ABC-transporter gene-polymorphisms are potential pharmacogenetic markers for mitoxantrone response in multiple sclerosis

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Escalation therapy with mitoxantrone (MX) in highly active multiple sclerosis is limited by partially dose-dependent side-effects. Predictors of therapeutic response may result in individualized risk stratification and MX dosing. ATP-binding cassette-transporters ABCB1 and ABCG2 represent multi-drug resistance mechanisms involved in active cellular MX efflux. Here, we investigated the role of ABC-gene single nucleotide polymorphisms (SNPs) for clinical MX response, corroborated by experimental in vitro and in vivo data. Frequencies of ABCB1 2677G>T, 3435C>T and five ABCG2-SNPs were analysed in 832 multiple sclerosis patients (Germany, Spain) and 264 healthy donors. Using a flow-cytometry-based in vitro assay, MX efflux in leukocytes from individuals with variant alleles in both ABC-genes (designated genotype ABCB1/ABCG2-L(ow), 22.2% of patients) was 37.7% lower than from individuals homozygous for common alleles (ABCB1/ABCG2-H(igh), P < 0.05, 14.8% of patients), resulting in genotype-dependent MX accumulation and cell death. Addition of glucocorticosteroids (GCs) inhibited MX efflux in vitro. ABC-transporters were highly expressed in leukocyte subsets, glial and neuronal cells as well as myocardium, i.e. cells/tissues potentially affected by MX therapy. In vivo significance was further corroborated in experimental autoimmune encephalomyelitis in Abcg2−/− animals. Using a MX dose titrated to be ineffective in wild-type animals, disease course and histopathology in Abcg2−/− mice were strongly ameliorated. Retrospective clinical analysis in MX monotherapy patients (n = 155)
used expanded disability status scale, relapse rate and multiple sclerosis functional composite as major outcome parameters. The clinical response rate (overall 121 of 155 patients (78.1%)) increased significantly with genotypes associated with decreasing ABCB1/ABCG2-function [ABCB1/ABCG2-H 15/24 (62.5%) responders, ABCB1/ABCG2-(intermediate) 78/98 (79.6%), ABCB1/ABCG2-L 28/33 (84.8%), exact Cochran-Armitage test P = 0.039]. The odds ratio for response was 1.9 (95% CI 1.0–3.5) with each increase in ABCB1/ABCG2 score (from ABCB1/ABCG2-H to –I-, and –I to –L). In 36 patients with severe cardiac or haematological side effects no statistically relevant difference in genotype frequency was observed. However, one patient with biopsy proven cardiomyopathy only after 24 mg/m² MX exhibited a rare genotype with variant, partly homozygous alleles in 3 ABC-transporter genes. In conclusion, SNPs in ABC-transporter genes may serve as pharmacogenetic markers associated with clinical response to MX therapy in multiple sclerosis. Combined MX/GC-treatment warrants further investigation.

Keywords: multi-drug resistance transporter; escalation therapy; pharmacogenetics; experimental autoimmune encephalomyelitis

Abbreviations: ABC = ATP-binding cassette transporter; ATP = adenosine triphosphate; CNS = central nervous system; EAE = experimental autoimmune encephalomyelitis; EDSS = expanded disability status scale; GC = glucocorticoid; MSFC = multiple sclerosis functional composite; MX = mitoxantrone; SNP = single nucleotide polymorphism

Introduction

The anthrancenedione mitoxantrone (MX) is recommended as an escalation therapy for highly active multiple sclerosis in case of failure or intolerance to previous immunomodulatory treatment or as first line therapy for malignant multiple sclerosis (Rieckmann et al., 2004; Morrisey et al., 2005; Boster et al., 2008). This clinical restriction accounts for the risk profile of MX, most importantly cardiotoxicity, bone marrow depression, therapy related acute leukaemia and gonadal dysfunction (Cohen and Mikol, 2004). Cardiotoxicity with symptomatic congestive heart failure in ~0.1% of patients is typically related to higher MX doses, restricting its lifetime cumulative use to a maximum of 140 mg/m² of body surface area (Ghalei et al., 2002). However, early cardiotoxicity at much lower dosages and late manifestations even several years after cessation of therapy have been described (Morrisey et al., 2005; Paul et al., 2007). Thus, biomarkers that allow individualized risk stratification are urgently needed. In addition, response predictors that permit individualized dosing may potentially lead to longer treatment intervals, an important issue since optimal therapy after lifetime MX dosage is exhausted is still under debate (Boster et al., 2008).

ATP-binding cassette (ABC)-transporters protect cells from endo- or exogenous toxic substances by energy-dependent efflux against a concentration gradient (Schinkel and Jonker, 2003; Robey et al., 2007). Widely expressed, ABC-transporters play a major role in drug absorption, distribution and excretion. In the central nervous system (CNS), they influence the accumulation of different relevant pharmacological substances e.g. in brain cancer, infections, epilepsy and psychiatric diseases; and current efforts aim at inhibiting ABC-mediated drug efflux with the rationale to enhance drug efficacy (Loscher and Potschka, 2005; Hermann et al., 2006; Uhr et al., 2008). Multi-drug-resistance phenotype was first described for chemotherapy-resistant cancer cells overexpressing P-glycoprotein encoded by the ABCB1 gene, for which MX is a known substrate. Subsequent description of non-P-glycoprotein-mediated drug resistance to MX led to the discovery of a novel gene, amongst others initially termed MX-resistance gene, now assigned the name ABCG2 (Robey et al., 2007). On the basis of our observation of differential immune cell death of MX-treated multiple sclerosis patients ex vivo (Chan et al., 2005), we hypothesized that variable ABC-transporter function could be involved in differential MX susceptibility on a cellular level but also in individual patients. Here we set out to investigate the functional role of ABCB1- and ABCG2-single nucleotide polymorphisms (SNPs) in different multiple sclerosis populations. The predictive role of these SNPs for clinical treatment, response and side effects to MX in multiple sclerosis was examined in a retrospective fashion and further supported by experimental data. Additional proof in vivo was obtained in chronic experimental autoimmune encephalomyelitis (EAE), an established animal model reflecting major pathogenetic hallmarks of multiple sclerosis.

