humans. In general, there are two fundamental approaches to the quantification of regional brain volume using MRI data: feature-based *segmentation* and *voxel-based registration*. Feature-based segmentation approaches use anatomical landmarks and standardized atlases to automatically identify boundaries between subregions. Mainstream software packages for segmentation include FreeSurfer[25], BrainSuite[26], and FSL-FIRST[27]. However, these packages provide only coarse parcellations of the cerebellum (e.g., labeling the whole grey matter and whole white matter in each hemisphere), thus overlooking the individual cerebellar lobules. These approaches are also prone to mis-segmentation, particularly overinclusion of the surrounding vasculature.

New machine-learning and multi-atlas labeling algorithms have been developed, which provide more accurate and finer-grained parcellation of the cerebellum, including Automatic Classification of Cerebellar Lobules Algorithm using Implicit Multi-boundary evolution (ACCLAIM[28,29]), CATK[30], Multiple Automatically Generated Templates (MAGeT[31]), Rapid automatic segmentation of the human cerebellum and its lobules (RASCAL[32]), graph-cut segmentation[33], and CEREbellum Segmentation (CERES[34]). In a recent paper comparing state-of-the-art fully automated cerebellum parcellation approaches, CERES2 was found to outperform other approaches relative to gold-standard manual segmentation of the cerebellar lobules[35]. More recently, Han and colleagues[36] developed a deep-learning algorithm called ACAPULCO (Automatic Cerebellum Anatomical Parcellation using U-Net with locally constrained optimization), which performs on par with CERES2, has broad applicability to both healthy and atrophied cerebellums, is available in open-source Docker and Singularity container format for 'off-the-shelf' implementation, and is more time-efficient than other approaches. ACAPULCO automatically parcellates the cerebellum into 28 anatomical regions.

In contrast to feature-based segmentation, voxel-based registration approaches operate by precisely mapping an MRI to a template image. To achieve this mapping, the voxels in the original image must be distorted in size and shape. The magnitude of this distortion effectively provides a measure of volume at each voxel relative to the gold-standard template. This form of volumetric assessment is known as 'voxel-based morphometry'[37]. Whole-brain voxel-based registration approaches, such as FSL-FLIRT[38]/FNIRT[39], SPM unified segmentation[40], and CAT12[41],

are commonly used for voxel-based morphometry. However, these approaches do not account well for the cerebellum, resulting in poor reliability and validity in infratentorial regions (cerebellum, brainstem[42]). To account for these limitations, the SUIT (Spatially Unbiased Infra-tentorial Template) algorithm was developed to optimize cerebellum registration and improve the accuracy of voxel-based morphometry[42,43].

Feature-based segmentation and voxel-based registration approaches for the estimation of regional cerebellar volume have fundamental strengths and weaknesses. Segmentation approaches are substantially more accurate for quantifying the volume of anatomically defined areas (e.g., lobules[35]). However, boundaries between distinct functional modules of the cerebellum do not map onto its anatomical folia and fissures (equivalent to gyri and sulci of the cerebrum[7]). As registration-based approaches are not constrained by anatomical landmarks, finer-grained spatial inference and high dimensional structure-function mapping of the cerebellum is possible[44]. Taken together, segmentation and registration approaches are complementary to one another and can be used to answer different research questions.

Here, a new standardized pipeline is presented, which integrates these existing, validated approaches to provide optimized and automated parcellation (ACAPULCO) and voxel-based registration of the cerebellum (SUIT) for volumetric quantification (**Figure 2**). The pipeline builds upon the established approaches to include quality control protocols, using qualitative visualization and quantitative outlier detection, and a rapid method for obtaining an estimation of intracranial volume (ICV) using Freesurfer. The pipeline is fully automated, with manual intervention only required for checking the quality control outputs, and can be run on Mac, Windows, and Linux operating systems. The pipeline is freely available with no restrictions of its use for noncommercial purposes and can be accessed from the ENIGMA Consortium Imaging Protocols webpage (under "ENIGMA Cerebellum Volumetrics Pipeline"), following the completion of a brief registration form[45].

All required software is listed in the **Table of Materials**, and detailed tutorials, including a live demonstration, are available upon download of the pipeline, in addition to the protocol described below. Finally, representative results are provided, from the implementation of the pipeline in a cohort of people with Friedreich ataxia (FRDA) and age-and sex-matched healthy controls, alongside recommendations for group-level statistical inferential analyses.

PROTOCOL:

NOTE: The data used in this study were part of a project approved by the Monash University Human Research Ethics Committee (project 7810). Participants provided written informed consent. While the pipeline can be run on Mac, Windows, or Linux operating systems, ACAPULCO, SUIT, and the QC pipelines have explicitly been tested on Linux (Ubuntu) and Mac (Catalina, Big Sur v11.0.1) operating systems.

1. Module 1: ACAPULCO (anatomical parcellation)

1.1. Data collection

1.1.1. Collect 3D T1-weighted MRI images of the whole brain at a resolution of 1 mm^3 or less at a high resolution and isotropic voxel dimensions (typically 1 mm x 1 mm x 1 mm), using a 3-Telsa (or greater) scanner. Consult with an imaging specialist at their radiography center to set up and acquire data that meet these specifications.

NOTE: T2-weighted images are sometimes useful for volumetric analyses; however, the pipeline presented here relies on T1-weighted data only, and some of the tools used are exclusive to this type of data. As such, T2-weighted images cannot be used.

1.1.2. Undertake a visual quality assessment of images to exclude gross cerebellar malformations (e.g., large lesions) or substantial motion artifacts that prevent identification of major cerebellar landmarks (e.g., the major anatomical fissures). Do not automatically exclude atrophied cerebella, even if substantial.

1.1.3. For group studies, also consider quantitative quality assessments using freely-available, standardized tools such as MRIQC[46] to further identify problematic data.

1.1.4. Convert all data to NIFTI-GZ format using a tool such as dcm2niix[47].

1.2. Recommended data organization

1.2.1. Obtain all necessary software as listed in the **Table of Materials**. Ensure Docker[48] or Singularity[49], Matlab[50], and SPM12[51] are installed prior to running the pipeline.

NOTE: Extensive written and video tutorials describing the pipeline are also available (see the **Table of Materials**).

1.2.2. Once all necessary software is installed, create folders in the working directory and label them 'acapulco,' 'suit,' and 'freesurfer.' Use the **mkdir** command from the command line.

1.2.3. In the 'acapulco' directory, create an **output** folder. In the **output** folder, create a directory for each subject in the study containing the T1-weighted image in NIFTI-GZ format.

NOTE: It is recommended to keep a copy of the original data elsewhere.

1.3. Anatomical cerebellar parcellation using ACAPULCO

1.3.1. Go to the **Table of Materials** and download the relevant scripts and containers required to run ACAPULCO (under **acapulco pipeline files**). In the 'acapulco' directory, place the (i) ACAPULCO Docker *OR* Singularity container ('acapulco_0.2.1.tar.gz' or '.sif', respectively), (ii) contents of the QC_scripts archive (3 files: 'QC_Master.R,' 'QC_Plots.Rmd,' and 'QC_Image_Merge.Rmd'), and (iii) 'R.sif' (singularity) *OR* 'calculate_icv.tar' (docker) file.

1.3.2. Open a terminal, and from the command line, run the ACAPULCO container on a single image (replace <<subject>> in the following). Wait for ~5 min for processing to complete.

1.3.2.1 Using Docker, type the command:

docker load --input acapulco_0.2.1.tar.gz
docker run -v $PWD:$PWD -w $PWD -t --user $(id -u):$(id -g) --rm acapulco:latest -i output/<<subject>>/<<subject>>.nii.gz -o output/<<subject>>

1.3.2.2. Using Singularity, type the command:

singularity run --cleanenv -B $PWD:$PWD acapulco-0.2.1.sif -i output/<<subject>>/<<subject>>.nii.gz -o output/<<subject>>

1.3.3. Loop across all subjects/scans in the cohort. See the **Table of Materials** for a link to the ENIGMA Imaging Protocols website for downloading the pipeline (under **ENIGMA Cerebellum Volumetrics Pipeline**) and the tutorial manual containing examples of how to create a for-loop for processing multiple subjects serially.

1.3.4. After processing, look for the following files generated in the subject-specific folders:

1.3.4.1. Identify "<subject>_n4_mni_seg_post_inverse.nii.gz": parcellated cerebellum mask in original (subject space).

1.3.4.2. Identify "<subject>_n4_mni_seg_post_volumes.csv": volumes (in mm^3) for each of the 28 subunits generated by acapulco;

1.3.4.3. Identify representative images (in 'pics' directory): sagittal, axial, and coronal.

1.4. Statistical outlier detection and quality control (QC)

1.4.1. From the terminal and in the 'acapulco' directory, ensure that the contents of QC_scripts are in the 'acapulco' directory. To run the QC scripts:

1.4.1.1 Using Docker, type the command:

docker load calculate_icv.tar
docker run -v $PWD:$PWD -w $PWD --rm -it luhancheng/calculate_icv:latest Rscript QC_Master.R output/

1.4.1.2 Using Singularity, type the command:

singularity exec -B $PWD:$PWD R.sif Rscript /path/to/QC_Master.R /path/to/acapulco/output

1.5. Examining the QC images generated by ACAPULCO

NOTE: There is a 3-step process for quality checking the ACAPULCO parcellated images.

1.5.1. Open the 'QC_Images.html' in a web browser and quickly (~10 s per subject) scroll through the images to identify obvious failures or systematic issues. Note the subject IDs of failed or suspect parcellated images for follow-up.

NOTE: See **Figure 3** for a guide on the neuroanatomy of the cerebellar lobules and **Figure 4, Figure 5,** and **Figure 6** in the representative results section below for examples of 'good' parcellations, 'subtle mis-parcellations,' and 'global failure' parcellations.

1.5.2 Open the 'Plots_for_Outliers.html' to check the boxplots for quantitative statistical outliers. Look for outliers (2.698 s.d above or below the mean) above or below the whiskers of the box plots. Hover over the data points to display the Subject ID. Identify the outliers denoted by a '1' in the relevant column in the 'Outliers.csv' file, and note the total number of segments identified as outliers for each subject in the final column in 'Outliers.csv.'

1.5.3. Manually inspect each image having one or more outliers. CRITICAL: Using a standard NIFTI image viewer (e.g., FSLEyes or MRICron), overlay the ACAPULCO mask onto the original T1w image to check the quality of the parcellation slice-by-slice.

1.5.3.1. To generate overlays for detailed QC from the command line using FSLEyes, i) change the directory to the 'acapulco' directory, ii) specify the subject to view (replace *<subject>):*

subj=<subject_name>

1.5.3.2. Copy/paste the following code to the terminal (without manually changing {subj} as this has been set by the previous line:

t1_image=output/${subj}/${subj}.nii.gz
acapulco_image=output/${subj}/${subj}_n4_mni_seg_post_inverse.nii.gz
fsleyes ${t1_image} ${acapulco_image} --overlayType label --lut random_big --outline --outlineWidth 3 ${acapulco_image} --overlayType volume --alpha 50 --cmap random

NOTE: A determination will need to be made whether to include the abnormal segment or not, i.e., is there a parcellation error, or is it just normal variability in the individual's anatomy? Each parcellated region is considered individually, so a few regions can be excluded for an image, while the remainder can be retained if correct.

