APPENDIX I: Assay methodologies

Euroimmun, Lubeck, Germany: Slide preparation

HEK293 cells were seeded on sterile cover glass and transfected (ExGen500, Fermentas, Germany) with plasmids encoding one of the following: human AQP4 (pEP7-M1-AQP4, pTriEx-1-M1-AQP4, pTriEx-1-M23-AQP4), MOG (pTriEx-1-MOG) or an empty vector (pTriEx-1). After cultivation at 37 °C for 48 hours, cells were washed in PBS, fixed with formalin, and air-dried. Using automated equipment, the cover glass was cut into millimetre-sized biochips that were fixed to microscope slides, with each reaction field containing a biochip from each of the transfections. Other sterile glass contains fixed rat or monkey tissue sections that are processed in a similar manner to create millimeter-sized biochips. Individual sera are then tested on mosaics of transfected HEK cells and/or animal tissue biochips.

Euroimmun Assay protocol:

All Euroimmun assays were performed according to the manufacturer's instruction. In brief, slides carrying the mosaics were incubated with patient samples (diluted 1:10, 1:32, 1:100, 1:320 and 1:1000 in PBS-Tween) for 30 min at room temperature. Subsequently, the slides were rinsed with a flush of PBS-Tween and incubated in PBS-Tween for 5 minutes. Bound antibodies were detected by adding fluorescein isothiocyanate (FITC)-labelled goat anti-human IgG for 30 minutes at room temperature. After washing in PBS-Tween as described before, the slides were embedded in mounting medium, and evaluated by fluorescence microscopy (Axio Imager.A1, Zeiss, Germany). Positive and negative controls were included in each analytical run. Samples showing reactivity at a dilution of 1:10 were considered positive (borderline results were evaluated as positive). Endpoint titers represent the dilution factor for which specific fluorescence was just identifiable. Semi-quantitative evaluation was also performed. Endpoint titre: <10: sample negative, =10: sample '+' positive, 32-100: sample '++' positive, 320-1000: '+++' positive, >1000: '++++' positive.

Assay No. 4 (Commercial M23-AQP4 CBA; CBA-EI), Assay No. 6 (Commercial M1-AQP4 CBA; CBA-EI) Euroimmun, Lubeck, Germany

Research and Development slide with three biochips. Each biochip contains HEK cells transfected with: 1) AQP4-M1, 2) AQP4-M23 and 3) MOG. This slide provided

data for two assays: AQP4-M1 and AQP4-M23. The MOG biochip provided the negative control for the AQP4-M1 and –M23 assays.

Assay No. 5 (Commercial M1-AQP4 CBA; CBA-EI), Assay No. 19 (Commercial TBA; TBA-IIF) Euroimmun, Lubeck, Germany

NEUROLOGY MOSAIC 6 slide with 12 biochips. The first 6 biochips are tissue sections: 1) rat hippocampus, 2) rat cerebellum, 3) monkey cerebellum, 4) monkey nerves, 5) monkey intestine, 6) monkey pancreas. The second 6 biochips are HEK cells transfected with: 7) GAD65, 8) NMDAR (NR1a), 9) GABAR (B1/B2), 10) AQP4-M1, 11) LGI1, 12) CASPR2. This slide provided data for two assays. For the tissue based assay an overall score from the rat hippocampus, rat cerebellum and the monkey cerebellum was given to each sera. Separately an individual score of the M1-AQP4 transfected cells was given. The HEK cells transfected with other antigens were used as negative controls.

Assay No.s 7, 8, 9, 10, 12, 13

Commercial M1-AQP4 CBA (CBA-EO)

The anti-AQP4 slide (FA 1128-1005-50) was used in these centres. It contains two biochips per well: human M1-AQP4 transfected HEK cells and empty plasmid transfected HEK cell as a negative control.

Assay No. 11

Commercial M1-AQP4 CBA (CBA-EO)

The 'NEUROLOGY MOSAIC 17' slide (FA 1111-XXXX-117), was used by centre 9. It contains 5 biochips per well: 1) monkey cerebellum, 2) monkey cerebrum, 3) monkey optic nerve, 4) M1-AQP4 transfected HEK cells and 5) empty plasmid transfected HEK cells. Standard protocol was followed except the initial serum dilution was 1:60.