Materials and Methods

ABCB1 and ABCG2 genotyping in multiple sclerosis patients and healthy controls

Genotyping of 832 multiple sclerosis patients from three sites in Germany (Göttingen, n = 166; Rostock, n = 423, Berg, n = 194) and one site in Spain (Barcelona, n = 49) (Table 1) was performed after informed consent according to the Declaration of Helsinki and approval by local ethics committees. Control samples were obtained from blood donors of the local blood bank (Göttingen, n = 191) (von Ahsen et al., 2000) and volunteers (Göttingen, Bochum n = 73) without history of neurological disease. TaqMan™ PCR was performed for ABCG2 M212M (reference SNP rs2231137) and Q141K (rs2231142) using Platinum qPCR SuperMix-UDG (Invitrogen, Karlsruhe, Germany) on a 7500 Real Time PCR system (Applied Biosystems, Darmstadt, Germany). ABCB1 3435C→T genotyping (rs1045642) (whole cohort) and ABCB1 2677G→T (MX-treated cohort, rs2032582) was performed as described before (von Ahsen et al., 2001) and detailed in the supplementary table.
**Quantification of ABCB1 and ABCG2 mRNA expression from human cells and tissues**

Primary human adult glial cells (microglia, oligodendrocytes, astrocytes) of >95% purity were isolated from surgical resectates obtained from the treatment of nontumour-related intractable epilepsy as described before (Chan et al., 2003). Peripheral blood mononuclear cells (PBMC) were isolated using BD vacutainer CPT/Na-citrate (Becton Dickinson, Heidelberg, Germany). Human CNS frontal cortex total RNA was purchased from Ambion (Huntingdon, United Kingdom). cDNA from cardiac tissue was from explanted organs of heart transplant recipients, hepatocyte cDNA originated from the resection margin of liver tumours. Relative quantification of ABC mRNA expression using the ΔΔCt method employed RNA polymerase II mRNA as endogenous control. Human epithelial breast cancer cell line MCF7 (DSMZ, Braunschweig, Germany) with stable ABC mRNA expression served as calibrator sample (Robey et al., 2007). For quantification of ABCG2 mRNA-expression QuantiTect Primer Assay Hs_ABCG2_SG_1 was used (Qiagen, Hilden, Germany) with QuantiTect SYBR Green PCR kit. ABCB1-/-RNA polymerase II mRNA expression was investigated using Platinum qPCR SuperMix-UDG (Invitrogen) supplemented with SYBR Green (Molecular Probes, Eugene, OR, USA, see also supplementary table).

### In vitro MX efflux/cell-death assay

MX efflux was investigated in freshly isolated CD56+ NK-cells (>98% purity, magnetic cell sorting (MACS), Miltenyi Biotec, Bergisch Gladbach, Germany) following a modification of a previously described protocol (Hitzl et al., 2001). The assay is based on flow cytometric quantification of intracellular MX (Chan et al., 2005) in the presence or absence of specific inhibitors of ABC-transporters at established concentrations (Elacridar/GF120918, specific for ABCB1 and ABCG2, 2.5 μM, GlaxoSmithKline, Collegerville, PA), PSC-833/Valspodar, ABCB1-specific (2.5 μM, PKF215-833, Novartis, Basel, Switzerland), fumitremorgin C, ABCG2-specific (10 μM, FTC, Alexis Biochemicals, Lörrach, Germany) (Schinkel and Jonker, 2003; Robey et al., 2007). 600 000 cells (RPMI 1640/10% FCS) were pre-incubated (15 min, 37°C/5% CO2) with respective inhibitors followed by 1 μM MX (Gry Pharma, Kirchzarten, Germany, 30 min, 37°C/5% CO2), corresponding to the serum concentration after intravenous administration of 12 mg/m² MX (Hu et al., 1992). For MX efflux, washed cells were resuspended in 4 ml 37°C pre-warmed medium, with or without inhibitor and incubated for another 10 min (37°C/5% CO2). Glucocorticosteroid effects (GC, methylprednisolone/MP, Sanofi-Aventis, Frankfurt, Germany; dexamethasone/DEx, Ratiopharm GmbH, Ulm, Germany) on MX accumulation and cell death were investigated after pre-incubation (25–100 μM, 60 min) (Pavek et al., 2005) followed by MX efflux as indicated above in the presence of respective GC. After washing, propidium-iodide staining (PI, 0.1 μg/ml, Sigma-Aldrich, Steinheim, Germany) excluded cells with impaired plasma membrane integrity (typically <1.6%). A minimum of 5000 PI-negative cells were analysed using FACS Calibur/Cell Quest software (Becton Dickinson). Cell-death analysis (AnnexinV-FITC, PI, Roche Biochemicals, Mannheim, Germany) was performed after MX incubation (30 or 60 min) and MX efflux followed by overnight incubation (37°C, 5% CO2).

### Induction of experimental autoimmune encephalomyelitis, MX treatment and histology

All animal experiments were approved by responsible authorities in Lower Saxonia, Germany. Abcg2−/− animals were backcrossed to C57BL/6 background for at least eight generations, control mice were purchased from Harlan (Borchen, Germany). Mice were anaesthetized with ketamine-hydrochloride (Inresa, Freiburg, Germany) and immunized subcutaneously (sc) with 50 μg myelin oligodendrocyte glycoprotein MOG35-55 peptide in PBS, emulsified in an equal volume of complete Freund’s adjuvant (CFA) containing Mycobacterium...
tuberculosis* H37RA (Difco, Detroit MI, USA, 1 mg/ml). Pertussis toxin
(List Biological Laboratories, Campbell, CA, USA; 200 ng i.p.) was given
on days 0 and 2. Animals were weighed and scored daily in a blinded
fashion using the following clinical score: 0 normal, 1 reduced tail
tone; 2 limp tail, impaired righting; 3 absent righting; 4 gait ataxia;
5 mild paraparesis; 6 moderate paraparesis; 7 severe paraparesis/
paraplegia; 8 tetraparesis; 9 moribund; and 10 death. MX was injected
intravenously (iv) after disease onset on days 12–14 and 16 (0.5 mg/
kg body weight). Three-micrometre spinal cord paraffin cross-sections
from animals perfused on day 23 or 29 were stained with Luxol fast
blue. Demyelinated areas were calculated as percentage from total
white matter (minimal 4, in most cases six spinal cord sections per
animal) as analysed by Image Processing software on a Olympus
microscope.