1.5.3.3. *Do one or more parcellated regions need to be excluded from the final dataset?*

If Yes (outlier is confirmed), exclude this parcellation(s) from the analysis by replacing the volume estimate with **NA** in the corresponding cell of the 'Cerebel_vols.csv' file for that subject.

1.5.3.4. *Do parcellation errors result in some of the cerebellum being excluded from the mask?*

If Yes, (for example, if particular cerebellar lobules are missing from the mask or appear 'cut off'), immediately exclude the subject from further analyses (i.e., do not proceed to run the SUIT module on those subjects).

2. Module 2: SUIT cerebellum-optimized voxel-based morphometry

2.1. Voxel-based morphometry analyses using SUIT

CRITICAL: This pipeline requires the ACAPULCO module to have already been run, as it relies on the generation of a subject-specific cerebellar mask for optimization of the registration and normalization of the cerebellum to the SUIT template. If the subject-specific mask generated by ACAPULCO does not include the whole cerebellum, this warrants exclusion from the SUIT module. For instructions on running SUIT standalone, see[52].

2.1.1. Obtain all necessary software listed in the **Table of Materials**. Ensure the SPM12 folder and all subfolders are in the MATLAB path. Ensure enigma_suit scripts are saved in 'spm12/toolbox' directory and added to the MATLAB path. To check the MATLAB path, type **pathtool** in the MATLAB command window, then click **Add with subfolders** to add the relevant folders.

2.1.2. Run the SUIT pipeline for one or more subjects. Wait for ~15–20 min (if using the graphical user interface [GUI]) and ~5–7 min if running from the terminal (bash/shell) for processing to complete.

2.1.2.1. To use the GUI (subjects will be run in serial), from the MATLAB command window, type the command:

suit_enigma_all

2.1.2.2. In the first pop-up window, select the subject folders from the **'**acapulco/output' directory to include in the analysis. Click on the individual folders on the right side of the window, or right-click and **Select All**. Press **Done**. In the second pop-up window, select the SUIT directory, where the analyses will be written.

2.1.2.3. Call the function from the MATLAB command line for a single subject, type the command:

suit_enigma_all('/path/to/acapulco/output/subjdir','/path/to/suitoutputdir')

2.1.2.4. Call the function from the terminal window, outside of MATLAB, for a single subject by typing the command:

matlab -nodisplay -nosplash -r "suit_enigma_all('/path/to/acapulco/output/subjdir','/path/to/suitoutputdir'), exit"

2.1.3. See the **Table of Materials** for a link to the ENIGMA Imaging Protocols website for downloading the pipeline (under **ENIGMA Cerebellum Volumetrics Pipeline**) and the tutorial manual containing examples of how to create a for-loop for processing multiple subjects serially.

2.1.4. Look for the following points regarding the script.

2.1.4.1. Ensure that the script copies the N4 bias-corrected, MNI-aligned (rigid-body) T1 image and the ACAPULCO cerebellum mask into the output directory.

2.1.4.2. Ensure that the script segments the grey and white matter of the cerebellum.

2.1.4.3. Ensure that the script corrects for overinclusion errors in the parcellation using the ACAPULCO mask.

2.1.4.4. Ensure that the script DARTEL normalizes and reslices the data into SUIT space with Jacobian modulation so that the value of each voxel is proportional to its original volume.

2.1.4.5. Check each subject's folder for the following final outputs: 'wd<subject>_seg1.nii' (grey matter) and 'wd<subject>_seg2.nii' (white matter).

2.2. Statistical outlier detection and quality control

2.2.1. Visually inspect the normalized, modulated images (wd*) for major failures. In MATLAB, type the command:

spm_display_4D

2.2.2. Manually select the 'wd*seg1' images from the suit subfolders, or navigate to the 'suit' directory; insert '^wd.*seg1' in the Filter box (no quotations) and press **Rec** button. Press **Done**.

2.2.3. Scroll through the images to ensure they are all well-aligned. See **Figure 7** for correctly normalized images from a healthy control (left) and an individual with a heavily atrophic cerebellum (right)**.**

NOTE: At this stage, the between-subject anatomy is very similar (as they have been registered to the same template), and volume differences are instead encoded by differing voxel intensities. Major failures will be obvious, e.g., blank images, large areas of missing tissue, unusual intensity gradients (i.e., bright voxels all at the top, dark voxels all at the bottom). These images should be excluded from subsequent steps.

2.2.4. Check spatial covariance for outliers. In MATLAB, type the command:

check_spatial_cov

2.2.4.1. Select the 'wd*seg1' images as per the previous step. When prompted, select the following options: **Prop scaling: Yes; Variable to covary out: No; Slice (mm): -48, Gap:1**.

2.2.4.2. Look at the boxplot displaying the mean spatial covariance of each image relative to all others in the sample. Identify data points that are >2s.d. below the mean in the MATLAB command window. For these, inspect the "<subj>_n4_mni.nii.gz" image in the SUIT folder for artifacts (motion, anatomical abnormalities), image quality issues, or preprocessing errors.

2.2.4.3. If the image quality and preprocessing are acceptable and visual inspection of the modulated images in the previous step does not indicate an issue with segmentation and normalization, retain these data in the sample. Otherwise, exclude these data.

3. MODULE 3 (optional): Intracranial Volume (ICV) estimation using FreeSurfer

NOTE: This module will use the FreeSurfer pipeline to calculate ICV. It does not need to be re-run if there are existing Freesurfer outputs for the cohort (any version).

3.1. Setting up FreeSurfer

3.1.1. Ensure FreeSurfer is downloaded and installed[53]. Go to the **Table of Materials** and download the relevant scripts to run this Module (under **ICV pipeline files**). When working with FreeSurfer, set the following variables:

export FREESURFER_HOME=<freesurfer_installation_directory>
source $FREESURFER_HOME/SetUpFreeSurfer.sh

3.1.2. Replace **<path>** in the following:
export SUBJECTS_DIR=<path>/enigma/Freesurfer

3.2. Running Freesurfer autorecon1

3.2.1. For a single subject, from inside the 'freesurfer' directory (processing time ~20 min), type the command:

cd <path>/enigma/freesurfer
recon-all -i ../input/<subject>.nii.gz -s <subject> -autorecon1

3.2.2. See the tutorial manual for examples of how to create a for-loop for processing multiple subjects serially.

3.3. Calculation of ICV

3.3.1. Data organization

3.3.1.1. In the 'freesurfer' directory, place the (i) Docker *OR* Singularity container used in Module 1 ('calculate_icv.tar' or 'R.sif,' respectively) and (ii) xfm2det script (see the **Table of Materials**). Then, do a git clone to clone the required ICV script:

git clone *https://github.com/Characterisation-Virtual-Laboratory/calculate_icv*

3.3.2. Running ICV extraction (processing time ~5 min)

3.3.2.1. From 'freesurfer' directory, with singularity ('R.sif') container, type:

singularity exec --cleanenv -B $PWD:$PWD R.sif calculate_icv/calculate_icv.py --freesurfer_dir=/path/to/freesurfer --acapulco_dir=/path/to/acapulco/QC/Cerebellvolsfile --output_csv_name=Cerebel_vols.csv calculate_icv

3.3.2.2. From 'freesurfer' directory, with docker container, type:

docker run -v $PWD:$PWD -w $PWD –rm -it luhancheng/calculate_icv:latest calculate_icv/calculate_icv.py --freesurfer_dir=/path/to/Freesurfer --acapulco_dir=/path/to/acapulco/QC/Cerebelvolsfile --output_csv_name=Cerebel_vols.csv calculate_icv

3.3.2.3. Running script without container—see the **Table of Materials** for additional required software and dependencies. From the 'freesurfer' directory, type:

./calculate_icv/ calculate_icv.py ---freesurfer_dir=/path/to/freesurfer --acapulco_dir=/path/to/acapulco/QC/Cerebelvolsfile --output_csv_name=Cerebel_vols.csv calculate_icv

NOTE: This will calculate the ICV for each subject and append a column with ICV to the end of the 'Cerebel_vols.csv' file.

REPRESENTATIVE RESULTS:

Cerebellum parcellation (ACAPULCO)

Quality control of cerebellum parcellated masks:

The following examples demonstrate the ACAPULCO parcellated outputs and guide decision-making about a) the quality of the parcellated mask at the individual level and b) subsequent inclusion or exclusion of a particular lobule(s) from the statistical analyses. Ultimately, the decision to include or exclude a subject is subjective; examples of 'good parcellations,' 'subtle

parcellation errors,' and 'global failures' from a variety of healthy and clinical groups are provided here.

Examples of 'good parcellations' are presented in **Figure 4**, including in healthy and heavily atrophied cerebella. In **Figure 5,** subtle over- and under-inclusions of individual cerebellum lobules are depicted. These are the most common type of parcellation error and may not be detected as statistical outliers in the quantitative QC protocol. These types of errors generally require the exclusion of the individual lobules that are affected, while the remainder of the parcellated cerebellum is unaffected and can be retained. In contrast, 'Global failures,' as depicted in **Figure 6**, require complete exclusion of the subject.

Statistical outlier detection:

To illustrate the pipeline, ACAPULCO was run on a sample of 31 people with FRDA (mean age= 36.5 years; SD= 13.0 years, 14 females) and 37 age- and sex-matched healthy controls (HC) (mean age= 37.1 years; SD=12.8 years, 17 females) as previously described[55]. Across the whole sample, 18 lobules were detected as statistical outliers (<1% of the total sample). After performing detailed QC on the images, 17 outlier lobules were removed from group-level analyses by removing the individual lobule volume for the respective subject(s) from the group-level cerebellar volumes file (i.e., the 'Cerebel_vols.csv' file). The remaining outlier was deemed not a segmentation error but rather due to variability in the individual's cerebellum anatomy and was therefore retained in the analysis. There were also two global parcellation failures (1 FRDA patient). The base rate of exclusion across all cerebellum lobules (i.e., global parcellation failures) was 1.5%. **Table 1** shows the exclusion rates for each of the 28 anatomical ROIs. Left lobule IX and Right lobule Crus I had the highest rates of exclusion.

Group-level statistical analysis:

A total of 66 subjects (30 FRDA patients) were included in the group-level analysis. Two-tailed Mann-Whitney independent samples tests were conducted to test for significant differences in cerebellum lobule volumes between FRDA and HC. Results showed significantly reduced white matter in the corpus medullare in FRDA vs. HC ($p < 0.05$, Bonferroni corrected for 28 comparisons. There were no other significant between-group differences. See **Supplemental Table S1** for the volumes of all 28 cerebellar subunits in the sample.

Cerebellum voxel-based morphometry analyses (SUIT)

Quality control:

Examples of well-aligned images and examples of exclusions for both healthy controls and FRDA are shown in **Figure 7**. SUIT analyses were run on a total of 64 subjects (28 FRDA) from the sample described above, following the exclusion of an additional two subjects due to incomplete whole cerebellar coverage in the cerebellum mask.