Reference:

Granieri L.*, Marnetto F.*, Valentino P., Frau J., Patanella A.K., Nytrova P., Sola P., Capobianco M., Jarius S., Bertolotto A. (2012) Evaluation of a multiparametric immunofluorescence assay for standardization of neuromyelitis optica serology. PLoS One. 7(6):e38896

Assay No. 1

Live M23-AQP4 CBA

HEK-293A cells were grown in 75cm^2 flasks (BD Biosciences, #353136) using DMEM culture medium containing 4.5g/l glucose, 10% FCS, 4mM L-glutamine (Life Technologies, #41965-039, #11140-035 and #10270) and passaged every 3 days. HEK-293A cells were seeded into 96-well cell culture plates (TPP Switzerland, #92096) at a density of 10.000 cells/150µl/well for AQP4 testing using the culture medium mentioned above. 24 h after seeding, transfection was performed using pcDNA6.2-hAQP4-M23-C-EmGFP and the transfection reagent Fugene HD (Promega, #E2312). Cells were maintained for 3 days in a humified incubator until commencing the live-cell staining immunofluorescence (IF) test. Plasmid construction for pcDNA6.2-hAQP4-M23-C-EmGFP is described elsewhere (Mader et al., 2011; Mader et al., 2010).

Live-cell staining immunofluorescence test for the detection of human antibodies directed against human Aquaporin 4 (AQP4)

Sera were first screened at a dilution of 1:20 and 1:40 using assay buffer (10% FCS heatinactivated, Sigma # F0804 in PBS, Sigma #P3813) as a diluent. After detection of a positive signal during the screening procedure, sera were retested using serial dilutions in two-fold steps starting at 1:20 (1:20, 1:40, 1:80 etc.). End-point titer levels were defined by disappearance of specific signal.

Sera showing strong background reactions were preabsorbed using acetone liver powder from calf produced in-house according to Coons et al (Coons et al., 1955). Briefly, sera were diluted 1:10 in assay buffer and consecutively incubated with 20mg, 15mg and 10mg of calf acetone liver powder. Incubation included two of the three steps for 1h at room temperature and one step at 4°C overnight on a rotator. Plates were removed from the incubator and the culture medium was replaced by blocking solution, consisting of 0.2 µg/ml goat IgG (Sigma, # I5256) in assay buffer. After 10 minutes, diluted samples were added, followed by an incubation for 1h at 4°C. Thereafter, cells were carefully washed three times using assay buffer, followed by incubation with the secondary antibody (Cy 3^{TM}-conjugated anti-human IgG, Jackson ImmunoResearch #109-166-088, 1:300 (AQP4) in assay buffer) for 30 minutes at room temperature. Cells were washed three times with assay buffer and finally DAPI (0.1 µg/ml in assay buffer, Sigma #D8417) was added to exclude dead cells. Screening and determination of titer levels was performed by two investigators,

both using individual result sheets and being blinded to the results of the other. Concordance rate was 100%.

Assay No. 21

M1-AQP4 ELISA (Aquaporin-4 Ab V2; Iason)