Retrospective clinical correlation of genotype and MX response

Patient samples were genotyped for *ABCB1* 2677G>T, 3435C>T,
*ABCG2* V12M and Q141K and retrospectively correlated with clinical
MX response. Genotyping and assessment of clinical responses
were performed blinded from each other by different investigators.
All patients (Table 3) had been diagnosed according to Poser-/ 
MacDonald-criteria (Poser and Brinar, 2004) and had been treated
with MX following established protocols (Edan et al., 1997; Hartung
et al., 2002). Due to presumably different pathogenesis and lack of
therapeutic MX efficacy, primary progressive multiple sclerosis
patients were not included in the analyses (Stüve et al., 2004). One hundred
and fifty-five patients (Göttingen n = 11, Rostock n = 37, Berg n = 107)
had received MX monotherapy every 3 months following the MIMMS
protocol (Hartung et al., 2002). In this cohort, GC pulse therapies
were only given during exacerbations. One hundred and fifty-
four patients had undergone MX/GC combination therapy
(Göttingen/Rostock n = 57, Barcelona n = 46, Berg n = 51). Of these,
German MX/GC patients had received methylprednisolone 1 g i.v.
over 3–5 days in combination with MX every 3 months. Spanish
MX/GC patients had received 4 mg dexamethasone i.v. immediately
before three MX infusions every month, followed by three monthly
intervals. Decision for MX/GC combination was on clinical grounds,
e.g. continuous clinical activity or better tolerability. To assess therapy
response, at least one of the following criteria had to be fulfilled:
expanded disability status scale (EDSS) stability/improvement (in case of
non-responders: EDSS deterioration of 1 point (EDSS < 6.0) or 0.5
points (EDSS > 6.0)) (number of patients classified according to this
criterion n = 294, n = 165 confirmed at minimum 2 different time
points); improvement of relapse rate (n = 110); multiple sclerosis func-
tional composite (MSFC) stability/improvement (n = 10) or magnetic
resonance imaging activity (Gd-enhancement, n = 15); and lack of dis-
ease progression as determined by the physician (n = 157, of these 150
fulfilled at least one other criterion). For most of the patients, assess-
ment was based on several of these criteria. Where applicable, mag-
netic motor evoked potentials (MEP, assessment of central motor
conduction time, amplitude and configuration, n = 63) served to con-
firm assignment to response groups. Treatment response was analys-
ed after 12 months on treatment (89.3% of the patients). If sufficient
data was lacking at 12 months, data after 9 months treatment (10%)
was analysed. If neither data after 12 nor 9 months was available,
response was assessed at the end of treatment (21–24 months,
0.7% of patients). Patients with severe cardiac (n = 28, median cumu-
lative MX dosage 72 mg/m², 56–93; 25th–75th percentile) and
patients with haematological side effects (n = 8, therapy associated
leukaemia, agranulocytosis, median cumulative MX dosage 72 mg/
m², 72–72), were retrospectively correlated with ABC-transporter gen-
yotype. Of these, samples from 10 patients with MX-associated cardi-
oc toxicity were collected separately from the Berlin site. In this group,
one patient had developed a severe, biopsy-proven cardiotoxicity
already after 24 mg/m² (Dörr et al., 2009), three patients a significant
decrease of echocardiographic LVEF (≥10%), and six patients a dia-
static dysfunction.

Statistical analysis

Descriptive statistics were reported as median and 25th and 75th
percentile unless stated otherwise. Continuous data were compared
using the exact Wilcoxon test. Apoptosis rates in vitro were analysed
by one way analysis of variance using the post-hoc Student–Newman–
Keuls test for grouping. P-values were reported two-sided if not
reported otherwise. Cross-tabulation of research data often led to
cells with expected frequencies of <5. Therefore exact statistics were
used for inference. Fisher’s exact test was used for the 2 x 2 case,
the Fisher–Freeman–Halton test for the 2 x 3 case and the exact
Cochran–Armitage test for trend on genotype scores in the additive
model 0-1-2. Exact statistics were calculated as implemented in SPSS
16 for Windows. The exact-like odds-ratio for the association between
response/non-response and the genetic burden score (*ABCB1/
ABCG2-H/-I/-L*) was calculated in R 2.9.0 using the elrm package
(Zamar et al., 2007) with a Markov chain size of 2.5e6. The
Cochran-Armitage test reports the one-sided P-value because only
one direction of trend was of interest and plausible given the
in vitro and experimental autoimmune encephalomyelitis in vivo
results (Zheng et al., 2003; 2008). Inhibition of MX efflux by GCs
was tested using the Kolmogorov–Smirnov (KS) statistic D (KS D)
(Leith et al., 1999). To determine differences in experimental autoim-
mune encephalomyelitis course by Mann–Whitney U-test, analysis
was performed from onset of disease until the end of observation rather
than for individual time points.

Results

*ABCB1* and *ABCG2* are highly polymorphic in multiple sclerosis
populations of central European ancestry with no major differences to
healthy donors

Clinical data of the 832 multiple sclerosis patients genotyped are
given in Table 1. Of five different *ABCG2* SNPs with potential
functional significance investigated, only reference SNP (rs) 2231137
(G>A) leading to a V12M substitution and rs2231142 (C>A) resulting in
a Q141K substitution were observed. As detailed in Table 2, 14.8% multiple sclerosis patients were
homozygous for the common alleles both for *ABCB1* and
*ABCG2* (wild type). In contrast, 22.2% were at least heterozygous for
variant alleles in both *ABCB1* and *ABCG2*. Total 63.1% of
the patients were at least heterozygous for variant alleles in
either *ABCB1* or *ABCG2*. All SNPs were found to be in Hardy–
Weinberg equilibrium. For *ABCB1* 2677G>T and 3435C>T no
difference could be detected between the multiple sclerosis populations and healthy donors. For ABCG2, the frequency of variant allele V12-codon was very low with 0.03% in multiple sclerosis patients and 0.05% in healthy donors (\(P = 0.023\)), whereas no difference was observed for Q141. There was no difference in genotype frequencies between unselected multiple sclerosis cohorts (Gottingen, Rostock) and MX-treated multiple sclerosis cohorts (Barcelona, Berg). A high degree of linkage disequilibrium was found between ABCG2 V12M and Q141K [linkage D' = 0.854, (Gaunt et al., 2007)]. These findings demonstrate that potentially functionally variant alleles for ABCB1 and ABCG2 occur frequently in our multiple sclerosis cohorts without indication of a role in disease susceptibility.