After testing the spatial covariance of all normalized images in the sample relative to each other, two scans were detected as statistical outliers based on their mean spatial covariance with the rest of the sample (**Figure 8**). However, visual inspection of both the original and normalized images indicated that although these individuals had some unique neuroanatomy, there were no significant artifacts in either image and the processing steps completed normally. As such, both subjects were retained in the analyses.

Group-level statistical analysis:

Images were smoothed using a Gaussian kernel of 3 mm full width at half maximum (FWHM). Nonparametric permutation tests were carried out in SnPM to test for significant between-group differences in cerebellum grey matter volumes. To this end, 5,000 permutations were run, with a cluster-forming threshold of $p < 0.001$. Images were explicitly masked with the SUIT grey matter atlas to limit inference to grey matter regions. To correct for head size, intracranial volume was entered as a covariate in the model. Final inference of group results was carried out at $p < 0.05$, family-wise error (FWE) cluster-level corrected.

Compared to HC, FRDA showed significantly reduced grey matter volume in bilateral anterior lobules I-V (left: x= -10, y= -46, z= -26; T= 5.61; Ke= 754; right: x= 10, y= -38, z= -21; T= 6.83; Ke= 569); and in medial posterior lobe regions, including Vermis VI, extending bilaterally into Lobule VI (x= 3, y= -65, z= -20; T= 7.25), and Vermis IX extending bilaterally into Lobule IX (x= 3, y= -65, z= -20; T= 6.46; Ke= 3974; **Figure 9**).

TABLE AND FIGURE LEGENDS:

Figure 1: The human cerebellum. (**A**) A flattened representation of the cerebellum and its major fissures, lobes, and lobules. Red = anterior lobe (lobules I–V); cream= posterior lobe (lobules IV–IX); purple= flocculonodular lobe. The cerebellum can be divided into midline "vermis" and lateral "hemisphere" zones. All lobules are identified in the vermis and hemispheres. In lobule VII, VIIAf in the vermis expands in the hemispheres to become Crus I, lobule VIIAt at the vermis becomes Crus II in the hemispheres, and lobule VIIB retains its label both in the vermis and hemispheres. (**B**) Top: Cerebellum flat map showing the anatomical subunits of the cerebellum in different colors. Bottom: a posterior view of the cerebellum. This figure was adapted from [56] and [57].

Figure 2: Schematic illustration of the pipeline. A high-resolution T1 MPRAGE image is required. There are three modules: ACAPULCO, SUIT, and ICV. The pipeline is fully automated (except for manual intervention required for QC of the outputs), is available in Docker and Singularity container format, and takes approximately 20 min to run from start to finish, per subject.

Figure 3: ACAPULCO parcellation demonstrating each of the 28 anatomical subunits. This figure was adapted from[36]. Abbreviations: CM = corpus medullare; Ver = vermis; R/L = right/left.

Figure 4: Examples of 'good' cerebellum parcellations. Sagittal (x= 0) and coronal (y= -57) slices are shown. (**A, B**) Sagittal and coronal slices showing parcellated masks from two healthy

cerebellar. The algorithm has localized the individual subunit boundaries correctly, and it has not overincluded the transverse sinus into the labeling and quantification of Crus I. (**C**) A heavily atrophied cerebellum of a SCA2 patient. Here, atrophy is apparent along the entire extent of the cerebellum, the sulci are broad, and there is a lot of missing tissue. There is a slight overinclusion of the vasculature in the posterior lobes that is more pronounced on the right side (yellow arrow). Aside from this overinclusion, ACAPULCO has worked well. Abbreviations: CM = corpus medullare; Ver = vermis; L/R = left/right.

Figure 5: Examples of cerebellar 'mis parcellations.' (**A**) Sagittal slice (x= 0) and coronal slice (y = -57) showing a parcellation error in an FRDA patient. The algorithm has not worked well along the midline, and underinclusions of Crus I and II (red arrow) are evident along the posterior extent. These lobules would be excluded from subsequent group-level analyses. (**B**) Sagittal slice (x= 8), coronal slice (y= -47) showing a parcellation error in a healthy cerebellar. The algorithm has completely missed left lobule VIIIb (red arrows). This lobule would be excluded from subsequent group-level analyses. (**C**) Sagittal slice (x= -24) and coronal slice (y= -47) showing a parcellation error in a healthy cerebellar. Some cerebellar atrophy is present, and there is an underinclusion of Crus I (red arrow). Abbreviations: FRDA = Friedreich ataxia; CM = corpus medullare; Ver = vermis; L/R = left/right.

Figure 6: Examples of 'global failures' of cerebellar parcellation. (**A**) Sagittal slice (x= 0) and coronal slice (y= -57) showing a parcellation failure. Here, the cerebellum is only partly segmented, and parts of the occipital lobe have incorrectly been labeled as the cerebellum. These types of failures are likely due to a problem with the header of the original image, which will affect the ACAPULCO affine transformation of the image into world coordinate and subsequent localization of the cerebellum. (**B**) Sagittal slice (x= 0) and coronal slice (x= -57) showing a parcellation failure in an FRDA patient. Here, the CM has been completely mis-segmented. The algorithm has labeled the CM at the back of the head (red oval), outside the brain. The boundaries of the white matter have not been captured and are mislabeled as grey matter, particularly affecting left lobules VIIIb and IX. Left lobule X has also been missed (red arrow on coronal slice). These examples warrant immediate exclusion from group-level analyses for ACAPULCO and SUIT analyses. Abbreviations: CM = corpus medullare; Ver = vermis; L/R = left/right.

Figure 7: Warped and modulated voxel-based morphometry maps. (**A, B**) Well-aligned cerebellar grey matter from two HCs. (**C**) A cerebellum from an HC that was detected as a statistical outlier but was retained in the analysis. (**D**) An atrophied cerebellum from a person with FRDA. The cerebellum has been warped correctly to the template; hence, this would not warrant exclusion. (**E**) An exclusion. There is a gradient in contrast from the top to the bottom of the image that reflects an error in the processing. (**F**) A hyperintense plane artifact in the lower right of the image of unknown origin necessitates exclusion. (**G**) An example of a heavily atrophied cerebellar of an SCA2 patient. The cerebellum has been warped correctly to the template; however, there is a lot of missing tissue, resulting in low contrast. This panel depicts another example of a heavily atrophied cerebellum. Here, the warping of the cerebellum to the template has worked well. This would not be an exclusion. (**H**) Example of poor masking

necessitating exclusion. Abbreviations: VBM = voxel-based morphometry; HC = healthy control; FRDA = Friedreich ataxia; SCA2 = Spinocerebellar ataxia 2.

Figure 8: Spatial covariance of SUIT voxel-based morphometry maps. Box plot illustrating the spatial covariance of the voxel-based morphometry maps for a cohort of 64 (28 FRDA) subjects. Spatial covariance is a measure of how well aligned each image is, relative to every other image in the sample. The data are tightly clustered together with an average spatial covariance correlation of ~0.95. Here, two outliers (1 FRDA, 1 HC) were detected, as >2 SDs below the mean. Abbreviations: FRDA = Friedreich ataxia; HC = healthy control; SD = standard deviation; cont = control.

Figure 9. Between-group results of a voxel-based morphometry analysis of cerebellar grey matter morphometry. (**A**) Sagittal, (**B**) coronal, and (**C**) salt map representations of voxel-level statistical maps in individuals with FRDA vs. controls, controlling for ICV. Only voxels that survive $p < 0.05$ FWE cluster-level corrected, are shown. Color bar indicates T statistic. Abbreviations: FRDA = Friedreich ataxia; ICV = intracranial volume; FWE = family-wise error.

Table 1: Cerebellar anatomical lobules derived from ACAPULCO and rates of exclusion (%) across a sample of 31 people with FRDA and 37 HCs. Abbreviations: FRDA = Friedreich ataxia; HC = healthy controls.

Supplemental Table S1: Volumes (mm^3) of 28 cerebellar anatomical lobules in Friedreich Ataxia and healthy control individuals.

DISCUSSION:

The cerebellum is critical to a wide range of human motor[3], cognitive[58], affective[10], and language[7,59] functions and is implicated in many neurological and psychiatric diseases. The availability of a standardized and easily implementable approach for the quantification of regional cerebellar volumes will contribute to increasingly detailed 'whole-brain' structure–function mapping, complete disease modeling, and improved opportunities to define and track cerebellar contributions to brain diseases. This standardized pipeline described here combines state-of-the-art approaches for automatic cerebellum parcellation and finer-grained spatial profiling of cerebellar grey matter morphometry in both health and disease.

The results of the cross-sectional cerebellum parcellation analysis using ACAPULCO presented here showed that people with FRDA (vs. HC) had significantly reduced white matter volumes. These findings support previous studies of FRDA, which consistently show early, robust, and progressive white matter volume loss, particularly in the dentate nuclei, in FRDA. Furthermore, the pattern and extent of progressive neurodegeneration in the superior and inferior cerebellar peduncles and the dentate nuclei have been shown to differ as a factor of onset age of FRDA[44]. The results from the SUIT analysis revealed additional findings. Specifically, there was significant voxel-level volume loss in FRDA (vs. HC) in anterior lobe regions corresponding to bilateral lobules I–IV and Right V, extending into lobule VI. In addition, the SUIT analysis revealed significant volume loss in FRDA (vs. HC) in the medial posterior lobe regions, including lobule IX, X, and

Vermis. This pattern of between-group differences is comparable to previously published work in the same cohort of FRDA patients, using a whole-brain VBM approach[55].

Defining cerebellar abnormalities in neurological and psychiatric diseases is a high-priority research area with translational impact. Instrumental to tracking and treating neurological diseases—particularly those where the cerebellum is a primary site of neurodegeneration—is the development of complete biological characterizations of cerebellum involvement. The pipeline presented herein allows for relationships between individual cerebellar lobule grey matter morphometry and clinical measures that are used as the "gold standard" for clinical endpoints of disease to be explored. Such research can have a significant translational impact. For example, in the rare cerebellar diseases space, the identification of a particular profile of cerebellar grey matter atrophy in a subgroup of patients that maps onto or predicts clinical symptoms would have implications for guiding clinical practice. The inclusion of the SUIT module further allows for interesting research questions to be addressed such as structure–function mapping of the cerebellum or analysis of functional gradients of the cerebellum[60].

General recommendations for group-level statistical analyses

ACAPULCO: Volumes of each cerebellar lobule (in mm^3) for each subject are recorded in *Cereb_vols.csv*. During statistical inference of group-level effects, intracranial volume (ICV; also recorded in *Cereb_vols.csv*) should be controlled for to account for variability in head size. Alpha-significance thresholds should be corrected to account for inference across multiple lobules.

SUIT: Grey matter cerebellar VBM can be performed on the wd<subject>seg1.nii images using standard MRI processing software such as SPM or FSL. See the CAT12 manual for an excellent introduction to VBM using SPM12[54]. ICV should be controlled for to account for variability in head size.

For VBM in the cerebellum, it is generally recommended to use a Gaussian spatial smoothing kernel of no more than 3 mm full width at half maximum (FWHM). Appropriate statistical correction must be applied to account for multiple comparisons across voxels. In general, it is recommended to use nonparametric approaches (e.g., SnPM or FSL-Randomise).

