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The role of the multidrug transporter proteins ABCB1 and ABCC2 in the diaplacental transport of talinolol in the term human placenta

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Abbreviations:

ABCB1	P-glycoprotein
ABCC2	Multidrug resistance related protein 2 (MRP2)

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Abstract

Placental syncytiotrophoblasts are known to express the efflux transporter proteins P-glycoprotein (ABCB1, P-gp) and multidrug resistance-associated protein 2 (ABCC2, MRP2), which are supposed to be a functional part of the human placental barrier. With advancing gestational age, expression of ABCB1 decreases progressively whereas ABCC2 is more expressed. To evaluate to which extent they contribute to placental barrier function at term, permeability of talinolol, a substrate of both carriers, was measured using a validated human placenta perfusion model. We identified in randomized, cross-over experiments an unidirectional transfer of talinolol in feto-maternal direction because the materno-fetal transfer was significantly lower (0.663 ± 0.188 versus 0.394 ± 0.067 relative to creatinine permeability, p=0.012). Materno-fetal permeability was increased by the ABCC2 inhibitor probenecid (0.59 ± 0.15 versus 0.68 ± 0.13, p=0.028) and the non-specific inhibitor verapamil (0.53 ± 0.09 versus 0.66 ± 0.16, p=0.028) but was not influenced by the ABCB1 inhibitor PSC833 (0.48 ± 0.11 versus 0.46 ± 0.09, p=0.345). Genetic polymorphisms of ABCB1 and ABCC2 lacked significant influence on expression of the carriers and permeability of talinolol, respectively. *Conclusion:* Materno-fetal transfer of talinolol is restricted by a unidirectional process that is influenced by inhibitors of ABCC2.

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Introduction

The human placenta brings the maternal and fetal blood circulations closely together, separated only by the single cell layer of the syncytiotrophoblast. It regulates the supply of nutrients and gases, the elimination of fetal waste products and the exposure to exogenous substances including drugs. There is growing evidence that energy-dependent transporter processes are involved in materno-fetal exchange of endogenous and xenobiotic substances. Meanwhile, more than 30 transport proteins were identified which are expressed to the maternal-facing brush-border apical membrane or to the fetal-facing basolateral membrane of the syncytiotrophoblast (Evseenko et al., 2006;Marzolini and Kim, 2005;Myllynen et al., 2007). There is evidence for some of them to be significantly involved in drug disposition, such as the maternal-facing efflux carriers ABCB1 (P-glycoprotein) and the multidrug resistance-associated protein 2 (ABCC2, MRP2), a second member of the ABC-transporter family (Ceckova-Novotna et al., 2006;Evseenko et al., 2006;Marzolini and Kim, 2005;St Pierre et al., 2000). ABCB1 and ABCC2 share a wide overlapping substrate spectrum and may be co-regulated, e.g. in the small intestine (Fromm et al., 2000;Jedlitschky et al., 2006). The information on their function in the human placenta, however, is contradictory and limited so far to ABCB1. Because placental ABCB1 expression decreases with advancing gestation age whereas ABCC2 undergoes gestational maturation, we hypothesized that ABCC2 in term placentas may be more important than ABCB1 for the transfer of drugs that are substrates of both carriers (Meyer zu Schwabedissen et al., 2005;Sun et al., 2006). A suitable probe drug to evaluate function of ABCB1 and ABCC2 in man is the non-metabolized β_1-selective blocker talinolol which is a high affinity substrate of ABCB1 as evidenced by in-vitro experiments and pharmacokinetic studies in man and of ABCC2 as

concluded from major changes in disposition in Abcc2-deficient GY/TR$^-$-rats (Bernsdorf et al., 2003;Gramatte and Oertel, 1999;Spahn-Langguth et al., 1998;Westphal et al., 2000). ß$_1$-selective blockers are used in the treatment of hypertension during pregnancy (Magee et al., 2000).

