Supplementary Material

Clinical Severity Score

Supplemental Table 1 shows the criteria used to calculate the clinical severity score.

Regions of significant difference between MBS and HC

Supplemental Tables 2-4 display information about regions of significant difference in volume and diffusion metrics between MBS and healthy controls. The coordinates for the voxel with the lowest p-value and number of voxels within each cluster was obtained using FSL cluster tool (Jenkinson *et al.*, 2012). In addition, to provide coordinates in a common reference atlas, we have performed diffeomorphic tensor based registration (Irfanoglu *et al.*, 2016) of our study template to the ICBM DTI-81 atlas (Mori *et al.*, 2008) which is in the ICBM-152 coordinates. Supplemental Figure 1 shows the spatial location of regions with significant difference in volume and MD between MBS and healthy controls. There were no FA differences in close proximity to where volume differences were observed.

Spatial Normalization

Supplemental Figure 2 shows areas of significant volumetric reduction in subjects with MBS compared to HC using two different study-specific templates as a reference template for spatial normalization: A- the study-specific template is constructed from 15 healthy controls, B- the study-specific template is constructed from 9 MBS, 9 CFW, and 9 HC subjects. Similar results are obtained using either template.

Structure and function of extraocular muscles

Area of volumetric reduction co-localizes with a portion of medial longitudinal fasciculus (MLF), a long thin fiber bundle that traverses in dorsal portion of the pons and plays a critical role in conjugate horizontal gaze. To appreciate its significance, we briefly review normal ocular motor circuitry and innervation of extraocular muscles (EOM).

Six extraocular muscles control the eye movement, these include the medial and lateral rectus, the inferior and superior rectus, and the inferior and superior oblique (Supplemental Figure 4). These muscles are innervated by three cranial nerves: the oculomotor (CN III), the trochlear (CN IV), and the abducens (CN VI). The abducens nerve innervates the lateral rectus muscles which are responsible for lateral movement of the eye, specifically abduction, while the trochlear innervates the superior oblique and allows downward movement of the eye towards the middle, and the remaining EOMs (superior, inferior, and medial rectus muscles) are controlled by the oculomotor nerve which allows for lateral downward and both lateral and middle upward movement of the eye as well as adduction and convergence. Please note a distinction about adduction and convergence. Convergence is the disjoint movement of both eyes towards the middle, whereas, adduction is defined when the pupil is directed towards the middle and is accommodated with abduction of contralateral side during bilateral horizontal gaze. Bilateral horizontal gaze is a coordinated lateral eye movement which involves coordination between the abducens and the oculomotor nerve.

Nuclei of CN VI contains at least three functional neuron types: motor neurons, internuclear neurons, and floccular-projection neurons. The motor neurons innervate the ipsilateral lateral rectus muscle to abduct the ipsilateral eye. The internuclear neurons cross the midline and travel via the MLF to connect to nucleus of CN III. CN III contracts the medial rectus of the contralateral side to adduct the contralateral eye and forming the anatomical basis for conjugate eye movements (Büttner-Ennever and Akert, 1981). The floccular-projection neurons belong to paramedian tract neurons (Büttner-Ennever and Horn, 1996; Horn and Adamczyk, 2012) which if their efferent or afferent inputs are damaged may contribute to a defect in gaze-holding (Nakamagoe *et al.*, 2000).

Supplemental Table 1. Criteria used to calculate clinical severity score. The scoring for the following categories: facial palsy, limited abduction/adduction, limited upgaze, clubfoot/other limb defects, and intellectual impairment ranged from 0 (least affected) to 3 (most affected). The scores for these subcategories were normalized to give the maximum score of 1 prior to calculating the clinical severity score. Additional neurological signs/symptoms were each given a score of 1 if present.