The most critical step for successful parcellation of the cerebellum using ACAPULCO is general quality-checking of the T1 images **prior** to **and** post processing. It is highly recommended that the user check for bad contrast images (e.g., an inconsistent gradient across the image) and severe tilting of the head and motion artifacts, all of which can affect the performance of the algorithm. In addition, while the ACAPULCO algorithm has been trained on atrophied cerebellar, it has not been trained on lesion data. It is anticipated that lesions in the cerebral cortex would not be expected to impact the performance of the algorithm and subsequent accuracy of the parcellation; however, large infarcts in the cerebellum would likely yield parcellation errors. Quality-checking the cerebellum mask post processing is **essential**. Minor parcellation errors (e.g., minor under- and overinclusions of cerebellum lobules) are sometimes not detected as statistical outliers; conversely, instances of incorrect nonoutliers can occur where data are within the normal range, despite an obvious parcellation error. If a subject is identified as an outlier, it

is essential to perform follow-up, detailed quality-checking of the cerebellum mask slice-by-slice to guide decision-making about whether to include or exclude the lobule(s) for that subject. Another critical step when running the SUIT pipeline (Module 2) is that it requires the ACAPULCO module to have already been run. Specifically, SUIT requires the cerebellum mask produced in ACAPULCO to run the cerebellum isolation and segmentation. It is important that the cerebellum mask is quality-checked to ensure full cerebellar coverage.

There are some limitations to the protocol. First, while ACAPULCO achieves state-of-the-art accuracy for cerebellar grey matter parcellation, it is not optimized for white matter parcellation; the corpus medullare covers the main body of the white matter but does not provide a measure of all the white matter. Second, the convolutional neural networks used to localize and segment the cerebellum in ACAPULCO do not generalize well to images with different contrasts or images that were not used in training. For example, because only 3T images were used in training, the parcellation quality using images acquired on a 1.5 T scanner is typically not as good; furthermore, there are no statistics with respect to ground truth that has been performed on these images. Finally, the pipeline controls for the confounding effects of head size on cerebellum volume estimates by providing an estimate of ICV that can be included as a regressor of no interest in group-level statistical analyses. However, an ideal approach would be to calculate ICV-corrected cerebellum volumes at the individual level prior to running QC, such that detected outliers reflect a true parcellation error and not natural variability in the subjects' neuroanatomy (e.g., having a large head).

In conclusion, we present a standardized pipeline for examining cerebellum grey mater morphometry, which has broad applicability to a range of neurological diseases. The pipeline is set up to allow for large, multisite studies and 'mega analyses' and is publicly available for use by research groups to facilitate reliability and reproducibility across the field. Ultimately, this pipeline provides a powerful methodological approach for further characterizing and tracking cerebellar structural changes with disease progression in neurological diseases. A longitudinal pipeline is currently being developed.

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The authors have no conflicts of interest to disclose.

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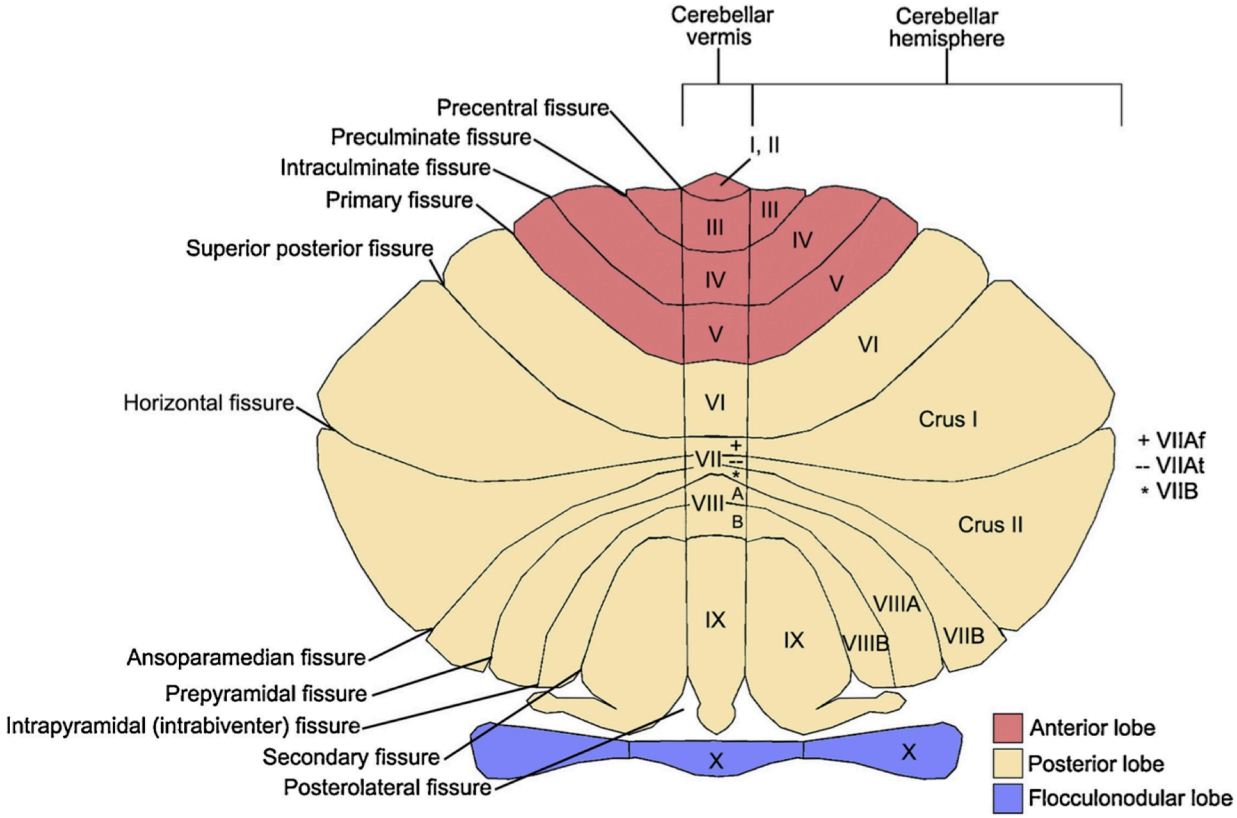
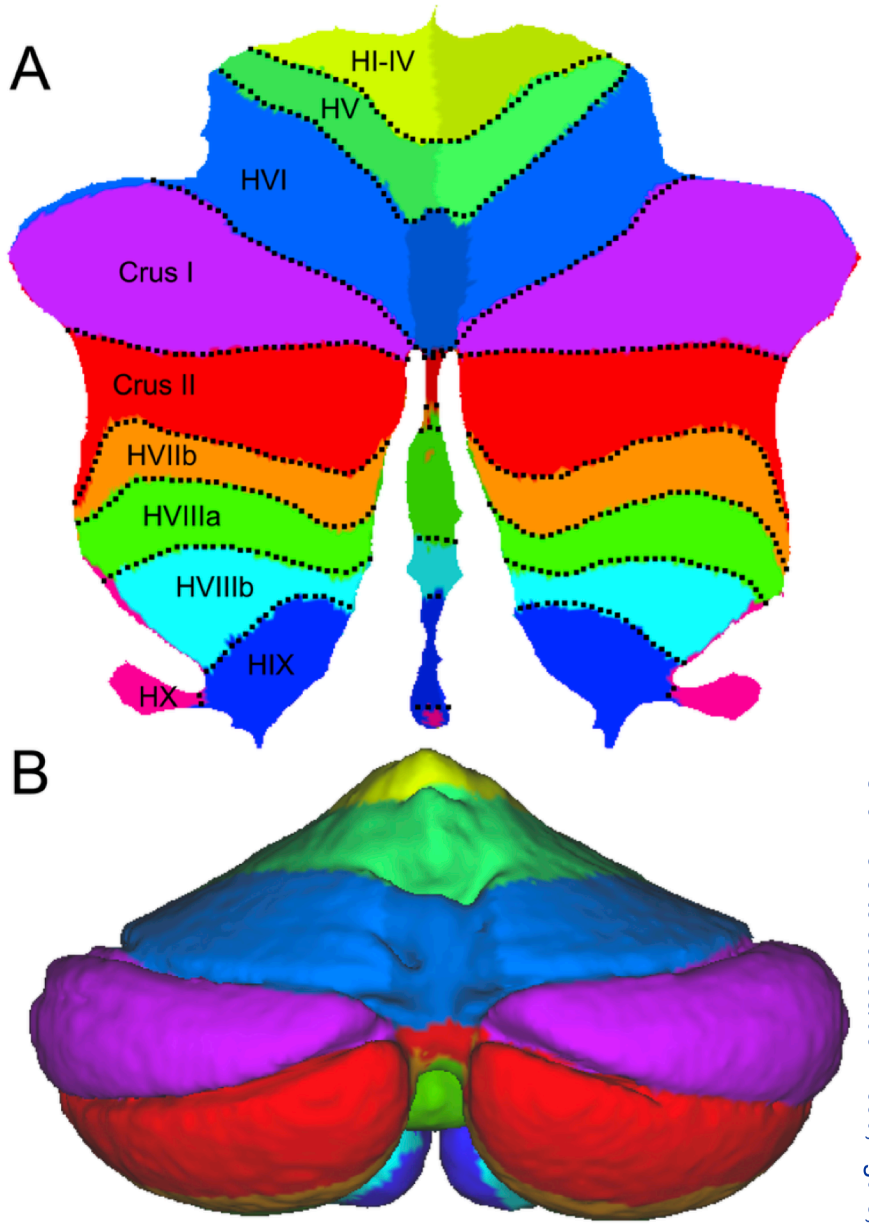
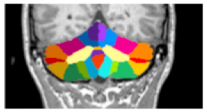




Module 1: ACAPULCO
Requirements: Single structural (e.g., MPRAGE) image
Docker or Singularity
Customized R scripts for QC (available through G-Drive link)

Cerebellum mask



Module 2: SUIT
Requirements: MATLAB
SPM12
SUIT toolbox
ENIGMA SUIT scripts (available through G-Drive link)

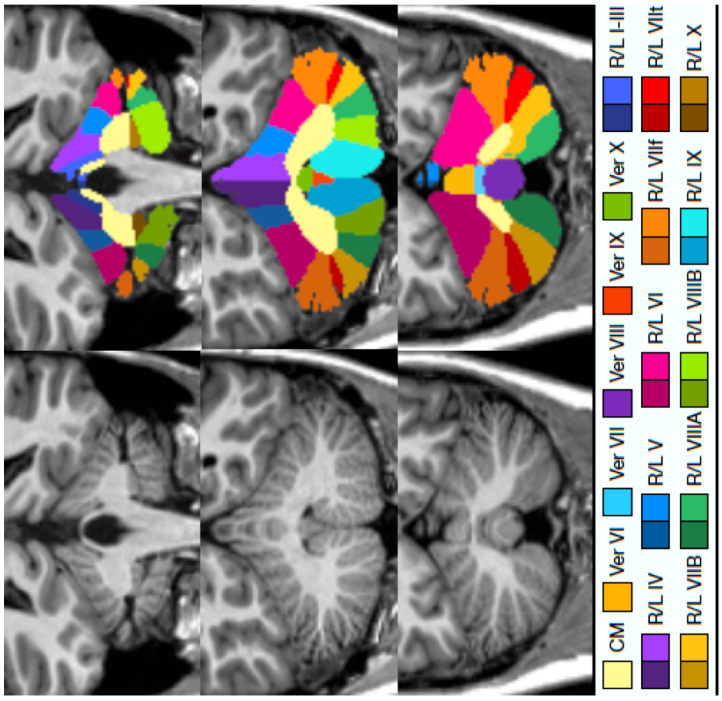
Module 3: ICV (optional)
Requirements: Freesurfer
Docker or Singularity
Git
Customized Python script (available through G-Drive link and git repo)