We provide evidence from perfusion experiments utilizing the dually perfused human placenta model that the placenta serves as a functional barrier for talinolol as caused by a maternal-directed efflux transport. Furthermore, we evaluated the influence of the most common *ABCB1* and *ABCC2* polymorphisms and of the inhibitors PSC833 (valspodar, for ABCB1), probenecid (for ABCC2) and verapamil (non-specific) on the materno-to-fetal transfer of the probe drug (Horikawa et al., 2002;Modok et al., 2006). Our data were obtained in quality-controlled perfusion experiments using human term placentas of carefully selected healthy women.

Material and Methods

Subjects:

To evaluate the influence of genetic and drug induced variability in function of ABCB1 and ABCC2 on the placental transfer of the probe drug talinolol, we used the human placenta perfusion model as initially described by Schneider et al.(Schneider et al., 1972). Seventy five human placentas from healthy parturient women after non-complicated vaginal or Cesarean delivery and written informed consent were prepared for perfusion. Per protocol analysis was possible with 26 of them (gestation 38-41 weeks, placenta weight 416-996 g, newborn body weight 2920-4470 g, APGAR 7-10). The ABCB1 and ABCC2 genotypes were as follows: *ABCB1* 2677G>T/A, 8 GG, 8 GT, 8 TT, 2 GT/A; *ABCB1* 3435C>T, 6 CC, 14 CT, 6 TT; *ABCC2* -24C>T, 22 CC, 4 CT, 1249G>A, 15 GG, 7 GA, 4 AA; 3972C>T, 16 CC, 6 CT, 4 TT. The study has been approved by the local ethical committee.

Placenta model:

Immediately after delivery, the fetal and maternal sides of the placenta were perfused as described recently (Bachmaier et al., 2007). The flow rate was 12 ml/min in the maternal and 4 ml/min in the fetal circuit resembling physiological flow rates. The fetal arterial pressure was maintained between 25 and 40 mmHg, the total perfusion volume in each circuit was 140 ml. At the beginning of all experiments, the placenta was open-loop perfused for 30 min for removal of blood followed by close-loop perfusion for 60 min for stabilization and detection of leakage or non-congruent maternal and fetal perfusion. There was no loss of perfusate from the fetal into the maternal compartment at the beginning of the experiments. Experiments were excluded, if fetal perfusion pressure was > 50 mmHg, loss of perfusate > 4 ml/h and in cases of non-congruence between the perfusion compartments. Tissue samples

were dissected from peripheral cotyledons before perfusion for genotyping and from the perfused cotyledon after perfusion for mRNA and protein quantification.

Study protocols:

The experiments were performed randomized, controlled, two-period, cross-over with 30 min wash-out perfusion. In the first study using eight placentas, permeability of talinolol in feto-maternal and materno-fetal direction was compared. After the stabilization period, talinolol (0.8 µM, AWD pharma, Dresden, Germany), antipyrine (0.4 mM, Sigma, Steinheim, Germany) and creatinine (1.3 mM, Arcos Organics, Geel, Belgium) were added randomly either to the maternal or to the fetal circuit (final concentrations). Samples (2 ml) were taken from both circuits after 5, 10, 15, 20, 30, 45, 60, 90, 120 and 150 min after administration and substituted by perfusion medium.

In our second experiment, the materno-fetal permeability of talinolol, antipyrine and creatinine was measured without and in the presence of verapamil (30 µM, Sigma, Steinheim, Germany), PSC833 (1.8 µM, Novartis, Basel, Switzerland) or probenecid (10 mM, Sigma, Steinheim, Germany) using in each case 6 placentas. All inhibitors were added to the perfusion medium of the maternal and fetal circulation in concentrations which have been shown in former studies to modulate the efflux carriers (Molsa et al., 2005;Naruhashi et al., 2002;Pauli-Magnus et al., 2000).

Genotyping, mRNA expression and protein content of ABCB1 and ABCC2:

The ABCB1 polymorphisms 2677G>T/A and 3435C>T and ABCC2 -24C>T, 1249G>A and 3972C>T were screened by PCR-RFLA. *ABCB1* and *ABCC2* mRNA expression was quantified by real-time RT-PCR analysis (Giessmann et al., 2004). Placental protein levels of ABCB1 and ABCC2 were measured by Western blot

analysis using for ABCB1 the monoclonal C219 (1:1000) and for ABCC2 the M_2III-6 (1:500) antibodies (Alexis Biochemicals, Grünberg, Germany).