Group	Subgroup	Subject #	Facial palsy	Limited abduction/adduction	Limited upgaze	Club foot/limb defect	Club foot	Other limb defects	Intellectual impairment	Other neuro involvement	Other system involvement	Clinical severity score
Moebius Syndrome (MBS)	Initial MBS cohort (MBS_i)	1	3	2	0	0			0	0	0	1.67
		2	3	2	3	1	✓		0	0	0	3.00
		3	3	3	2	1	✓		0	1 Mirror movements	0	4.00
		4	2	3	2	0			0	2 Night terrors, ADHD	0	4.33
		5	3	3	0	1	✓		0	3 Mirror movements, night terrors, balance	0	5.33
	MBS + mirror movements (MBS_m)	6	3	3	1	1	✓		0	1 Mirror movements	0	3.67
		7	3	3	0	3		✓short 5th finger, B	0	1 Mirror movements	0	4.00
		8	2	3	1	1			0	1 Mirror movements	1 Short stature	4.33
		9	3	3	1	0			0	2 Mirror movements, sleep disorder	1 Syncopal episodes	5.33
		10	3	3	0	1	✓		0	2 Mirror movements, sleep disorder	1 Syncopal episodes	5.33
		11	3	3	0	1	✓		1	3 Mirror movements, sleep disorder, ADHD	0	5.67
		12	3	3	0	0			0	3 Mirror movements, night terrors, hearing loss	2 Cleft palate, contractures	7.00
	MBS + limb deformities (MBS_l)	13	2	3	0	3	✓	✓brachy syndactyly, B	0	0	0	2.67
		14	1	2	0	3	✓	✓brachy syndactyly B, absent thumb	0	0	1 Atrial septal defect	3.00
		15	3	2	2	2		✓ transverse R arm	0	0	0	3.00
		16	1	2	0	2		✓ brachydactyly L	1	2 Night terrors, ADD	0	4.00
		17	2	2	3	2		✓Poland, brachysyndactyly R	0	3 Night terrors, ADHD, seizures	1 Syncopal episode	7.00
		18	3	3	0	3		✓ transverse lower limbs, B	1	3 Mirror movements, hearing loss, syringomelia	1 Vocal cord paralysis	7.33
		19	3	3	1	3	✓	✓ brachysyndactyly B	2	4 Epilepsy, hearing loss, syringomelia, ASD	3 Adrenal insufficiency, tracheostomy, submucosal cleft	11.00
	MBS + other (MBS_o)	20	3	3	0	0			1	3 Dysautonomia, seizure, depression	2 Contractures, vocal cord paralysis	7.33
		21	3	3	0	1	✓		3	3 Night terrors, seizures, ASD	3 Growth hormone deficiency, hypothyroidism, cleft palate	9.33
Congential Facial Weakness (CFW)	Isolated CFW (iCFW)	22	2	0	0	0			0	0	0	0.67
		23	2	0	0	0			0	0	0	0.67
		24	3	0	0	0			0	0	0	2.00
		25	3	0	0	0			0	0	0	2.00
		26	3	1	1	0			0	0	1 Pneumothorax	2.67
	CCDD NOS	27	1	0	0	3		✓ oligodactyly, B	0	0	0	1.33
	Myopathic CFW	28	2	3	3	0			0	0	0	2.67
		29	3	2	1	1	✓		0	0	2 Contractures, cleft palate	4.33
		30	3	1	2	0			0	0	3 High arched palate, contractures, gastrointestinal immobility	5.00

ADD: Attention-Deficit Disorder, ADHD: Attention-Deficit/Hyperactivity Disorder, ASD: Autism Spectrum Disorder, B: Bilateral, CCDD NOS: Congenital Cranial Dysinnervation Disorders Not Otherwise Specified. R: Right, L: Left.

Supplemental Table 2: Information about a cluster with significant volumetric reduction in subjects with MBS_t compared to HC (FWE corrected $p < 0.01$)

Cluster Size	Voxel coordinates (study specific template)			ICBM-152 coordinates		
	x	y	z	x	y	z
23	97	104	35	-1	-39	-38

x, y, z represent right/left, anterior/posterior, and inferior/superior axes respectively.
Clusters of more than two voxels are shown.