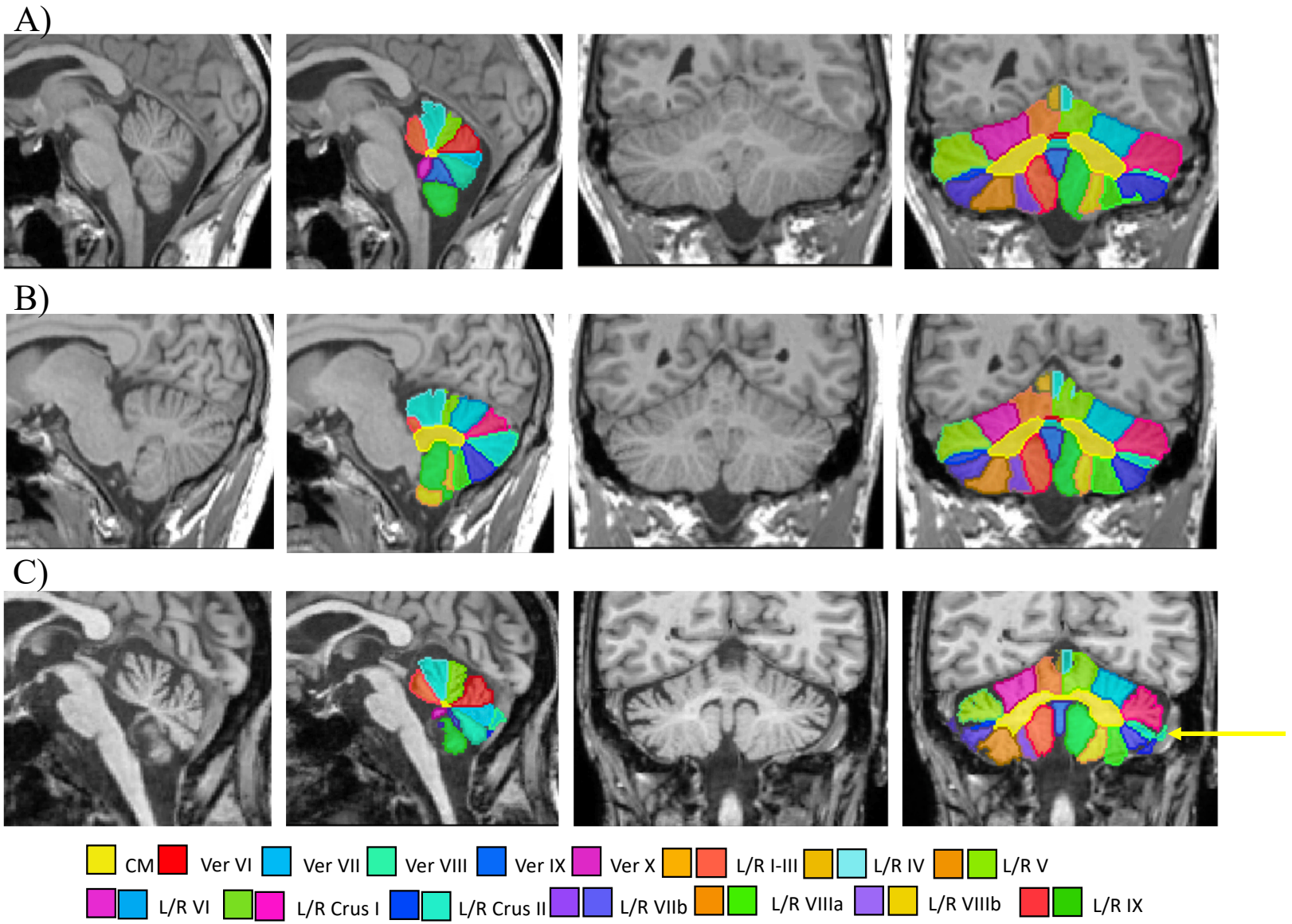
Output:

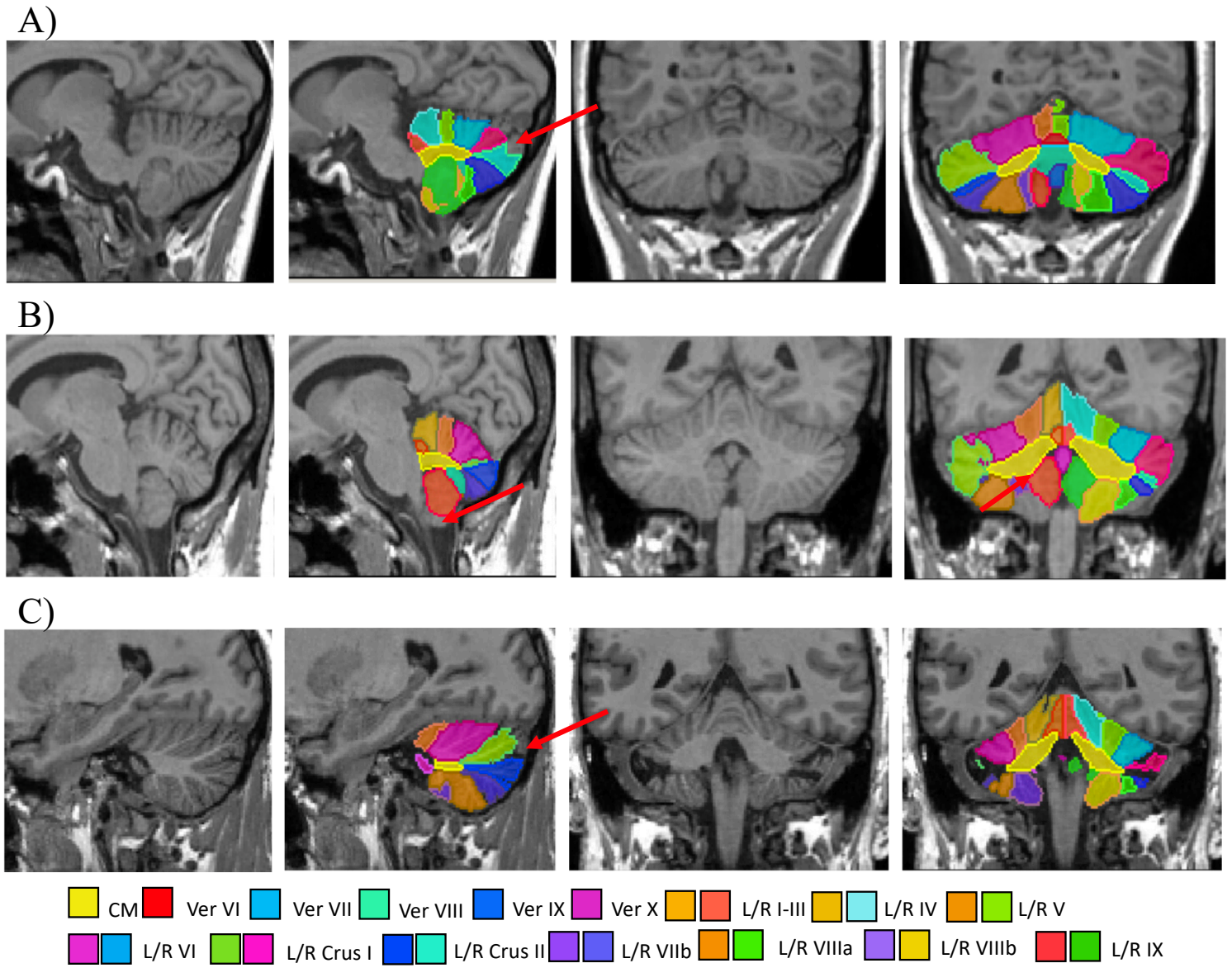
1. csv file: aggregated subject x cerebellar lobule volume matrix for group-level statistical analyses*
2. QC outputs
3. Grey matter Voxel Based Morphometry maps for group-level statistical analyses

* If Module 3 is run, an estimation of ICV is obtained for each subject, and appended to the .csv file