Additionally to the IF testing, samples were assayed using the commercially available ELISA EIASON^{R} Aquaporin-4 Ab V2 (lot 2KAQE 4, by IASON GmbH, Austria) according to the manufacturer's instruction. Iason is the Austrian distributor of the RSR AQP4 ELISA (RSR, UK). For the whole analysis five plates were used, all of which were from the same lot. On consecutive days, 2 assays per day were performed. No significant difference in control samples performance was observed between the plates (results see below). All reagents and plates were brought to room temperature. Sera were thawed, mixed and centrifuged for 5 min at 10.000 g. Lyophilized reagents were reconstituted and concentrated solutions diluted exactly as described. Briefly, 50µl/well of the calibrators (CAL1-5), controls (CO1, CO2, NC) and test sera (undiluted) were added to the plate in duplicate, leaving two wells empty for blank (BLANK). This was followed by the immediate addition of 25µl/well AQP4 Biotin ("BIO"), except for the BLANK wells. This step was performed using a multi-channel pipette. The plate was covered and incubated for 2 hours at room temperature on a ELISA plate shaker (IKA, MTS 2/4 digital). After incubation, wells were washed 3 times (300µl/well) using an automated plate washer (HydroFlex, Tecan). The plate was tapped on a dry absorbent to remove residual liquid and 100µl/well of Streptavidin peroxidase ("SAPOD") were added (except BLANK wells), followed by an incubation of 20 min at room temperature on a ELISA plate shaker. After another three washing steps and removal of droplets, 100µl/well substrate ("SUB") was added and the plate left for 20 min in the dark at room temperature. Thereafter, 100µl/well Stop Solution ("STOP") was added and the plate immediately mixed on a shaker for a few seconds to fully distribute the stop solution in the wells. Measurement of absorbance (450nm, reference wave length 620nm) was performed within 2 min after stopping the reaction (Beckman Coulter, DTX880 multimode detector).
Duplicate OD-values of samples/calibrators/controls were within the expected range and therefore all calculations were performed with the mean value. Calculation of units/ml (U/ml) was performed with a polynomic calibration curve using CAL1-4 (R^2=1 in all plates assayed). These calibrators showed acceptable OD_{450nm}-value

behavior in terms of slope compared to the manufacturers values. CAL 5 was excluded from the calibration curve due to obviously lower OD value behavior. Therefore, samples higher than CAL 4 were additionally calculated with a point-to-point calibration curve. However, as the goal was to determine positivity of samples, the exact U/ml in such highly positive samples was not considered important for final analysis. Although the polynomic curve was excellent for the critical unit range around 3 U/ml, we decided to compare a different calculation approach to exclude possible differences concerning positivity/negativity of samples. Therefore, samples showing borderline values were additionally calculated using a point-to-point calibration curve. However, no relevant discrepancies in borderline values were detected. Final results were determined according to the manufacturer‘s cut-off value at 3 U/ml. Provided control samples were always within range: CO1 (quality control certificate: 12±4.0 U/ml): 9,5 (±0,4) U/ml, CO2 (quality control certificate: 28±8 U/ml): 25,5 (±1,2) U/ml

Assay No. 16

M23-AQP4 flow cytometry

Human embryonic kidney cells HEK293A (ATCC, LGC Standards GmbH, Wesel, Germany) were seeded in 3ml of DMEM medium with 4.5 g/l glucose (Gibco, Invitrogen #41965-039), 1% non essential amino acid (NEAA, Gibco # 11140-035), 200mM L-glutamine (Gibco #11140-035) and 10% fetal calf serum (FCS, Gibco #10270) in 6-well plates (TPP Switzerland, #92006) at a density of $4x10^5$ cells per well. Approximately 24 hours later, cells were transiently transfected with full length M23 human AQP4 protein fused at the C-terminus with EmGFP protein (Mader et al., 2010). CD2 fused to EmGFP transfected cells served as control. Cells were transfected with FuGENE HD (Promega E2312):DNA ratio of 3.5:1 according to manufacturer`s protocol. Briefly, 18µl of FuGENE HD was added to 153µl pre-warmed OptiMEM (Gibco) using 4.95µg of AQP4 plasmid DNA and 3.3µg of CD2 plasmid DNA. Subsequently, solutions were vortexed briefly and incubated for 5 minutes to allow complex building. 150µl DNA-Fugene complex was added to each well. Cells were kept under optimum growth conditions at 37°C and 5% CO2 in a cell incubator. Two days post transfection cells were harvested and FACS analysis was performed. Therefore, the medium was removed and cells washed with 3 ml PBS (Sigma #P3813). Cell layer was detached using 150µl of 0.05 % trypsin-EDTA