Assays for glucose, lactate, creatinine, talinolol and antipyrine:

Glucose and lactate concentrations were measured amperometrically using the Super GL Ambulance[8] (Ruhrtal Labor Technik, Möhensee, Germany) and creatinine using the kit Dimension® CREA (Dade Behring, Marburg, Germany).

Talinolol was quantified with an HPLC method as described recently for human serum (Westphal et al., 2000). The method was validated between 0.005 and 1.0 µg/ml perfusion medium. Within-day accuracy of the method was between –2.8 and 8.3 % of the nominal concentrations and precision 2.2 - 6.5 % of means. The following between-day variability was assessed with quality control samples containing 0.025, 0.25 and 0.75 µg/ml talinolol: accuracy -2.9 to 4.1 %, precision 4.3 to 10.6 % of the nominal and mean values, respectively.

Antipyrine in concentrations between 0.5 and 50 µg/ml was assayed by isocratic HPLC. In brief, 100 µl perfusion medium was mixed with 400 µl distilled water, 100 µl 4 N sodium hydroxide and 100 µl internal standard solution (0.027 mg/ml phenacetine) and extracted twice with 3 ml diethylether. After evaporation to dryness, the residue was dissolved in 140 µl of the mobile phase (MTSS buffer pH 3.0, mixed with 35 % methanol). Fifty µl were injected into the HPLC (Merck-Hitachi, Düsseldorf, Germany) equipped with the column Merck LiChroCart 125-4 HPLC cartidge filled with LiChrospher 100 RP 18e (temperature 30 °C, flow 1 ml/min). The following quality parameters were obtained: within-day accuracy and precision, -7.8 to 9.5 % and 2.8 to 11.2 %, respectively; between-day accuracy -0.4 to 2.0 % and precision 5.2 to 11.4 % of the nominal and mean values, respectively.

Biometrical evaluation:

Permeability (P) of talinolol, creatinine and antipyrine was calculated according to the equation $P = C_{fet}/(weight_{cot} \times [AUC_{mat}-AUC_{fet}])$ with C_{fet} to be the concentration in the fetal circuit at the end of perfusion, AUC_{mat} the area under the concentration-time curve (AUC) in the maternal circuit, AUC_{fet} the AUC in the fetal circuit, and $weight_{cot}$ the wet weight of the cotyledon (Bajoria and Fisk, 1998). Ratios of the talinolol permeability over the creatinine permeability were calculated to normalize for individual differences caused by paracellular transfer (Brownbill et al., 2000). Means ± standard deviations (SD) or medians, minimums and maximums are given. Sample statistics was done using the Wilcoxon and Mann/Whitney tests and Spearman´s rank correlation as appropriate.

Results

Method validation: Placental carbohydrate metabolism remained unchanged during the time of perfusion and was also not influenced by talinolol, PSC833, verapamil or probenecid as confirmed by monitoring glucose consumption and lactate production. Furthermore, the passive placental transport was also not significantly influenced by the experimental conditions as verified by the permeability data for creatinine, a surrogate for paracellular transfer, and for antipyrine, a measure for non-ionic simple diffusion (Table) (Brownbill et al., 2000;Schneider et al., 1972). Because of the lower perfusion rate in the fetal circulation, antipyrine permeability in maternal direction was expectedly lower than in fetal direction (p=0.069). Therefore, permeability of talinolol in maternal direction might have been underestimated.

Unidirectional transfer of talinolol: We identified a significant unidirectional placental transfer of talinolol in feto-maternal direction. The permeability of talinolol from the maternal to the fetal circulation was significantly lower than that from the fetal to the maternal side of the placenta (0.006 ± 0.002 µmol $\times$ $min^{-1} \times g^{-1}$ versus 0.013 ± 0.007 µmol $\times$ $min^{-1} \times g^{-1}$; p=0.012). Similarly significant differences in permeability were obtained after considering the differences in paracellular transfer by normalization of the data to creatinine permeability (0.39 ± 0.07 versus 0.66 ± 0.19, p=0.012) (Figure 1).