### Table 2 Genotyping of multiple sclerosis patients and healthy controls for ABCB1 and ABCG2

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Healthy donors</th>
<th>Multiple sclerosis patients</th>
<th>P</th>
<th>HapMap data</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABCG2-12VV</strong></td>
<td>237 (89.8%)</td>
<td>784 (94.2%)</td>
<td>0.95</td>
<td>58 (96.7%)</td>
</tr>
<tr>
<td><strong>ABCG2-12VM</strong></td>
<td>26 (9.8%)</td>
<td>47 (5.6%)</td>
<td>0.010</td>
<td>2 (3.3%)</td>
</tr>
<tr>
<td><strong>ABCG2-12MM</strong></td>
<td>1 (0.4%)</td>
<td>1 (0.1%)</td>
<td>0.014</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Frequency of WT allele (SD)</td>
<td>0.95 (0.010)</td>
<td>0.97 (0.004)</td>
<td>0.023</td>
<td>58 (100.0%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>264 (100.0%)</td>
<td>832 (100.0%)</td>
<td>0.023</td>
<td>60 (100.0%)</td>
</tr>
<tr>
<td><strong>ABCG2-141QQ</strong></td>
<td>214 (81.1%)</td>
<td>658 (79.1%)</td>
<td>0.010</td>
<td>89 (78.8%)</td>
</tr>
<tr>
<td><strong>ABCG2-141QK</strong></td>
<td>46 (17.4%)</td>
<td>163 (19.6%)</td>
<td>0.010</td>
<td>23 (20.4%)</td>
</tr>
<tr>
<td><strong>ABCG2-141KK</strong></td>
<td>4 (1.5%)</td>
<td>11 (1.3%)</td>
<td>0.008</td>
<td>1 (0.9%)</td>
</tr>
<tr>
<td>Frequency of WT allele (SD)</td>
<td>0.90 (0.014)</td>
<td>0.89 (0.008)</td>
<td>0.746</td>
<td>60 (100.0%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>264 (100.0%)</td>
<td>832 (100.0%)</td>
<td>0.746</td>
<td>60 (100.0%)</td>
</tr>
<tr>
<td><strong>ABCB1-2677GG</strong></td>
<td>63 (30.7%)</td>
<td>105 (29.8%)</td>
<td>0.025</td>
<td>22 (19.5%)</td>
</tr>
<tr>
<td><strong>ABCB1-2677GT</strong></td>
<td>99 (48.3%)</td>
<td>178 (50.6%)</td>
<td>0.025</td>
<td>62 (54.9%)</td>
</tr>
<tr>
<td><strong>ABCB1-2677TT</strong></td>
<td>43 (21.0%)</td>
<td>69 (19.6%)</td>
<td>0.025</td>
<td>29 (25.7%)</td>
</tr>
<tr>
<td>Frequency of WT allele (SD)</td>
<td>0.55 (0.025)</td>
<td>0.55 (0.019)</td>
<td>0.856</td>
<td>60 (100.0%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>205 (100.0%)</td>
<td>352 (100.0%)</td>
<td>0.856</td>
<td>60 (100.0%)</td>
</tr>
<tr>
<td><strong>ABCB1-3435CC</strong></td>
<td>58 (22.0%)</td>
<td>187 (22.6%)</td>
<td>0.021</td>
<td>33 (29.2%)</td>
</tr>
<tr>
<td><strong>ABCB1-3435CT</strong></td>
<td>136 (51.5%)</td>
<td>414 (50.1%)</td>
<td>0.021</td>
<td>63 (55.8%)</td>
</tr>
<tr>
<td><strong>ABCB1-3435TT</strong></td>
<td>70 (26.5%)</td>
<td>225 (27.2%)</td>
<td>0.021</td>
<td>17 (15.0%)</td>
</tr>
<tr>
<td>Frequency of WT allele (SD)</td>
<td>0.48 (0.021)</td>
<td>0.48 (0.012)</td>
<td>0.939</td>
<td>60 (100.0%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>205 (100.0%)</td>
<td>352 (100.0%)</td>
<td>0.939</td>
<td>60 (100.0%)</td>
</tr>
<tr>
<td>Common alleles ABCB1 and ABCG2 (ABCB1/ABCG2-H)</td>
<td>28 (13.7%)</td>
<td>52 (14.8%)</td>
<td>0.834</td>
<td>60 (100.0%)</td>
</tr>
<tr>
<td>At least heterozygous for variant allele in either ABCB1 or ABCG2 (ABCB1/ABCG2-I)</td>
<td>135 (65.9%)</td>
<td>222 (63.1%)</td>
<td>60 (100.0%)</td>
<td></td>
</tr>
<tr>
<td>At least heterozygous for variant alleles in ABCB1 and ABCG2 (ABCB1/ABCG2-L)</td>
<td>42 (20.5%)</td>
<td>78 (22.2%)</td>
<td>60 (100.0%)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>205 (100.0%)</td>
<td>352 (100.0%)</td>
<td>0.834</td>
<td>60 (100.0%)</td>
</tr>
</tbody>
</table>

Data from the International HapMap CEU database are shown for comparison, n (%), Fisher's exact test for genotypic difference between multiple sclerosis patients and healthy controls.

High ABCB1-/ABCG2 mRNA expression in different cells/organs potentially affected by MX therapy

Analysis of ABC-transporter mRNA expression showed 3.9-fold higher ABCG2 expression of CD56⁺ cells than unsorted peripheral blood mononuclear cells and even more in comparison to CD19⁺ B-cells (Fig. 1A). High ABCG2 mRNA expression was also observed in human CNS-cells, especially human frontal cortical brain tissue (53-fold in comparison to peripheral blood mononuclear cells), oligodendrocytes (19-fold), and microglia (17-fold). Also human myocardium (16-fold in comparison to peripheral blood mononuclear cells) and liver tissue (76-fold) demonstrated high expression. As depicted in Fig. 1B, all investigated cells exhibited also high ABCB1 mRNA expression, with highest values in CD56⁺ cells, oligodendrocytes, liver and cortex.

**ABCBC1/ABCG2 genotype determines in vitro MX efflux and MX-induced cell death in multiple sclerosis patients and healthy donors**

As depicted in Fig. 2A, ABC-transporter-mediated MX efflux was compared in CD56⁺ cells of a multiple sclerosis patient with the common ABCB1/ABCG2 alleles (ABCB1-2677GG, -3435CC, ABCG2-12VV, -141QQ, wild type) with a patient carrying variant alleles in both genes (ABCB1-2677TT, -3435TT, ABCG2-12VM, -141QQ). Using the dual ABCB1-/ABCG2-inhibitor Elacridar wild-type sequences determined higher MX efflux in comparison to the latter genotype. The wild-type genotype was therefore designated...
ABCB1/ABCG2-IGH efflux in contrast to genotypes with at least heterozygous variant alleles in both genes, designated genotype ABCB1/ABCG2-L (low efflux). The same effect of genotype-dependent MX efflux was observed in healthy individuals (Fig. 2A), enabling us to investigate additional healthy controls after proof of principle in multiple sclerosis patients. Figure 2B depicts cumulative data for 12 individuals (six pairs of different genotypes) with 37.7% lower ABC-mediated MX efflux in ABCB1/ABCG2-L genotypes [mean fluorescence intensity (MFI) 164.3] than in respective individuals with -H genotype (MFI 263.5, P = 0.031). In preliminary experiments, individuals polymorphic in either ABCB1 or ABCG2 exhibited intermediate MX efflux [ABCB1/ABCG2-I (intermediate)]
In comparison to the dual ABCB1/ABCG2-inhibitor Elacridar, genotype dependent reduction of MX efflux was less pronounced using the ABCB1-selective inhibitor PSC-833 (P = 0.062, n = 5 pairs of different genotypes). No clear effect was observed using the ABCG2-specific inhibitor FTC alone (data not shown).