(Gibco, #25300-054). Trypsinization was stopped by adding 1 ml medium to the cells and the suspension was transferred to a 50 ml falcon tube and centrifuged at 500 g for 5 minutes at room temperature (RT). After removing the supernatant, the cell pellet was resuspended in 10ml FACS buffer (PBS + 10% FCS + 1mM EDTA) supplemented with 4µg goat IgG (Sigma, I5256) and blocked for 20 minutes by shaking carefully at RT. Meanwhile serum dilutions were prepared. Therefore, each serum sample was diluted 1:50 in 500 µl FACS buffer. Control serum samples (K1: AQP4-IgG titer 1:1280; K2: AQP4-IgG titer 1:80; K3: AQP4-IgG negative) were included in each FACS analysis. Serum was plated on a round-bottom 96-well plate (TPP Switzerland, #92097), 100µl of serum dilution per well and duplicates were used for all samples. After blocking, cells were centrifuged (500g, 5 min) and the cell pellet was resuspended in 10ml FACS buffer. For cell counting, 50µl trypan blue solution (Sigma-Aldrich) and 30µl PBS were mixed with 20µl cells. A Neubauer counting chamber (Glaswarenfabrik Kerl Hecht) was filled with 20µl of the cell mixture and viable cells were counted four times in the 4x4 squares of the chamber. Cells / ml = total viable cells counted in 4 squares / 4 x dilution factor (50) x 10,000. Approximately, 150,000-200,000 cells in 100µl FACS buffer were added to the serum dilutions (reaching the final serum dilution of 1:100) in each well. Cell/serum mixture was in incubated for 60 minutes by shaking carefully at room temperature and the samples were protected from light exposure. Subsequently, cells were washed with FACS buffer three times by centrifugation for 4 minutes at 500g and decanting the supernatant carefully. Cells were incubated for 30 minutes with secondary antibody 1:100 diluted Allophycocyanin-conjugated AffiniPure F(ab`)2 Fragment Goat Anti-Human IgG (H+L) (Dianova, # 109-135-098) by shaking at room temperature in darkness. Subsequently, cells were washed with FACS buffer three times by centrifugation for 4 minutes at 500g and decanting the supernatant carefully. Finally, cells were diluted in 100µl FACS buffer and transferred to 500µl PCR soft tubes (Biozym) supplemented with 3µl Via-Probe (7-AAD, BD Biosciences # 555816). FACS analysis was performed with the BD Accuri C6 flow cytometer and the BD Accuri C6 software using the following gates (Figure 1):