Unidirectional transfer of talinolol is influenced by PSC833, probenecid and verapamil: The materno-fetal permeability of talinolol normalized to creatinine permeability was slightly but significantly increased in the presence of probenecid (0.68 ± 0.13 versus 0.59 ± 0.15, p=0.028) and verapamil (0.66 ± 0.16 versus 0.53 ± 0.09, p=0.028); the verapamil effect seemed to be stronger than of

probenecid. PSC833 did not significantly influence talinolol permeability (0.48 ± 0.11 versus 0.46 ± 0.09, p=0.345) although we observed an increase of the materno-fetal transfer in five of our six experiments with PSC833 (Figure 2).

Placental expression of ABCB1 and ABCC2 and permeability of talinolol: There were no significant correlations between placental mRNA expression and protein content of ABCB1 and ABCC2, respectively. Expression of the transporters on the mRNA and protein level was not correlated to materno-fetal permeability of talinolol. Evaluation of our data with reference to the haplotypes of *ABCB1* and *ABCC2* showed that genetic polymorphisms did neither influence mRNA and protein expression of the efflux carriers nor permeability of talinolol (data are not shown).

Discussion

We provided valid experimental data on unidirectional placental transfer of talinolol using dually perfused human placentas which were obtained from carefully selected healthy women. The metabolic conditions during the perfusion for seven hours were stable as oxygen consumption and lactate production have not changed. Nevertheless, we used randomized, controlled, cross-over designs to minimize study-related intra-subject differences such as time-dependent changes in expression of the transporters. Furthermore, we corrected our data with talinolol to the permeability of creatinine to minimize influence of paracellular transfer (Brownbill et al., 2000). In all our materno-fetal transfer studies, there were also no significant differences in antipyrine permeability which is an accepted surrogate to characterize placental passive diffusion (Schneider et al., 1985). As expected because of the lower perfusion rate in the fetal circulation, antipyrine permeability in maternal direction was somewhat lower even though not statistically different. Therefore, permeability of talinolol in maternal direction might have been underestimated if perfusion is also rate-limiting for the transfer of talinolol.

In our inhibition experiments, only the materno-fetal transfer was measured using a cross-over design because this is the clinically relevant transport route. However, it is recommended for future studies to evaluate whether inhibition of efflux carriers in the syncytiotrophoblast leads to decrease of the feto-maternal permeability.

It is largely accepted that endogenous and xenobiotic compounds are exchanged via the human placenta by active transporters localized to the basal and apical membrane of the syncytiotrophoblast such as the efflux pumps ABCB1, ABCC1 (MRP1), ABCC2 and ABCG2 (BCRP) or certain members of the organic anion transport polypeptides (OATP1A2, OATP2B1), the organic cation transporters

(OCT3, OCTN1, OCTN2) or the organic anion transporters (OAT1, OAT3) (Evseenko et al., 2006;Marzolini and Kim, 2005). Their function in the human placenta, particularly the interplay between uptake and efflux carriers to initiate unidirectional substance transfer, however, is so far nearly unknown. The only exception is ABCB1 which was shown to be involved in the maternal directed efflux of saquinavir, methadone, paclitaxel and quetiapine (Molsa et al., 2005;Nanovskaya et al., 2005;Rahi et al., 2007). Therefore, ABCB1 mediated efflux seems to be the mechanism behind that what is called “placenta barrier” for many drugs which reach much lower blood concentrations in the fetus than in the mother despite low plasma protein binding; e.g. digoxin, calcium channel blockers, ß-receptor blockers, antidepressants, antiretroviral drugs (Evseenko et al., 2006;Marzolini and Kim, 2005). We provide for the first time evidence that the multidrug transporter ABCC2 may be also a functional part of the “placenta-barrier”. In late pregnancy, ABCC2 may be more important than ABCC2 because it is increasingly expressed with advancing pregnancy, in the contrary to ABCB1 for which a progressive 2-fold decline in expression was observed between the early pregnancy and term (Meyer zu Schwabedissen et al., 2005;Sun et al., 2006). This hypothesis is confirmed by our data with talinolol which is a substrate of ABCB1 and ABCC2 (Bernsdorf et al., 2003;Spahn-Langguth et al., 1998). Placental transfer of talinolol at term was in our study more influenced by ABCC2 than ABCB1 as concluded from its higher permeability in fetal direction in the presence of the ABCC2 inhibitor probenecid but not in the presence of the ABCB1 inhibitor PSC833 (Horikawa et al., 2002;Modok et al., 2006).