Supplemental Table 3: Information about clusters corresponding to areas of significant increase in MD in subjects with MBS compared to HC (FWE corrected $p < 0.01$)

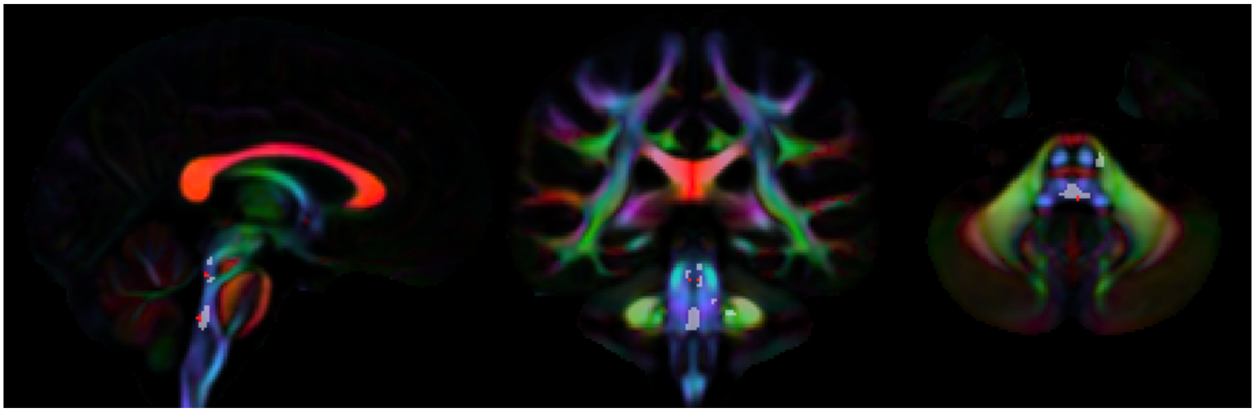
Cluster Size	Voxel coordinates (study specific template)			ICBM-152 coordinates		
	x	y	z	x	y	z
4	97	103	36	-1	-40	-37
3	97	105	51	-1	-37	-22

x, y, z represent right/left, anterior/posterior, and inferior/superior axes respectively.
Clusters of more than two voxels are shown.

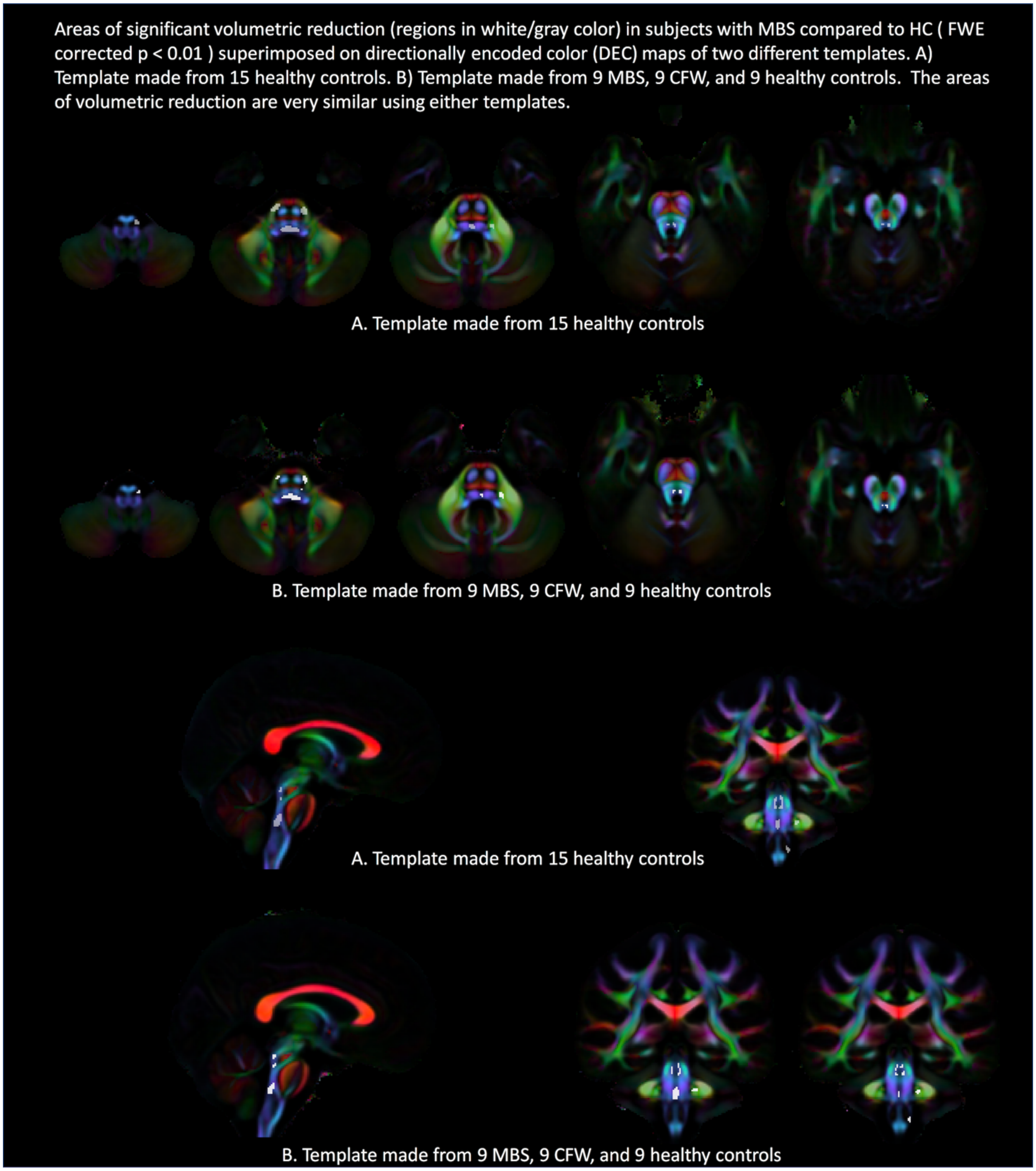
Supplemental Table 4: Information about clusters with significant volumetric reduction in subjects with MBS compared to HC (FWE corrected $p < 0.01$)