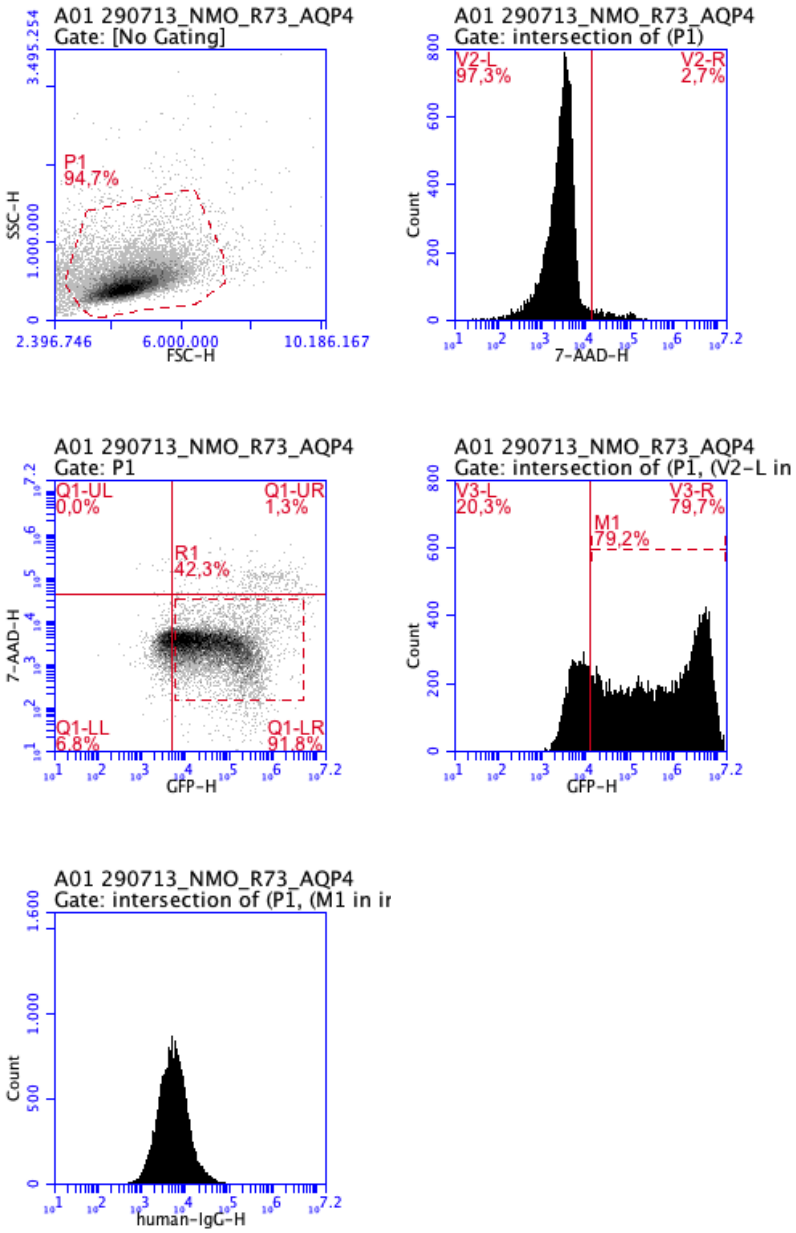


Figure 1: P1 gate for HEK293A cells. V2-R cells stained with 7-AAD as death cell marker are excluded from further analysis. Record gate R1 shows EmGFP positive cells. 10,000 events were recorded. M1 shows percentage of transfected cells. Threshold is set at 104 defined by Fugene transfected cells without plasmid DNA. Cell count of APC Goat Anti-Human IgG as intersection of P1 and M1).

Assay qualification and statistical analysis

Precision was determined by running all samples in duplicates in order to calculate the intra-assay variability. Samples we replicated if the coefficient of variation was higher than 15%.

The binding ratio was calculated by subtracting the mean value of the median of the CD2 transfected cells from the mean value of the median of the AQP4 transfected cells. From 143 controls from Medical University of Innsbruck (56 patients with multiple sclerosis, 40 patients with other neurological diseases and 47 healthy controls) a cut-off value of a delta median MFI of 12539 (100% specificity) was established which corresponded to the delta median MFI value of the K2 control serum sample in the same experiment. To avoid inter-day variability the cut off was therefore set by the K2 control serum sample in each serial data set. Therefore, the binding ratio of each sample was divided by the binding ratio of the K2 sample. For the quantitative analysis, all samples with a value above 1.0 were considered as positive.

4. Cut-off values and semi-quantitative data for all assay used

	IF AQP4-IgG	IF MOG-IgG	FACS AQP4-IgG	ELISA AQP4-IgG
Cut-off	1:20	1:160	1	3
-	<20	<1:160	<1	<3
+	20-80	160-320	1-4	3-20
++	160-640	640-1280	4-8	20-40
+++	1280-2560	2560-5120	8-16	40-80
++++	>2560	>5120	>16	>80

References

Coons, A.H., Leduc, E.H., Connolly, J.M., 1955. Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. J Exp Med 102, 49-60.