Figure 3







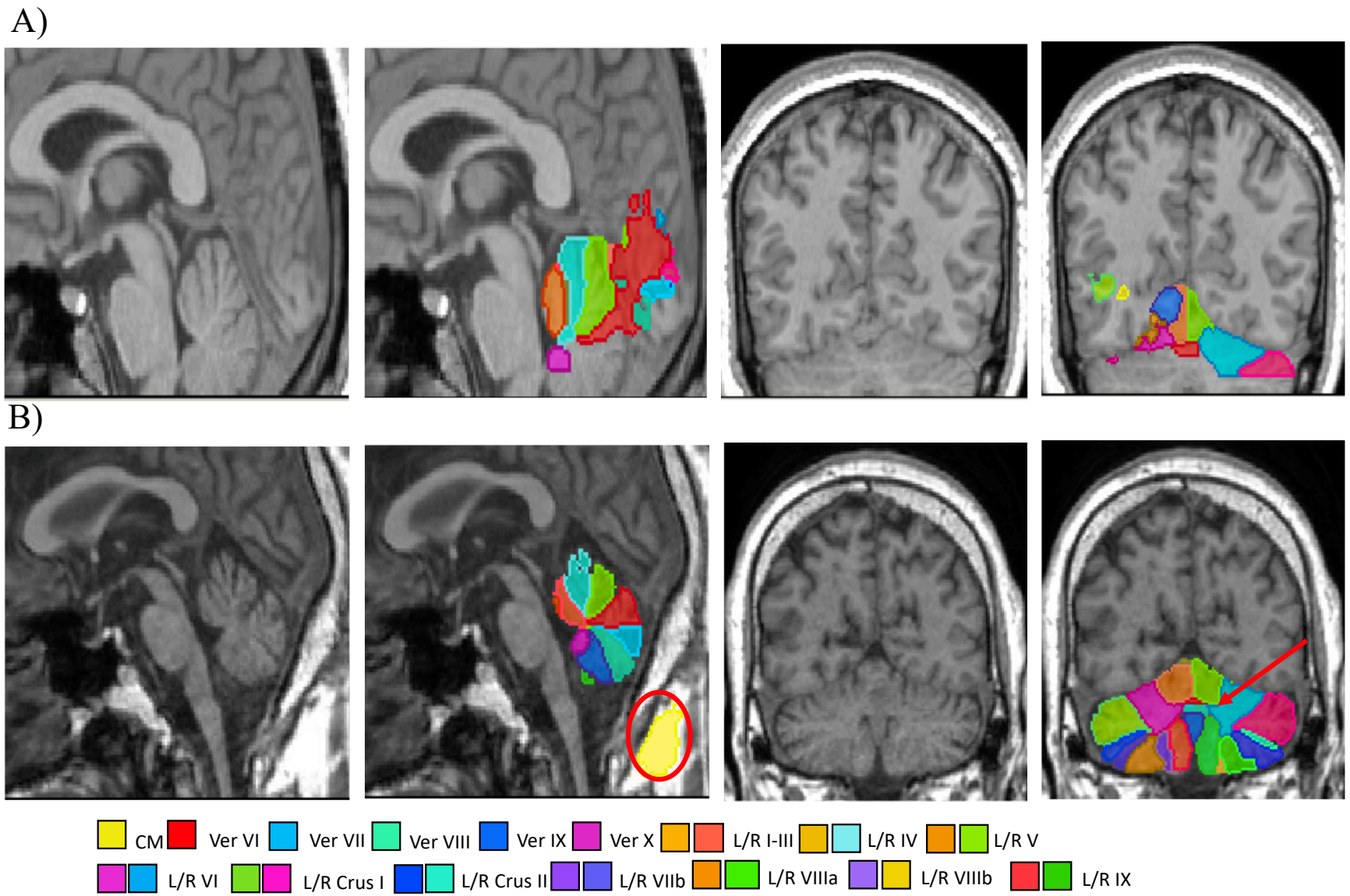


Figure 7

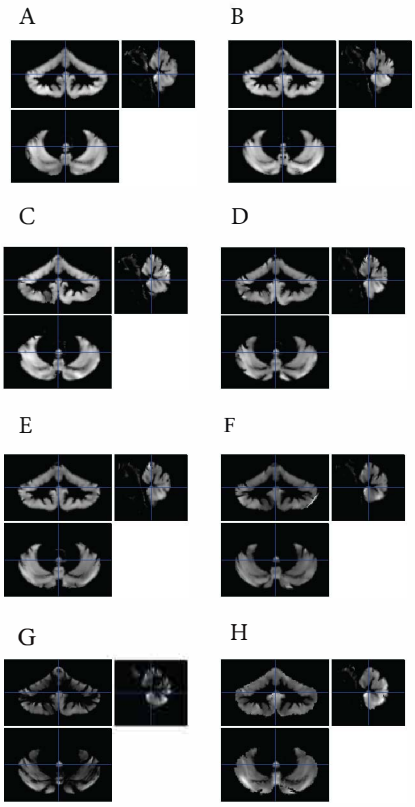


Figure 8

Click here to access/download;Figure;Figure8.pdf

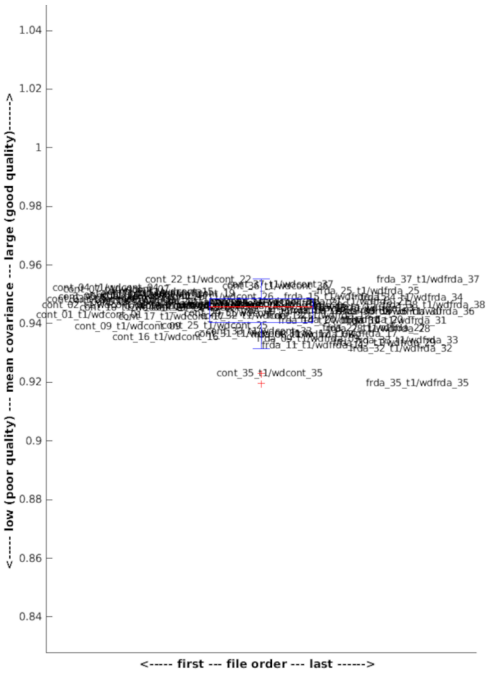
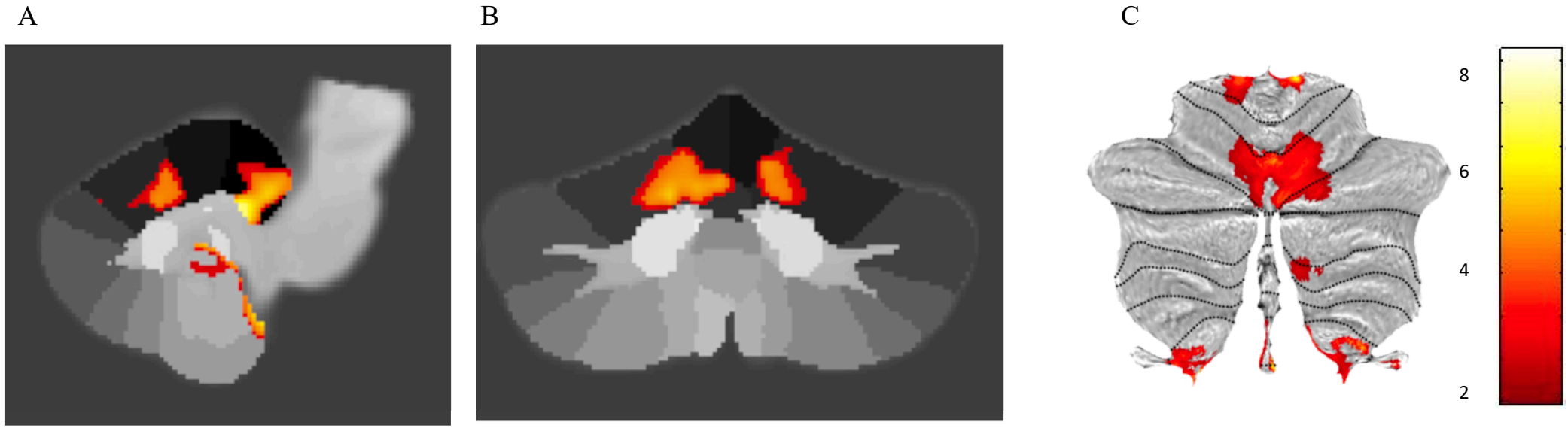


Figure 9



Lobule	% excluded
CM	1.5
Left Crus I	2.9
Left Crus II	2.9
Left I III	2.9
Left IV	1.5
Left IX	**4.51**
Left V	2.9
Left VI	1.5
Left VIIB	1.5
Left VIIIA	1.5
Left VIIIB	2.9
Left X	2.9
Right Crus I	**6.02**
Right Crus II	2.9
Right I III	2.9
Right IV	1.5
Right IX	2.9
Right V	1.5
Right VI	1.5
Right VIIB	1.5
Right VIIIA	2.9
Right VIIIB	1.5
Right X	1.5
Vermis IX	1.5
Vermis VI	2.9
Vermis VII	1.5
Vermis VIII	1.5
Vermis X	1.5

Response to the editor and author's comments

JoVE manuscript 63340

Editor comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
   We have thoroughly read through the revised manuscript to ensure there are no spelling or grammar issues.

2. Please provide email for each author.
   We have provided an email for each author on the title page of the manuscript.

3. Please provide at least 6 keywords or phrases.
   We have added 6 key words to the title page of the manuscript.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
   We have revised the manuscript and removed instances of any personal pronouns.

5. Please define all abbreviations at the first instance.
   All abbreviations have been defined at the first instance.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). Any text that cannot be written in the imperative tense (e.g., provide extraneous details, optional steps, or recommendations) may be added as a "Note."
   We thank the editor for making this point. We have ensured that all text in the protocol is written in the imperative tense, and where appropriate, added "Notes" to provide additional detail. These changes can be found to the protocol on pages 4-9 of the revised manuscript.
   Examples of this can be found on the following pages:

   page 6, step 3.1. The text now reads: "Go to the table of materials and download the relevant scripts and containers required to run ACAPULCO (under "acapulco pipeline files")."
   Page 6, step 4.1. The text now reads: "From the terminal, ensure that you are in the acapulco directory and that the contents of QC_scripts are in the acapulco directory. To run the QC scripts:
   4.1.1 (using Docker). Type the command…"
   Page 7, step 5.1 and page 7, step 5.3.1 have been changed to remove text that was not using imperative tense and has instead been added as a note.
   Page 9, step 2.1 has been changed to remove text that was not using imperative tense, and has instead been added as a note.
   Page 11, section 3.3.3. Text now reads:

"3.3.3 Running script without container- see Table of Materials for additional required software and dependencies. Ensure to be in the Freesurfer directory. Then type: ./calculate_icv/ calculate_icv.py ---freesurfer_dir=/path/to/Freesurfer --acapulco_dir=/path/to/acapulco/QC/Cerebelvolsfile --output_csv_name=Cerebel_vols.csv calculate_icv"

7. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.
We have attempted to simplify the protocol whilst still retaining sufficient detail that the reader could replicate the protocol. To achieve this we have simplified each step by reducing it to a maximum of 3-4 sentences, and where we felt necessary, added additional steps, to capture the information that is important and necessary to replicate the protocol.

Examples of this can be found on the following pages:

Page 5, step 2.2 is now divided into 2.2 and 2.2.1, and reads as follows:

"2.2 Once all necessary software is installed, in the working directory, create folders labelled 'acapulco', 'suit', and 'freesurfer'. From the command line, this is done using the "mkdir" command.

2.2.1 In the 'acapulco' directory, create an 'output' folder. In the 'output' folder, create a directory for each subject in the study containing the T1-weighted image in NIFTI-GZ format."

Page 7, step 5.2 has been divided into further steps. Additional information has been added to step 5.2 to address the "how" question and make the instruction more explicit. The text now reads:

"5.2 Open the "Plots_for_Outliers.html" to check boxplots for quantitative statistical outliers. Outliers (2.698 s.d above or below the mean) will appear above or below the whiskers of the box plots. Hover over the data-points to display the Subject ID. Outliers are also denoted by a '1' in the relevant column in the Outliers.csv file. The final column in Outliers.csv contains the total number of segments identified as outliers for each subject.
5.3. Each image having one or more outliers should be manually inspected. Using a standard NIFTI image viewer (e.g., FSLEyes or MRICron), overlay the ACAPULCO mask onto the original T1w image."

Page 9, step 2 has been divided into 2.1 and 2.2.

In addition, a detailed tutorial document, which extensively describes the protocol and how to run it from start to finish step-by-step, is available on the ENIGMA Consortium Imaging Protocols webpage under "Cerebellum Volumetrics Imaging Pipeline" located here: http://enigma.ini.usc.edu/protocols/imaging-protocols/
We have also clarified this in the revised manuscript, on page 4 and provided the URL for the user to access, in order to download the pipeline including the tutorial document.

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
We thank the editor for this comment. We have attempted to add more detail to address the "how" question with performing every step. We have also added additional steps, to capture the information that is important and necessary to replicate the protocol. These changes can be found throughout the manuscript on the following pages:

Page 5, step 2. The text now reads: "2.1 Obtain all necessary software as listed in the Table of Materials. Ensure Docker[48] or Singularity[49], Matlab[50] and SPM12[51] are installed prior to running the pipeline. Extensive written and video tutorials describing the pipeline are also available (see Table of Materials)."

Page 5, step 2.2. The text now reads: "2.2 Once all necessary software is installed, in the working directory, create folders labelled 'acapulco', 'suit', and 'freesurfer'. From the command line, this is done using the "mkdir" command."