As depicted in Fig. 3A, increased intracellular MX accumulation in an ABCB1/ABCG2-L multiple sclerosis patient results in greater...
Figure 3 (A) ABCB1-/ABCG2 genotype determines MX-induced cell death in multiple sclerosis patients and healthy donors. CD56+ cells of an ABCB1/ABCG2-L multiple sclerosis (MS) patient demonstrate higher proportion of MX-containing, dying cells (MX+/AnnexinV+) than cells of an ABCB1/ABCG2-H multiple sclerosis patient. Similar results are obtained for healthy donors (HD) of respective genotypes. Twenty-four hours after MX efflux, representative experiments. (B) ABCB1-/ABCG2 genotype determines MX-induced cell death in multiple sclerosis patients and healthy donors. ABCB1/ABCG2-L gene carriers exhibit higher proportions of MX-containing, dying cells (MX+/AnnexinV+) than ABCB1/ABCG2-H genotype individuals (P<0.05, Anova). This is due to ABCB1/ABCG2 genotype, since inhibition of MX efflux in CD56+ cells of ABCB1/ABCG2-H gene carriers by dual ABCB1/ABCG2-inhibitor Elacridar leads to higher proportion MX+/AnnexinV+ cells (P<0.05) than in ABCB1/ABCG2-L patients (P=NS). Each genotype: 1 multiple sclerosis patient, 4 healthy donors (24 h after MX efflux). (C) GCs inhibit MX efflux. MX efflux from CD56+ cells of an ABCG2-/ABCB1-H healthy individual is inhibited by methylprednisolone (MP) or dexamethasone (DEX). Histograms of intracellular MX in the presence of MP or DEX (50 μM, black histogram) in comparison to MX efflux without GC (grey filled histograms). Light grey histogram: control without MX. Δ: difference of mean geometric fluorescence intensity representing intracellular MX concentration as calculated by efflux inhibited by GC minus non-inhibited efflux. D: Kolmogorov–Smirnov test statistics. One representative experiment of three (two healthy donors, one multiple sclerosis patient). *P<0.05.
proportion of cell death (MX+/AnnexinV+-cells) as compared to an ABCB1/ABCG2–/– multiple sclerosis patient. Again, similar effects were observed in healthy donors (Fig. 3A). Analysing one pair of multiple sclerosis patients and four pairs of healthy controls, individuals with ABCB1/ABCG2–/– genotypes had 22.9% more MX+/AnnexinV+ (Fig. 3B, P < 0.05) and 16.1% more MX+/PI+ double positive cells (data not shown) than individuals carrying the ABCB1/ABCG2–/+ genotype. Inhibition of MX efflux by dual ABCB1/ABCG2-inhibitor Elacridar led to increased cell death in ABCB1/ABCG2–/+ individuals by 21.9% (MX+/AnnexinV+ cells, P < 0.05), whereas this increase was only 3.5% in the L-genotype individuals (NS), arguing for differential susceptibility to MX-induced cell death dependent on respective ABCB1/ABCG2-transporter genotypes presumably via differential MX accumulation.

In clinical practice, MX is often combined with GCs (Morrissey et al., 2005). Thus, we next examined the influence of the GCs methylprednisolone (MP) and dexamethasone (DEX) on intracellular MX accumulation and MX-induced cell death. In CD56+ cells of individuals with ABCB1/ABCG2–/+ gene sequences, both methylprednisolone and dexamethasone significantly inhibited MX efflux (Fig. 3C). After 24 h the addition of methylprednisolone led to an increase of 8% MX+/AnnexinV+ cells in ABCB1/ABCG2–/+ gene carriers (n = 2). This increase was presumably due to inhibition of MX efflux and not to direct GC-mediated cell death, since only negligible changes (+0.52% MX+/AnnexinV+ cells) were seen in ABCB1/ABCG2–/– individuals (n = 2).

**Abc-transporter genotype determines therapeutic response to MX in experimental autoimmune encephalomyelitis**

To further elucidate functional significance of Abc-transporter genotype in vivo, treatment response to MX in Abcg2–/– mice was compared to respective wild-type animals in chronic MOG35–55 EAE. MX therapeutically administered after onset of disease was down-titrated to a dose just not efficient in wild-type animals as compared to respective sham-treated controls (Fig. 4A). In contrast, using this suboptimal MX dose, experimental autoimmune encephalomyelitis in Abcg2–/– mice was strongly ameliorated (P < 0.001). Effects on clinical course were also mirrored histologically with strongly reduced spinal cord demyelination in MX-treated

Figure 3 Continued.

Figure 4 (A) MX in suboptimal dose ameliorates clinical EAE in Abcg2–/– but not wild-type mice. Clinical disease course in C57Bl/6 wild-type (wt) animals is not altered using suboptimal MX dose (0.5 mg/kg body weight, i.v., Days 12–14 and 16, indicated by arrows) but strongly ameliorated in Abcg2–/– mice in comparison to respective PBS-treated controls. n = 9/10 for MX-treated Abcg2–/– and C57Bl/6 wt mice, n = 6 for PBS-treated Abcg2–/– controls. Data pooled from two independent experiments showing analogous results. NS: P not significant (wt MX versus wt PBS), ***P < 0.001 (Abcg2–/– MX versus Abcg2 PBS), Mann–Whitney U-test. (B) MX in suboptimal dose ameliorates demyelination in abcg2–/– EAE but not wild-type mice. Spinal cord demyelination (Luxol fast blue staining) in wt animals is not significantly altered using suboptimal MX dose, but strongly reduced in Abcg2–/– mice in comparison to respective PBS-treated controls. Data from two independent experiments, NS: P not significant, **P < 0.01, Mann–Whitney U-test.
Effects of ABC-transporter genotype on therapeutic response to MX