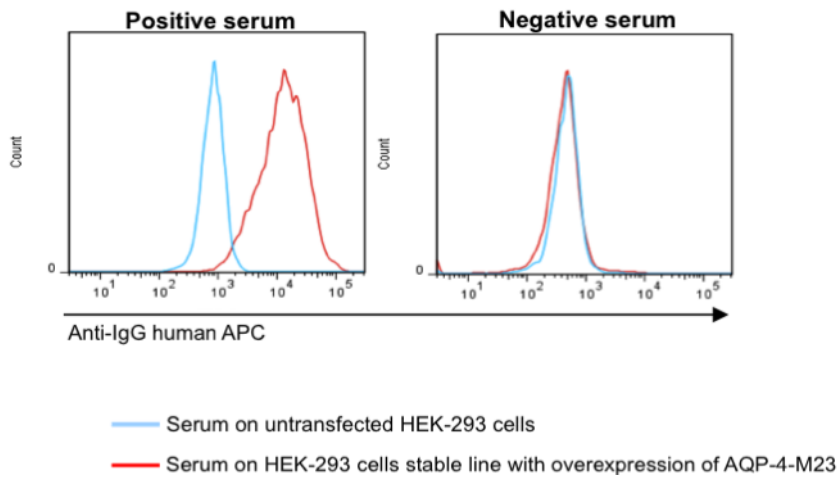
Mader, S., Gredler, V., Schanda, K., Rostasy, K., Dujmovic, I., Pfaller, K., Lutterotti, A., Jarius, S., Di Pauli, F., Kuenz, B., Ehling, R., Hegen, H., Deisenhammer, F., Aboul-Enein, F., Storch, M.K., Koson, P., Drulovic, J., Kristoferitsch, W., Berger, T., Reindl, M., 2011. Complement activating antibodies to myelin oligodendrocyte glycoprotein in neuromyelitis optica and related disorders. J. Neuroinflamm. 8, 184.

Mader, S., Lutterotti, A., Di Pauli, F., Kuenz, B., Schanda, K., Aboul-Enein, F., Khalil, M., Storch, M.K., Jarius, S., Kristoferitsch, W., Berger, T., Reindl, M., 2010. Patterns of Antibody Binding to Aquaporin-4 Isoforms in Neuromyelitis Optica. PLoS One 5, -.

Assay No. 15

M23-AQP4 Flow cytometry

HEK293 cells stably transfected with human M23-AQP4 and untransfected HEK cells were detached from T75 flask with 5 mL accutase (PAA L11-007) for 5 min at 37^{o}C. Each sera is tested on 100,000 cells. Cells were washed with DMEM (DMEM Life technologies 41965062) with 10% FCS (PAA A15-104) and centrifuged for 5 min at 1400 rpm at 20^{o}C. The supernatant was removed and the cells were blocked with 500 µl of PBS with 8% normal goat serum (NGS; Vector Lab S1000) for 30 min at RT. Cells were washed again, resuspended and aliquoted to wells of a 96 well plate (100,000 cells/20 µl). Centrifuge sera for 10 min at 10,000 rpm at 4^{o}C. Dilute sera 1:50 in PBS with 8% NGS, and add 20 µl to each well giving a final dilution of 1:100. Incubate for 20 min at 4^{o}C. Wash cells twice with 100 ul of DMEM. Centrifuge for 5 min at 1400 rpm at 20^{o}C. Fix cells in PBS with 1% PFA (PFA 20% Elect Micro Sciences 15713-S) for 15 min at RT. Centrifuge for 5 min at 1400 rpm at 20^{o}C, remove supernatant and add 50 ul goat anti-human IgG Allophycocyanin (APC; Jackson IR 109-136-170) diluted 1:100 in PBS with 8% NGS for 20 min at RT in the dark. Wash cells with 100 ul PBS, centrifuge for 5 min at 1400 rpm at 20^{o}C, resuspend in 100 ul PBS and keep at 4^{o}C until flow cytometry analysis (FACS BD Canto II). Each experiment contains one negative serum and two different positive sera. A serum is considered positive when the APC MFI of a patient's serum on the M23-AQP4 stable HEK cells is twice the APC MFI of the same sera on untransfected HEK cells.