Page 6, step 3.1 The text now reads: "3.1 Go to the table of materials and download the relevant scripts and containers required to run ACAPULCO (under "acapulco pipeline files")."
Similarly, step 3.2 follows as "3.2 Open a terminal, and from the command line, run the ACAPULCO container on a single image (replace <<subject>> in the following). Processing takes ~5 minutes. To do this:…"

Page 7, step 5.3.1 We have added the following text: "To generate overlays for detailed QC from the command line using FSLEyes:

- *change directory to the acapulco directory*
- *specify the subject you wish to view (replace <subject>):*
  subj=<subject_name>
- *Then copy/paste the following code to the terminal (no need to manually change {subj} as this has been set by the previous line:*
  t1_image=output/${subj}/${subj}.nii.gz
  acapulco_image=output/${subj}/${subj}_n4_mni_seg_post_inverse.nii.gz
  fsleyes ${t1_image} ${acapulco_image} --overlayType label --lut random_big --outline --outlineWidth 3 ${acapulco_image} --overlayType volume --alpha 50 --cmap random"

Page 8, section 1.1. Text has been added that reads "To check the MATLAB path, type "pathtool" in the MATLAB command window, then click "Add with subfolders" to add the relevant folders."

Finally, as stated in our response above to point 7, a detailed tutorial manual is available which, alongside the manuscript, provides very detailed instructions for the user to follow along and easily replicate the protocol.

9. Please add more details to your protocol steps:

   Lines 175-179: where are these files located? Please point to relevant resource/directory in the Table of Materials.
   We thank the editor for pointing this out. The change can be found on page 6 of the revised manuscript, step 3. The text now reads: "3.1 Go to the table of materials and download the relevant scripts and containers required to run ACAPULCO (under "acapulco pipeline files")."

   Lines 181-190: are these run on a command line or a different platform? Please specify.
   We have made this clearer. This is reflected on page 6 of the revised manuscript, step 3.2 The text now reads: "3.2 Open a terminal, and from the command line…."

   Line 192: please point to Table of Materials for tutorial manual.
   We have revised the text to point the reader to the Table of Materials for the tutorial manual. The text on page 6, step 3.3 of the revised manuscript reads: "3.3 Loop across all subjects/scans in the cohort. See the Table of Materials for a link to the ENIGMA Imaging Protocols website for downloading the pipeline, and tutorial manual that contains examples of how to create a for-loop for processing multiple subjects serially."

   We have also ensured that, throughout the manuscript, we point to the corresponding scripts/software and tutorial manual in the Table of Materials, to make it clear for the reader where the relevant files can be found.

   Line 231: Please avoid mentioning in the manuscript any program/software that is not open-source and freely available. JoVE cannot publish manuscripts containing commercial language. Please include all the commercial terms in the Table of Materials and refer them in the manuscript text wherever needed.
   We thank the editor for reminding us of this. We have removed one instance of text where we referred to MATLAB which is not open-source and freely available. We have instead added text to the Table of Materials to state that a license is required to operate MATLAB, and cited the relevant URL for the software.

   The text on page 4 of the revised manuscript now reads: "The pipeline is freely available with no restrictions of its use for non-commercial purposes, and can be accessed from the ENIGMA Consortium Imaging Protocols webpage (under "ENIGMA Cerebellum Volumetrics Pipeline") following the completion of a brief registration form: http://enigma.ini.usc.edu/protocols/imaging-protocols/[45]. All required software is listed in the Table of Materials, and detailed tutorials including a live demonstration, are available upon download of the pipeline, in addition to the protocol described below."

   Line 243: How is the subject excluded using the SUIT module? Throughout the protocol, please ensure to answer the "how" question, i.e., how a step is performed.
   We apologise for not being clear. When acapulco is run, if there is not full cerebellar coverage (e.g., due to a number of cerebellar lobules missing or cut-off from the mask), then SUIT cannot be run, as it relies upon the generation of a subject-specific

cerebellar mask, for the optimisation of the registration and normalisation of the cerebellum to the SUIT template.

We have altered the text in the revised manuscript in two places, to clarify this:

Page 8, step 5.2.2: The text now reads: "Do parcellation errors result in some of the cerebellum being excluded from the mask? If Yes, (for example, if particular cerebellar lobules are missing from the mask or appear 'cut off'), the subject will need to be immediately excluded from further analyses using the SUIT module."

Page 8 under the heading Module 2: SUIT Cerebellum-Optimised Voxel-Based, the text now reads: "***CRITICAL**: This pipeline requires the ACAPULCO module to have already been run, as it relies on the generation of a subject-specific cerebellar mask, for optimisation of the registration and normalisation of the cerebellum to the SUIT template. If the subject-specific mask generated from acapulco does not include the whole cerebellum, this warrants exclusion from the SUIT module. For instructions on running SUIT standalone, see*[52].

Line 255: Please point to the corresponding scripts/resources in the Table of Materials. Please do the same wherever a tool/resource/tutorial manual is mentioned in the text.
We have ensured that we point to the corresponding scripts and resources where appropriate, throughout the revised manuscript. Examples of this can be found on the following pages:

Page 5, step 2.1. The text now reads "2.1 Obtain all necessary software as listed in the Table of Materials."

Page 6, step 3.3. The text now reads: "3.3 Loop across all subjects/scans in the cohort. See the Table of Materials for a link to the ENIGMA Imaging Protocols website for downloading the pipeline and tutorial manual that contains examples of how to create a for-loop for processing multiple subjects serially."

Page 9, step 1.3. The text now reads: "See the Table of Materials for a link to the ENIGMA Imaging Protocols website for downloading the pipeline and tutorial manual that contains examples of how to create a for-loop for processing multiple subjects serially."

Page 10, step 1. We have added: *"Ensure Freesurfer is downloaded and installed: https://surfer.nmr.mgh.harvard.edu/fswiki/DownloadAndInstall. Go to the Table of materials and download the relevant scripts to run this Module (under "ICV pipeline files"). When working with Freesurfer, the following variables must be set…"*

10. Please insert single line spacing between individual steps and sub-steps in the Protocol.
We have added a single line spacing between individual steps and sub-steps in the Protocol of the revised manuscript.

11. Please highlight no more than 3 pages of protocol including headings and spacings for scripting and filming the video. Please highlight complete sentences in the protocol.

We can only film action steps written in imperative tense. Do not highlight Notes (extraneous details, optional steps, or recommendations). Please ensure that the highlighted steps form a cohesive narrative with a logical flow and is in line with the Title of the manuscript.
We have ensured that a maximum of 3 pages in total of the revised protocol are highlighted. This contains the most critical steps of the pipeline that are written in an imperative sense, and which are required for the user to replicate the protocol.

12. Please provide practical details of how to collect 3D T1-weighted MRI images of the whole brain; alternatively, provide reference to a previously published protocol for the same.
We thank the editor for their comment. We would like to clarify that the collection of T1-weighted MRI images would need to be done by (or in direct consultation with) a radiographer who can help the user set up and acquire the T1 images that meet these specifications. Whilst we can give general recommendations for the acquisition (e.g., 1mm isotropic voxel dimensions, the use of a rapid acquisition gradient echo sequence like MPRAGE, use of a 3T scanner), the exact process of the collection of MRI data is well beyond the scope of this protocol.

We have added this detail to the revised manuscript. Page 5, step 1 under "Data Collection" we have added: "Collect 3D T1-weighted MRI images of the whole brain at a resolution of 1mm^3 or less. High resolution, isotropic voxel dimensions (typically 1 x 1 x 1mm) and use of a 3-Telsa (or greater) scanner are recommended. The researcher should consult with an imaging specialist at their radiography centre to set-up and acquire data that meet these specifications."

13. Please add all the websites/URLs in the protocol as citations. You can include them in the Reference section.
We have added all websites and URL's in the protocol as citations, including the ENIGMA website where the link to download our pipeline, can be found.
Note to the editor: we were not sure how to list the URL's in the References section, as information such as title, year, author etc are not available for URL's. Please feel free to alter the detail of the URL's in the Reference list, accordingly.

Examples of some of citations can be found on the following pages of the revised manuscript:

Page 5, step 2.1 the text now reads: "Obtain all necessary software as listed in the Table of Materials. Ensure Docker[48] or Singularity[49], Matlab[50] and SPM12[51] are installed prior to running the pipeline."

Page 8, Module 2, Step , under "Critical…" the text now reads: For instructions on *running SUIT standalone, see[52].*

Page 10, step 1. The text now reads: "Setting up Freesurfer
Ensure Freesurfer is downloaded and installed[53]. Please refer to the Table of materials for all necessary scripts to run this Module (under "ICV pipeline files").

14. Please mention any sponsor/funding or other acknowledgements in the "Acknowledgments" section.

We have added the relevant funding to the acknowledgments section of the revised manuscript.

15. Please include all the Figure Legends after the Representative Results. Each Figure Legend should include a title and a short description of the data presented.
We have moved the Figure Legends to after the Representative Results section. We have altered the legend of each figure to have a short title and then description of the data presented. Whilst we have attempted to keep the description short, we feel as though particular figures require additional text, in order to adequately capture the data being presented.

16. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable).
We have added a color bar to Figure 9 (between group findings from SUIT analysis) to display the T statistic. We have also added a colour legend to Figures 4, 5 and 6.

17. Figures 4 and 5: There are 4 rows and 3 columns given how the individual panels are spaced. Instead of using "top", "middle" and "bottom" to indicate rows, please use labels such as A, B, C, etc. for clarity and provide the explanation accordingly in the manuscript text and Figure legends.
We have changed Figures 4 and 5 to include "a", "b" and "c" labels. We have also changed the figure legends accordingly.

18. Figures 6: There are 4 rows and 2 columns given how the individual panels are spaced. Instead of using "top", and "bottom" to indicate rows, please use labels such as A, B, C, etc. for clarity and provide the explanation accordingly in the manuscript text and Figure legends.
As above, we have changed the labels in Figure 6 to read "a" and "b". We have also changed the figure legend accordingly.
19. Figure 7: Please identify Sagittal, coronal and salt map representations with labels.
We believe this comment was intended for Figure 9 as Figure 7 does not contain salt map representations of the cerebellum. We have edited Figure 9 with labels "a", "b" and "c" to reflect the labelling of these images in the figure legend.
20. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been reprinted/adapted/modified from [citation]."
Copyright permission to reuse Figures 1 and 3 had already been obtained, under the Elsevier open access and copyright policy. We have cited the creative commons attribute license in the Figure legend for Figures 1 and 3. We have also added the link to the policy in a word document that we have uploaded to our editorial manager account.
21. Please do not abbreviate the journal names in the References.
We apologise. We have corrected this.

Reviewer comments

Reviewer 1:

I understand the authors have advanced the codes and pipelines from 2007 NeuroImage and 2019 Nat Neuroscience. However, it was not truly easy for me because of the lack of excellence.
"and takes approximately 20 minutes from start to finish" Change the expression because it sounds as if the entire process is very easy for everybody, please. It should be up to the PC spec and environment.
Relative expressions should be omitted or reconsidered not to exaggerate the convenience, while I recognize the codes and pipelines would be so excellent and powerful.
We thank the reviewer for their comments. We now recognise that the tone used in the sentence "and takes approximately 20 minutes from start to finish", may be perceived by the user that the pipeline is easy to run, which may not be the case for everybody. We have edited this sentence accordingly. The changes are reflected in the abstract and introduction of the revised manuscript on pages 2 and 4 respectively.