Of 353 fully genotyped MX-treated patients, data of 339 patients fulfilled strict assessment criteria for response classification. Given presumably different pathogenesis/MX response, PPMS patients were not included in the analyses. Thus, 309 MX-treated patients from four centres were retrospectively analysed using quantitative parameters such as EDSS, relapse rate and MSFC as main outcome parameters. Of patients from all cohorts (MX monotherapy, MX/GC combination therapy), 68.6% responded to MX, whereas 31.4% were classified as non-responders. Among patients receiving MX monotherapy (n = 155) 78.1% were responders (Table 3, panel A). In MX monotherapy patients carrying the ABCB1/ABCG2- H genotype had the lowest responder rate (15/24, 62.5%), carriers of variant alleles in either ABCB1 or ABCG2 (ABCB1/ABCG2-I) exhibited an intermediate response (78/98, 79.6%), whereas 28/33 (84.8%) with ABCB1/ABCG2-L genotype were MX responders (P = 0.039). Odds ratio for positive treatment response between ABCB1/ABCG2-H and -I-genotype was 1.9 (95% CI 1.0–3.5) and accordingly 3.5 between ABCB1/ABCG2-H and -L-genotype. As shown in Table 4, baseline data for the MX monotherapy group demonstrated a lower MX dosage for treatment responders at the time point of response classification. Other potential confounders such as age or disease duration at MX treatment, baseline expanded disability status scale or disease course/activity were not significantly different between responders and non-responders. Given profound effects of GCs on in vitro MX efflux as well as markedly different treatment protocols and baseline characteristics between MX-mono- and MX/GC combination therapy groups (Table 4), the MX/GC combination therapy group was analysed separately. Patients receiving MX/GC combination had a 59.1% response rate (Table 3, panel B). In the MX/GC combination therapy cohort no clear genotype-dependent effect could be observed (Table 3, panel B). However, major differences in treatment protocols and baseline characteristics already within this group preclude further firm conclusions. Since altered MX efflux could also be related to organ-specific side effects, genotype distribution was analysed for severe cardiac or haematological side effects. While 30 of these 36 patients were at least heterozygous for variant alleles in at least one ABC-gene, in this relatively small cohort there was no statistically relevant difference in genotype frequency compared to the total multiple sclerosis population investigated. MX/GC combination therapy was often found in the cases with severe side effects: 11 of 28 patients with cardiac side effects had received GC/MX combination therapy and the same held true for seven of eight patients with severe haematological side effects. In one case, unusually severe side effects were associated with an ABC-genotype rarely found in the total multiple sclerosis-cohort: a 28-year-old female RRMS patient developed severe cardiac failure already after 24 mg/m² MX [biventricular ejection fraction 10%, multi-organ failure, biopsy exclusion of other causes (Dörr et al., 2009)]. The patient exhibited a genotype with homozgyous variant alleles in two ABCB1- and one ABCG2-locus examined, in addition to a variant allele in the ABC-transporter ABCG2 (rs717620) (ABCB1 -2677TT, -3435CT, ABCG2-141KK, ABCG2-116CT). This genotype was very unusual among our unselected multiple sclerosis patients (0.55%, Table 2).

Table 3 Association of ABC-transporter genotype with therapeutic response to MX monotherapy, exact Cochran-Armitage test (P = 0.039) (panel A), or MX/GC combination therapy (P = 0.348) (panel B)

<table>
<thead>
<tr>
<th></th>
<th>Total, n (%)</th>
<th>Responder, n (%)</th>
<th>Non-Responder, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A: MX monotherapy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCB1/ABCG2-H</td>
<td>24 (15.5)</td>
<td>15 (62.5)</td>
<td>9 (37.5)</td>
</tr>
<tr>
<td>ABCB1/ABCG2-I</td>
<td>98 (63.2)</td>
<td>78 (79.6)</td>
<td>20 (20.4)</td>
</tr>
<tr>
<td>ABCB1/ABCG2-L</td>
<td>33 (21.3)</td>
<td>28 (84.8)</td>
<td>5 (15.2)</td>
</tr>
<tr>
<td>Total</td>
<td>155</td>
<td>121 (78.1)</td>
<td>34 (21.9)</td>
</tr>
<tr>
<td><strong>B: MX/GC combination therapy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCB1/ABCG2-H</td>
<td>21 (13.6)</td>
<td>12 (57.1)</td>
<td>9 (42.9)</td>
</tr>
<tr>
<td>ABCB1/ABCG2-I</td>
<td>100 (64.9)</td>
<td>58 (58.0)</td>
<td>42 (42.0)</td>
</tr>
<tr>
<td>ABCB1/ABCG2-L</td>
<td>33 (21.4)</td>
<td>21 (63.6)</td>
<td>12 (36.4)</td>
</tr>
<tr>
<td>Total</td>
<td>154</td>
<td>91 (59.1)</td>
<td>63 (40.9)</td>
</tr>
</tbody>
</table>

Retrospective analysis using EDSS, relapse rate and MSFC as main outcome parameters to define responders and non-responders, respectively. Analysis for patients carrying ABCB1/ABCG2-H genotype (common alleles in both genes), ABCB1/ABCG2-4 (at least heterozygous for variant alleles in either ABCB1 or ABCG2), or ABCB1/ABCG2-L (at least heterozygous for variant allele in ABCB1 and ABCG2).

Discussion

Our main findings are a high prevalence of functionally relevant variant alleles in ABC-transporter genes in multiple sclerosis patients that determine MX efflux and MX-induced cell death in immune cells. High ABC-transporter mRNA expression argues for a functional role also in CNS- and cardiac tissue. In vivo relevance was corroborated in MX-treated Abcg2–/– experimental autoimmune encephalomyelitis mice. ABC-transporter genotype was associated with clinical response to MX therapy in multiple sclerosis. GCs that are clinically often used in combination inhibit in vitro MX efflux, suggesting synergistic effects at least in a sub-population of patients.

We have focussed on the most prominent ABC-transporters previously demonstrated to influence MX transport (Schinkel and Jonker, 2003). The ABCB1 3435C>T polymorphism is associated with intestinal expression levels and function and has the best validated effect on drug disposition (Hoffmeyer et al., 2000). For ABCG2, only V12M and Q141K polymorphisms could be detected, in vitro leading to disruption of apical membrane localization (V12M) or decreased ATPase function (Q141K).
No major differences in genotype frequencies were found in comparison to healthy donors and our data compared well to the HapMap CEU population (The International HapMap Consortium, 2007). Small differences in frequency of **ABCG2** variant allele V12 may best be explained by high variation that is expected for a low frequency allele in a relatively small sample. Therefore, our data does not indicate a role for ABC-transporters for disease susceptibility.