Assay No. 17

M1-AQP4 Flow cytometry assay

LN18-AQP4 or LN18-CTR cells were diluted in RPMI-1640 growth medium and added to wells of a 96-well plate (20,000 cells/20 µL) that contained 20 µL of NMO-IgG–positive serum (1:100). The plates were incubated on ice for 25 min on an orbital shaker. Cells were then washed twice with FACS buffer (PBS containing 1% fetal bovine serum), resuspended in FACS buffer containing alexa-fluor 488 goat anti-human IgG (H+L) antibody (diluted 1:100) and incubated on ice for 25 min. Cells were washed twice, resuspended in 175 µL of FACS buffer, and transferred into FACS tubes (BD Biosciences). Cells were analyzed with a CyAn ADP high-performance Flow Cytometer (Dako Corp, Glostrup, Denmark). The median fluorescence intensity (MFI) of an individual sera binding to the control cells was subtracted from the MFI of the M1-AQP4 stable line to generate a ΔMFI for each serum.

Assay No. 14

M23-AQP4 Flow cytometry assay

Human embryonic kidney cells (HEK293T) were cultured in IMDM medium (Bio Whittaker, Verviers, Belgium) containing 10% fetal calf serum (Bodinco, Alkmaar, The Netherlands), penicillin 100 U/ml / streptomycin 100 ug/ml (Gibco, Merelbeke, Belgium), and 50 µM 2-mercaptoethanol (Sigma-Aldrich, Steinheim, Germany). Plasmid containing human AQP4-M23 complementary DNA and AcGFP (M23-pIRES2- AcGFP1) was kindly provided by Dr. Patrick Waters (University of Oxford, Oxford, England). HEK293T cells were transiently transfected with M23-pIRES2-AcGFP1 plasmid using standard transfection techniques. Two days after transfection, AQP4-transfected HEK293T cells were harvested after trypsinization and washing

into ice-cold PBAE buffer (PBS/0.5% BSA (vol/vol, Celliance, Kanakee, Illinois, USA) /0.1% azide /1 mM EDTA (all from Sigma) containing 10% (vol/vol) normal goat serum (NGS, Sanquin, Amsterdam, The Netherlands). AQP4-transfected cells (100,000 per well) were incubated with patient or control samples (final sample dilution1:20 in ice-cold PBAE buffer) in 96 wells round-bottom microtiter plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) for 30 min on ice. After washing thrice with PBAE cells were incubated with goat anti-human IgG Allophycocyanin (APC) conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Brunschwig Chemie B.V., Amsterdam, The Netherlands) at 1:500 in PBAE buffer containing 10% NGS for 25 min on ice. Cells were than washed 4 times in PBAE and analyzed immediately by FACS. Binding of human IgG was determined by measuring APC fluorescence after setting an acquisition gate of 10,000 events on AcGFP-positive cells (cut-off: mean fluorescence intensity 20,000). To correct for non-specific staining of HEK293T cells, the APC-channel MFI from non-transfected/weakly transfected GFP-negative cells was subtracted from that of AQP4-transfected cells for each individual sample.

In each assay, two positive control sera and 8 individual negative control sera (healthy lab workers) were tested. Anti-AQP4 IgG was considered positive if the ΔMFI of a sample was higher than the assay cut-off value (assay cut-off = average ΔMFI + 10 standard deviations of 8 individual negative control sera).

Assay No. 18

Tissue Based Assay (TBA-IHC)

Adult female Wistar rats that were sacrificed in a CO2 chamber and followed the protocol described previously. Briefly, non-perfused rat brains were removed, sagittaly sectioned and fixed for 1h in 4% paraformaldehyde at 4°C. Subsequently, brains were cryoprotected with 40% sucrose for 48 hr, embedded in freezing medium, and snap frozen in isopentane chilled with liquid nitrogen. Seven micron thick cryostat-cut sections were defrosted for 20 minutes, washed once with PBS and then incubated with 0.3% hydrogen peroxide for 15 minutes. After washing 3x with PBS, slides were incubated with 5% goat serum in PBS for 1 hour, and then labeled with patients´or control sera (1:200, diluted in 5% goat serum) at 4°C overnight. The next day, sections were washed 3x in PBS, labeled with biotinylated goat antihuman IgG (Vector lab) (1:2000, diluted in 5% goat serum) for 2h, washed 3x in PBS, and

incubated with avidin-biotin peroxidase for 1h at room temperature. The reactivity was developed with diaminobenzidine (Vector lab) for 7 minutes.