Cluster Size	Voxel coordinates (study specific template)			ICBM-152 coordinates		
	x	y	z	x	y	z
202	96	104	36	0	-39	-37
76	104	115	34	-10	-28	-41
48	88	119	33	9	-24	-42
26	102	108	21	-6	-39	-57
24	98	107	55	-2	-35	-18
21	107	107	39	-13	-37	-35
17	109	118	42	-14	-25	-33
16	94	107	49	3	-36	-24
13	103	105	44	-6	-37	-30
3	99	102	24	-3	-43	-53

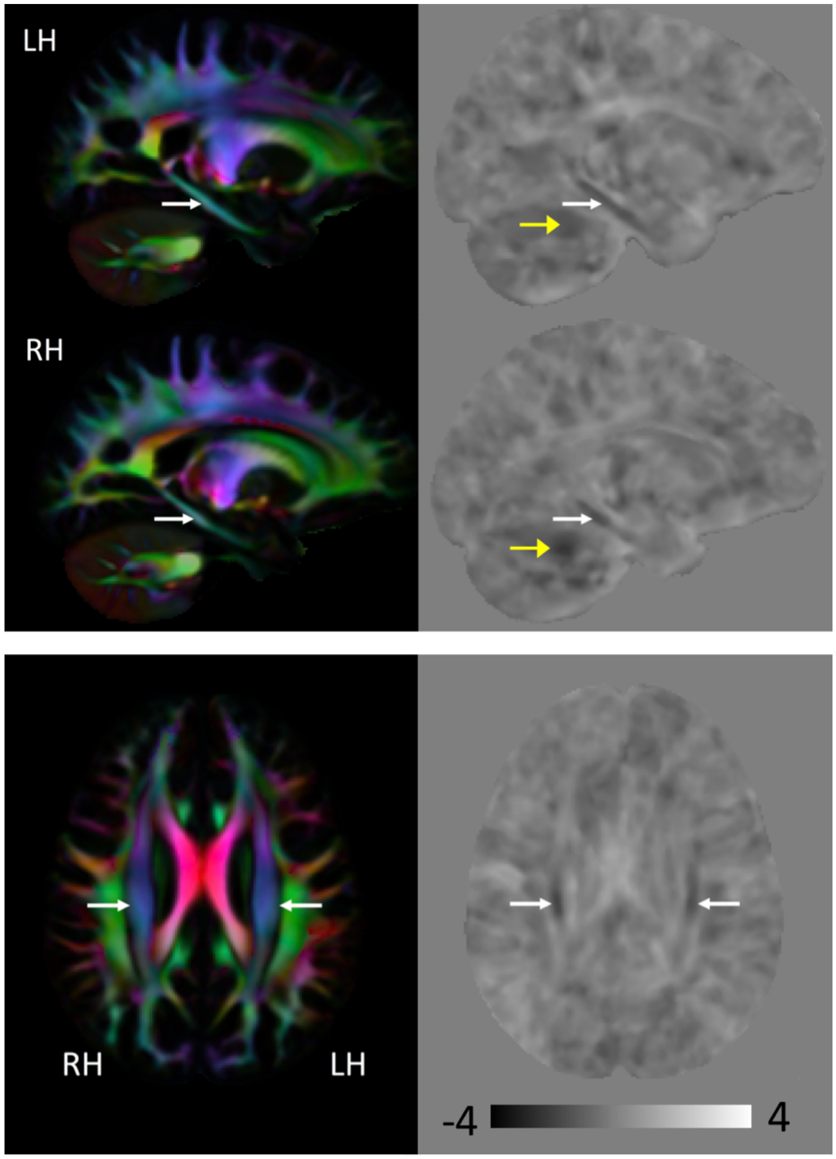
x, y, z represent right/left, anterior/posterior, and inferior/superior axes respectively.
Clusters of more than two voxels are shown.