Functional significance of **ABC**-transporter genotypes was demonstrated in immune cells from multiple sclerosis patients and healthy controls. Cells from **ABCB1/ABCG2**-L genotype patients had lower in vitro MX efflux in comparison to **ABCB1/ABCG2**-H carriers. Higher MX-induced cell death in **ABCB1/ABCG2**-L in comparison to **ABCB1/ABCG2**-H and increase of MX-induced cell death after inhibition of both transporters only in **ABCB1/ABCG2**-H but not **ABCB1/ABCG2**-L further supports functional relevance of investigated SNPs. Given rapid completion of the apoptotic cell death programme within 4–5 h, at least in certain tissues and under defined conditions, even small quantitative differences in cell death at a given time point as shown here will account for major cell loss over time (Bursch et al., 1990). Genotype-dependent effects were demonstrated after inhibition of both **ABCB1/ABCG2**, while differences using monospecific inhibitors were less pronounced. This indicates an additive role of **ABCB1** and **ABCG2** in MX efflux, similar to effects in transgenic mice in which these transporters provide additive, functionally non-redundant protection against MX-induced cell death (Zhou et al., 2003).

No conclusion can be drawn from our data with regard to potential predominance of an **ABC**-transporter and the participation of additional mechanisms (e.g. other MDR transporters). However, even in animals deficient only in **Abcg2** we could observe strong effects on therapeutic MX efficacy, arguing for a major role of this transporter.

(Mizuarai et al., 2004).

### Table 4 Baseline characteristics according to MX monotherapy and MX/GC combination therapy, respectively

<table>
<thead>
<tr>
<th></th>
<th>MX monotherapy</th>
<th></th>
<th></th>
<th></th>
<th>MX/GC combination therapy</th>
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<tr>
<td></td>
<td>Responder</td>
<td>Non-Responder</td>
<td>P</td>
<td>Responder</td>
<td>Non-Responder</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 121 (78.1%)</td>
<td>n = 34 (21.9%)</td>
<td>0.310</td>
<td>n = 91 (59.1%)</td>
<td>n = 63 (40.9%)</td>
<td>0.033</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Male, n (%)</td>
<td>45 (37.2)</td>
<td>9 (26.5)</td>
<td></td>
<td>33 (36.3)</td>
<td>34 (54.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>76 (62.8)</td>
<td>25 (73.5)</td>
<td></td>
<td>58 (63.7)</td>
<td>29 (46.0)</td>
<td></td>
<td></td>
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<tr>
<td>Centre</td>
<td></td>
<td></td>
<td>0.041</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
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</tr>
<tr>
<td>Barcelona, Spain</td>
<td>0</td>
<td>0</td>
<td></td>
<td>23</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Göttingen, Germany</td>
<td>6</td>
<td>5</td>
<td></td>
<td>8</td>
<td>1</td>
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<td></td>
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<tr>
<td>Rostock, Germany</td>
<td>33</td>
<td>4</td>
<td></td>
<td>41</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Berg, Germany</td>
<td>82</td>
<td>25</td>
<td></td>
<td>19</td>
<td>32</td>
<td></td>
<td></td>
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<tr>
<td>Age at disease onset, median</td>
<td></td>
<td></td>
<td>0.367</td>
<td></td>
<td></td>
<td>0.028</td>
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<tr>
<td>Age at disease diagnosis, a</td>
<td></td>
<td></td>
<td>0.494</td>
<td></td>
<td></td>
<td>0.094</td>
<td></td>
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<tr>
<td>Disease course, n (%)</td>
<td></td>
<td></td>
<td>1.000</td>
<td></td>
<td></td>
<td>0.230</td>
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</tr>
<tr>
<td>Relapsing-remitting</td>
<td>17 (14.0)</td>
<td>4 (11.8)</td>
<td></td>
<td>23 (25.3)</td>
<td>10 (15.9)</td>
<td></td>
<td></td>
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<tr>
<td>Secondary progressive</td>
<td>102 (84.3)</td>
<td>30 (88.2)</td>
<td></td>
<td>68 (74.7)</td>
<td>53 (84.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atypical</td>
<td>2 (1.7)</td>
<td>0 (0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active patients, n (%)</td>
<td>64 (52.9)</td>
<td>16 (47.1)</td>
<td>0.566</td>
<td>31 (34.1)</td>
<td>30 (47.6)</td>
<td>0.097</td>
<td></td>
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<td>Age at first MX infusion, median (25th–75th percentile)</td>
<td>43 (37–50)</td>
<td>42 (37–48)</td>
<td>0.445</td>
<td>42 (33–49)</td>
<td>39 (31–47)</td>
<td>0.189</td>
<td></td>
</tr>
<tr>
<td>Duration of disease at time point of first MX infusion (disease onset), median (25th–75th percentile)</td>
<td>11 (6–18)</td>
<td>9 (3–15)</td>
<td>0.112</td>
<td>16 (7–23)</td>
<td>9 (6–18)</td>
<td>0.027</td>
<td></td>
</tr>
<tr>
<td>Duration of disease at time point of first MX infusion (disease diagnosis), a median (25th–75th percentile)</td>
<td>7 (3–10)</td>
<td>6 (1–9)</td>
<td>0.477</td>
<td>4 (0–11)</td>
<td>0 (0–3)</td>
<td>0.001</td>
<td></td>
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<tr>
<td>EDSS at treatment onset, median (25th–75th percentile)</td>
<td>6.0 (4.0–6.5)</td>
<td>5.5 (4.3–6.0)</td>
<td>0.288</td>
<td>6.0 (4.5–6.5)</td>
<td>5.5 (4.0–6.0)</td>
<td>0.093</td>
<td></td>
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<tr>
<td>Number of cycles at time point of response-classification, median (25th–75th percentile)</td>
<td>5 (5–5)</td>
<td>5 (5–5)</td>
<td>0.459</td>
<td>6 (5–6)</td>
<td>5 (5–6)</td>
<td>0.580</td>
<td></td>
</tr>
<tr>
<td>MX dosage at time point of response classification mg/m² body surface, median (25th–75th percentile)</td>
<td>60 (49–60)</td>
<td>60 (60–60)</td>
<td>0.030</td>
<td>60 (48–72)</td>
<td>60 (60–72)</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>Cumulative MX dosage mg/m² body surface, median (25th–75th percentile)</td>
<td>90 (60–114)</td>
<td>90 (60–119)</td>
<td>0.920</td>
<td>72 (54–100)</td>
<td>72 (72–119)</td>
<td>0.419</td>
<td></td>
</tr>
</tbody>
</table>