Reference

Höftberger R, Sabater L, Marignier R et al. An optimized immunohistochemistry technique improves NMO-IgG detection: study comparison with cell-based assays. PLoS One 2013;8:e79083

Assay No. 3

Live M23-AQP4 Cell Based Assay

Thirty-six hours after transfecting HEK293 cells with human M23-AQP4 (a gift from Dr. R. Marignier) live cells were incubated at RT with centrifuged serum 1:20 (diluted in DMEM with HEPES and 1% bovine serum albumin) for 30 minutes. After removing the media and washing with the same buffer, HEK cells were fixed with 1% paraformaldehyde for 15 minutes at RT and permeablized with 0.3% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA). HEK cells were then immunolabeled with a rabbit polyclonal anti-AQP4 antibody (1:500; Sigma-Aldrich) for 1 hr at RT, followed by the corresponding Alexa Fluor secondary antibodies against human and rabbit IgGs (1:1000; Molecular Probes, Invitrogen, Eugene, OR, USA).

Reference

Höftberger R, Sabater L, Marignier R et al. An optimized immunohistochemistry technique improves NMO-IgG detection: study comparison with cell-based assays. PLoS One 2013;8:e79083

Assay No. 20

Tissue Based Assay (TBA-IIF)

Snap frozen mouse cerebellum, hippocampus, kidney and liver were cut at 7 μM, washed once with PBS, fixed with 4% PFA for 1 hr, washed thrice with PBS and incubated with 10% normal goat serum (NGS) in PBS (blocking buffer) for 1 hr at RT. Serum was incubated with the sections at a dilution of 1:60 in blocking buffer overnight at 4ºC. Commercial rabbit anti-AQP4 polyclonal antibody (AB3594 Chemicon International Inc) diluted 1:200 and incubated with sections for 1 hr at RT was used as a positive control. Tissue incubated without a primary antibody was used as a negative control. The sections were washed thrice in PBS and incubated with goat

anti-human IgG-FITC (Southern Biotechnology Associates) for 1 hr at RT. After a final wash (x3 with PBS), the tissue was mounted in Vectashield mounting medium and stored at 4ºC before reading. The tissue was scored as negative, equivocal or weak-strong positive. A positive staining pattern required a minimum of stain on cerebellar pia, midbrain pia or capillary stain with or without kidney distal collecting tubules.

Assay No. 2

Live M23-AQP4 CBA

HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS (TCS Cellworks Ltd, Buckingham, UK), penicillin G and streptomycin (each 100 units/mL; Invitrogen, Paisley, UK), at 37°C in an atmosphere of 5% CO2/95% air. Cells were plated at a density of $0.35x10^6$ cells/well on 3 poly-l-lysined cover slips per well of a six well plate. The next day the cells were transiently transfected with 3 µg of plasmid DNA mixed with 1.25 µL of 20% glucose, and 1.5 µL of PEI per well. After replacing the medium at 16 hours, the cells were grown for another 24-30 hr before use. Coverslips were moved to a 24 well plate and incubated with serum diluted 1:20 in 250 µl DMEM with 20 mM HEPES buffer (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) and 1% BSA for 1 hour at RT, washed 3 times with the same buffer, and fixed immediately with 3% formaldehyde in PBS for 15 minutes at RT. Cells were then rinsed twice and incubated with alexa fluor 568 goat anti–human IgG (Invitrogen–Molecular Probes, Paisley) at 1:750 in the same buffer for 45 minutes at RT. The coverslips were then washed 4 times in phosphate-buffered saline and embedded in vectashield mounting medium that contained DAPI (1:1000) to counterstain the nuclei. A sample was considered positive if it showed a typical surface stain on cells transfected with AQP4 and no stain on cells transfected with a control antigen.

Reference: Waters PJ, McKeon A, Leite MI, *et al.* Serologic diagnosis of NMO: a multicenter comparison of aquaporin-4-IgG assays. *Neurology* 2012; **78**: 665–71.