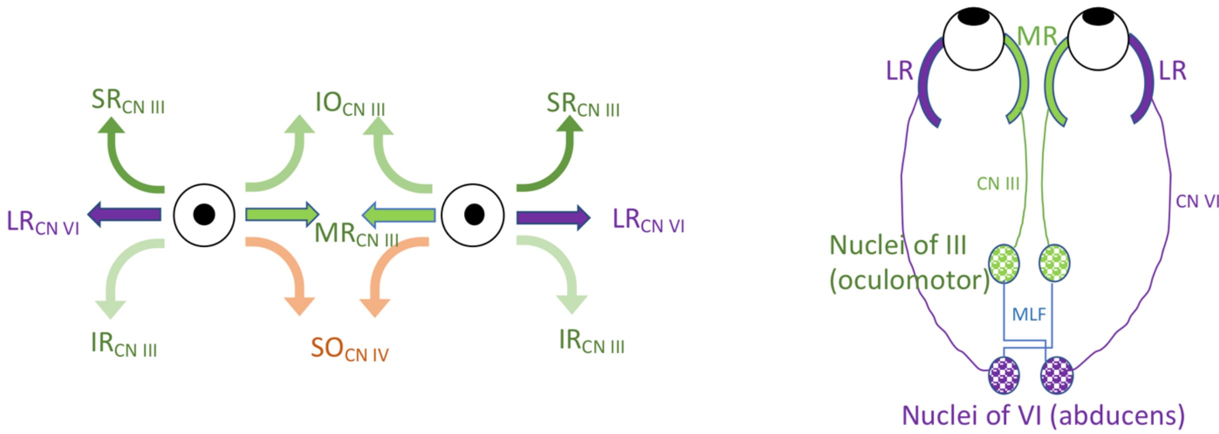
Supplemental Figure 1. Areas of significant volumetric reduction (shown in gray) and increase in MD (shown in red) in subjects with MBS compared to HC (FWE corrected $p < 0.01$) superimposed on the directionally encoded color (DEC) map.



Supplemental Figure 2. Effect of using different study-specific templates on the DTBM results.



Supplemental Figure 3. Top: Volumetric reduction in the parahippocampal portion of cingulum (indicated by the white arrows) and lobules V and VI of the cerebellum (yellow arrows) in subjects with MBS compared to HC. Bottom: Volumetric reduction in a specific region of the centrum semiovale (indicated by the arrows) in subjects with MBS compared to HC. Left: Directionally encoded color (DEC) map. Right: Effect size of lnJ maps comparing MBS subjects to HC. Dark areas indicate regions that are smaller in MBS subjects, whereas bright areas indicate areas that are larger in these subjects. In the effect size map black corresponds to -4, white to +4, and the gray background is equal to 0. LH: left hemisphere, RH: right hemisphere.



Supplemental Figure 4. Left: schematic diagram of muscles and cranial nerves involved in eye movement. Right: schematic diagram of cranial nerves and muscles involved in conjugate horizontal gaze. SR: superior rectus, IO: inferior rectus, LR: lateral rectus, IR: inferior rectus, SO: superior oblique, MR: medial rectus. Motor neurons of CN VI project ipsilaterally to LR to abduct the eye, whereas the interneurons cross the midline via medial longitudinal fasciculus (MLF) and project contralaterally to MR to adduct the eye.

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