Atypical disease course includes neuromyelitis optica.

a Data not available for centre Berg.
Thus, together with unchanged mRNA expression of these Abc-transporters in experimental autoimmune encephalomyelitis, no further evidence of redundancy could be observed. In our animal experimental approach we tried to mirror the hypothesis that individuals with ABC-L genotype may respond to lower MX dosages than other genotypes. Since MX is highly efficacious in different EAE-models (Weilbach et al., 2004) we down titrated the concentration to a dosage not effective any more in wild-type animals. However, this dosage is fully effective in respective Abcg2-ko animals. Whereas the animal knock out situation may not be equivalent to gene polymorphisms, both functionally relevant ABCG2-SNPs identified here interfere with either expression/localization and/or activity of the transporter. The level of functional inactivation differs widely (approximately factor 2–5) in a cell-type specific manner (e.g. malignant cells, transfecants) (Mizuarai et al., 2004). Thus, we believe that mechanistic conclusions can be drawn from our in vivo animal model.

Presumed drug targets of MX in multiple sclerosis and experimental autoimmune encephalomyelitis include peripheral immune cells, the blood–brain barrier and CNS effects in situ (Kopadze et al., 2006; Duddy et al., 2007). Initial observations of preferential cell death of CD19+ cells from MX-treated multiple sclerosis patients with relative sparing of CD56+ cells (Chan et al., 2005) led to our hypothesis that ABC-transporters critically influence MX effects. Here, CD56+ cells showed highest ABC-transporter expression in comparison to CD19+ cells and unsorted peripheral blood mononuclear cells. Recently, preferential MX-induced cell death of CD19+/CD27+ memory B cells has been described, presumably altering the dysregulated B-cell cytokine network found in multiple sclerosis patients (Duddy et al., 2007), however ABC-transporter expression has not been investigated in these cells yet. High ABCB1/ABCG2- mRNA expression in CNS-tissue, myocardium and liver indicate relevant functional roles at these sites, where MX is detected up to 9 months after infusion (Morrissey et al., 2005).

MX is approved for highly active relapsing and secondary progressive multiple sclerosis, with some authors promoting early use to halt aggressive disease (Gonsette, 2007). Whereas clinical characteristics that are associated with favourable therapeutic response have been identified (e.g. age at start of treatment, disease course/relapse rate, baseline EDSS) (Le Page et al., 2007), thus far there are no biomarkers that predict clinical efficacy and individual risk profile. Here, in MX monotherapy, ABCB1/ABCG2-L patients were overrepresented among MX responders, whereas ABCB1/ABCG2-H genotype carriers were less likely MX responders. Different MX dosages between treatment responders and non-responders in MX monotherapy argue for validity of ABC-genotype effects, since lower dosage was found in the responder group. No statistically significant differences were observed for other potential confounders (e.g. duration of disease, baseline expanded disability status scale, disease course/activity) (Le Page et al., 2007). However, potential confounders were not controlled for in this retrospective association with limited patient numbers available for extensive analysis even from four major academic multiple sclerosis centres. Clinical assessment was conservative with semiquantitative parameters, assisted by electrophysiological or magnetic resonance imaging data. As a result, of the genotyped MX-treated patients, data from 95.7% of patients were eligible. Due to strong effects of GC on MX efflux in vitro, as well as profoundly different treatment regimen and baseline characteristics, subgrouping of MX monotherapy and MX/GC combination therapy cohorts was performed. This is further supported by demonstration of complex GC interactions with ABC-transporters (e.g. inhibition, acting as substrate, regulation of ABC-transporter expression) (Pavek et al., 2005). Finally, the most stringent statistical approach using exact tests to strictly control for type I error was chosen. A prospective, multicentre confirmation study with recruitment of a large and homogenous patient group with control of potential confounders and unbiased treatment protocols will be implemented in Germany in 2009 by our group with a study duration of 3 years.

The clinically widely used GC/MX combination follows the empirical rationale of potentially increased efficacy and tolerability (Morrissey et al., 2005). Here, we demonstrate an inhibitory effect of methylprednisolone and dexamethasone on MX efflux in vitro, leading to increased MX-induced cell death without additional GC-induced cell death. Hypothetically, this could lead to higher efficacy but also higher risk of side effects, at least in a subpopulation e.g. characterized by ABC-genotype. Different response rates between MX monotherapy and MX/GC combination therapy groups, as well as different baseline characteristics precluded confirmation of this hypothesis. However, the occurrence of major cardiac or haematological side effects during MX/GC combination therapy in conjunction with respective genotypes clearly warrants prospective evaluation. Statistical analysis in our patient group with severe side effects is limited by small numbers and heterogeneous treatment schemes. On a descriptive level a case of very early biopsy proven MX-associated cardiotoxicity with variant, in majority homozygous alleles in three ABC-transporter genes was identified (Dör et al., 2009). At least in our multiple sclerosis cohorts, frequency of this particular genotype was extremely low, further raising interest in the association with this unusually severe side effect. ABC-transporter genotype-dependent effects have been described in anthracycline-induced cardiotoxicity in non-Hodgkin lymphoma patients (Wojnowski et al., 2005). However, in this cohort multiple other risk factors for cardiotoxicity may play a role (e.g. mediastinal irradiation, cotreatment with cardiotoxic agents and older age). In contrast, multiple sclerosis patients are typically younger, not treated in a polypragmatic approach and cardiologically otherwise healthy. Therefore, a larger prospective trial will be necessary to address these issues in multiple sclerosis appropriately. The corroboration of ABC-genotypes as risk biomarkers could be supported by in vitro investigations. While this appears feasible for cardiomyocyte function, thus far there is no in vitro model for haematological side effects. In this context, recently described potential genetic hotspots in treatment-related leukaemia in multiple sclerosis are of interest (Hasan et al., 2008).

In conclusion, our data opens the possibility to optimize individual risk/benefit profiles based on pharmacogenetic markers which may have a direct impact e.g. for individualized titration of dosages, intervals and safety monitoring. This is of particular interest since MX is not expected to be replaced by other substances in the near future and approaches to optimization of MX treatment such as addition of cardioprotective agents are still experimental (Weilbach et al., 2004; Bernitsas et al., 2006).
**Supplementary material**

Supplementary material is available at [Brain](https://academic.oup.com/brain/article-abstract/132/9/2517/357418) online.

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**References**