The abstract on page 2 now reads: "The pipeline has broad applicability to a range of neurological diseases and is fully automated with manual intervention only required for quality control of the outputs. The pipeline is freely available, with substantial accompanying documentation and can be run on Mac, Windows and Linux operating systems."

The text on page 4 now reads: "The pipeline is fully automated, with manual intervention only required for checking of the quality control outputs, and can be run on Mac, Windows and Linux operating systems. The pipeline is freely available with no restrictions of its use for non-commercial purposes, and can be accessed from the ENIGMA Consortium Imaging Protocols webpage (under "ENIGMA Cerebellum Volumetrics Pipeline") following the completion of a brief registration form: http://enigma.ini.usc.edu/protocols/imaging-protocols/[45]"

In addition, we have also changed relative expressions and made the protocol more explicit with added details of how to carry out each step. We have also made reference to the Table of Materials throughout the revised manuscript, to point the reader to the relevant resources including the tutorial manual available online, which extensively details the pipeline step-by-step. Some examples of these changes include:

Page 5, under "Recommended Data Organisation", the text now reads:
"Obtain all necessary software as listed in the Table of Materials. Ensure Docker (https://docs.docker.com/) or Singularity (https://www.sylabs.io/docs/), Matlab (https://au.mathworks.com/), and SPM12 (http://www.fil.ion.ucl.ac.uk/spm/software/spm12/) are installed prior to running the pipeline. Extensive written and video tutorials describing the pipeline are also available (see Table of Materials)."

Page 6, step 4.1, we have attempted to be more explicit in how to run the QC step. The text now reads: "From the terminal, ensure that you are in the acapulco directory and that the contents of QC_scripts are in the acapulco directory. To run the QC scripts:

4.1.1 (using Docker). Type the command:

docker load calculate_icv.tar

docker run -v $PWD:$PWD -w $PWD --rm -it luhancheng/calculate_icv:latest Rscript QC_Master.R output/

Page 7, step 5.2 we have added some detail. The text now reads: "Open the "Plots_for_Outliers.html" to check boxplots for quantitative statistical outliers. Outliers (2.698 s.d above or below the mean) will appear above or below the whiskers of the box plots. Hover over the data-points to display the Subject ID…"

Page 7, step 5.3.1, we have added detail to describe how to generate overlays for detailed QC using FSLeyes. The text now reads: "To generate overlays for detailed QC from the command line using FSLEyes:

- *change directory to the acapulco directory*
- *specify the subject you wish to view (replace <subject>):*
  subj=<subject_name>
- *Then copy/paste the following code to the terminal (no need to manually change {subj} as this has been set by the previous line:*
  t1_image=output/${subj}/${subj}.nii.gz
  acapulco_image=output/${subj}/${subj}_n4_mni_seg_post_inverse.nii.gz

fsleyes ${t1_image} ${acapulco_image} --overlayType label --lut random_big --outline --outlineWidth 3 ${acapulco_image} --overlayType volume --alpha 50 --cmap random

Reviewer 2:

Manuscript Summary:
The manuscript proposes a standardized pipeline for examining human cerebellar grey matter morphometry using structural MRI images. It is an interesting paper because many popular softwares such as FSL and SPM have been widely used to segment and quantify the brain into different compartments but they are not optimized for cerebellum. The paper has provided good motivations to show the importance of their work.

Major Concerns:
Overlapped with their previous publications.

Minor Concerns:
- Should list the Operating System used because they are many Windows users, not all are familiar with Linux.
- None Linux users may have issues with the Module 3, especially the various paths in the commands. In addition, many programs have to be installed prior to running the analysis. Perhaps a pre-installed virtual machine can be shared so that the users can use all the commands listed with no error. This is useful if the main aim to publish the paper in JOVE is to promote the authors' work.
- Line 415: please provide more details regarding the removed outlier.
- Line 421: Please display the cerebellum lobule volumes of FRDA and HC in a table, include the p values for each comparison.
- Line 522: Besides T1 weighted image, T2 weighted image is sometimes used as the structural MRI images for analysis. Please discuss this and whether the proposed approach is equivalently applicable to T2.
- Figure 8 caption, line 759: two outliers (1 FRDA) ... how about the one or both are from the same patient?
- Figure 9 caption: Please show the colorbar or explain why different colour is used in the image.
- Cant see the comments clearly, some of the texts are missing.

We thank the reviewer for their comments. Below are our responses to each of the comments:

Major concerns: Overlapped with their previous publications.

We recognise that the methodological approaches presented in our manuscript, specifically ACAPULCO and SUIT, do overlap with previous publications by senior authors on this manuscript. However, we would like to emphasise that the purpose of this manuscript is not to replicate previous work using these existing validated methods, but rather to build upon the methodological approaches. We achieve this by synthesizing a pipeline that combines ACAPULCO and SUIT, and adds customized quality control procedures, that collectively, provides a state-of-the-art pipeline for cerebellar parcellation, that is superior to current leading approaches. The proposed pipeline is standardized and freely available for use and has broad applicability to a range of neurological and psychiatric disorders, making it a valuable contribution to the field.

- Should list the Operating System used because they are many Windows users, not all are familiar with Linux.
We thank the reviewer for their comment. Whilst out pipeline can be run on Mac, Windows and Linux OS's, the pipeline has explicitly been tested on Mac and Linux OS's only. We have made this clear in the revised manuscript, with the following changes:

Abstract, Page 2. We have added the sentence: "The pipeline can be run on Mac, Windows and Linux operating systems."

Page 4, under "Protocol" we have added the following note: "Whilst the pipeline can be run on Mac, Windows or Linux operating systems, acapulco, SUIT and the QC pipelines have explicitly been tested on Linux (Ubuntu) and Mac (Catalina, Big Sur v11.0.1) operating systems."

-None Linux users may have issues with the Module 3, especially the various paths in the commands. In addition, many programs have to be installed prior to running the analysis. Perhaps a pre-installed virtual machine can be shared so that the users can use all the commands listed with no error. This is useful if the main aim to publish the paper in JOVE is to promote the authors' work.
We recognise the reviewers point and understand that Module 3 may be difficult for some users because of the various paths that are required in the command line. We thank the reviewer for the suggestion of alternatively using a pre-installed virtual machine, however one of the major advantages of Docker/Singularity is that it circumvents the need for a virtual machine, which comes at a high computational cost. We have successfully rolled the pipeline out across several disease-specific working groups that collectively include over 40 sites around the world within the ENIGMA Consortium, and have received overwhelming positive feedback. A detailed tutorial document is also available, which extensively describes the protocol and how to run it from start to finish step-by-step. In addition, we have a troubleshooting document that accompanies the pipeline files, where common errors associated with each Module are described, and corresponding troubleshooting steps that can be taken. We have made reference to the link to the pipeline files (including the detailed tutorial manual and troubleshooting guide) throughout the revised manuscript, by pointing the reader to the table of Materials.

- Line 415: please provide more details regarding the removed outlier.
Sorry if we were not clear. 18 lobules in total (across the sample) were detected as statistical outliers. For each identified outlier lobule, the cerebellar mask was carefully inspected slice-by-slice in FSLeyes. 17 of the identified outliers were determined to be a true outlier; that is, there was an evident under or overinclusion of the lobule in the mask, which affected the volume quantification of the lobule. The remaining outlier lobule was not deemed to be a true outlier, and rather was due to normal variability in the individual's cerebellum anatomy.

We have added the following text to page 12 of the revised manuscript, under "Statistical Outlier detection" to clarify how outliers are removed, and to provide further information on why one outlier lobule was retained in the analysis: "…17 of the outlier lobules were removed from group-level analyses, by removing the individual lobule volume for the respective subject(s) from the group-level cerebellar volumes file (i.e., the Cerebel_vols.csv file). The remaining outlier was deemed not a segmentation error, but rather, due to variability in the individual's cerebellum anatomy, and was therefore retained in the analysis."

-Line 421: Please display the cerebellum lobule volumes of FRDA and HC in a table, include the p values for each comparison.
We have added a Table to supplement the manuscript (Supplementary Table 1) that lists the acapulco cerebellar volumes for each of the 28 anatomical lobules, as well as the p value for each of the Mann Whitney U Tests performed.

- Line 522: Besides T1 weighted image, T2 weighted image is sometimes used as the structural MRI images for analysis. Please discuss this and whether the proposed approach is equivalently applicable to T2.
T2-weighted MRI images are sometimes useful for volumetric analysis, either standalone or when paired with T1-weighted data. However, the pipeline presented here relies on T1-weighted data only, and some of the tools used are exclusive to this type of data. As such, there are no options for using T2w images as either an alternative or an add-on in this context. We have now made this clear in the manuscript on page 5 of the revised manuscript where we have added the following note:

"Note: T2-weighted images are sometimes useful for volumetric analyses, however, the pipeline presented here relies on T1-weighted data only, and some of the tools used are exclusive to this type of data. As such, T2-weighted images can not be used."

- Figure 8 caption, line 759: two outliers (1 FRDA) ... how about the one or both are from the same patient?
Sorry if this was not clear. Of the two outliers, 1 was an individual with FRDA, and one was a HC. We have clarified this in the revised caption for Figure 8.

Figure 9 caption: Please show the colorbar or explain why different colour is used in the image.
We have added a colour bar to Figure 9, to show the T statistic derived from our second-level analyses. We have also added the labels to the figure and explained what each part of the figure displays, in the revised caption of Figure 9.

Supplemental Table S1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);SupplementaryTable1.xlsx

	Friedrich Ataxia		Control		
Cerebellar lobule/region	**Mean**	**SD**	**Mean**	**SD**	***p* value (Mann-Whitney *U* Test)**
Corpus Medullare	10008.77	2087.55	12282.83	2156.77	<.001
Left Crus I	13951.13	1854.55	14447.22	2047.5	0.21
Left Crus II	8239.83	1444.27	8712.42	1421.08	0.17
Left I-III	850.97	159.92	915.08	211.51	0.01
Left IV	3708.1	594.15	3935.36	528.36	0.13
Left IX	3107.77	799.8	3273.11	842.07	0.13
Left V	3582.93	377.16	377.162	454.03	0.59
Left VI	9855.6	1356.6	10209.53	1255.59	0.24
Left VIIB	5777.43	1057.97	6157.94	1035.18	0.09
Left VIIIA	5166.03	1444.46	5402.81	1193.93	0.26
Left VIIIB	2875.4	560.47	2955.64	749.77	0.24
Left X	462.07	111.29	497.72	95.37	0.24
Right Crus I	13863.43	3162.99	14434.69	2875.84	0.17
Right Crus II	7795.27	1205.17	8331.22	1262.73	0.11
Right I-III	909.97	250.51	944.81	274.34	0.11
Right IV	4127.3	580.18	4339	561.39	0.07
Right IX	3223.4	803.15	3491.75	599.9	0.23
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