Obviously, ABCC2 dominates drug efflux in late pregnancy to a higher extent than ABCB1. This is supported by observations with digoxin which is a substrate of

ABCB1 but not of ABCC2 (Lowes et al., 2003). Therefore, the transplacental transfer of digoxin at term was not influenced by the ABCB1 inhibitors verapamil and quinidine in the dually perfused placenta model (Holcberg et al., 2003). One might speculate that ABCC2 contributes also to placental barrier functions for saquinavir and placlitaxel which are substrates of ABCB1 and ABCC2. For both drugs was already shown by competition experiments with specific ABCB1 inhibitors that at least ABCB1 is involved (Janneh et al., 2005;Lagas et al., 2006;Molsa et al., 2005;Nanovskaya et al., 2005).

However, the unidirectional placental transfer of drugs seems to be an extremely complex process as also evidenced by our results. In the presence of the widely used ABCB1 inhibitor verapamil, the materno-fetal talinolol permeability was significantly increased, even to a higher extent than in the presence of probenecid, although the specific modulator PSC833 lacked markedly influence (Naito and Tsuruo, 1989). R-verapamil is also known to modulate ABCC1 and OATPs as confirmed for the uptake of fexofenadine (Cvetkovic et al., 1999;Perrotton et al., 2007). ABCC1 seems to be localized to the basal and brush-border membrane of the syncytiotrophoblast and to the blood vessel endothelia and it undergoes gestational maturation. The OATP transporters OATP2B1 and OATP1B3 are localized to the basal membrane of the syncytium and are obviously involved in uptake of steroid sulfates and unconjugated bilirubin (Evseenko et al., 2006). Functional interaction of basolateral uptake transporters (e.g. OATP2B1, OATP1B3) with apical efflux carriers (e.g. ABCB1, ABCC2, ABCG2) might be the way how steroids, bilirubin and drugs may pass the placenta in feto-maternal direction. This conception is in line with the recent observation, that the expression of OATP2B1 and ABCG2 in the human placenta are significantly correlated (Grube et al., 2007). There is evidence that

OATPs are involved in disposition of talinolol (Schwarz et al., 2005). Therefore, verapamil may have increased placental talinolol permeability by inhibition of basolateral OATPs. Whether inhibition of ABCC1 by verapamil is also involved in modulation of placental talinolol transfer needs further investigation. In this contexts should be mentioned that probenecid is also an inhibitor of ABCC1 in-vitro which might have contributed to facilitated talinolol permeability in fetal direction (de Jong et al., 2003). In our study was not evaluated whether the high abundant ABCG2 is also involved in diaplacental transfer of talinolol as shown for glyburide using placenta membrane vesicles and the ABCG2 inhibitor novobiocin (Gedeon et al., 2007). Furthermore, ABCC1, ABCB1, ABCC2 and ABCG2 have a wide spectrum of substrate in common. In future mechanistic studies, more specific and potent inhibitors would be required to clearly demonstrate the function of placental drug transport proteins.

There is evidence from literature, that the genetic polymorphisms *ABCB1* G2677T and C3435T and *ABCC2* G1249A are associated with altered expression of the transporters in the human placenta (Hitzl et al., 2004;Meyer zu Schwabedissen et al., 2005). The statistical power in our study, however, was too low to confirm functional relevance of this genetic variability for placental talinolol transfer, because of the low sample size (N=26) and the high inter-subject variability of the permeability (35 %).

Conclusions:

The materno-fetal transfer of talinolol is restricted by a unidirectional process that is influenced by inhibitors of ABCC2. There is evidence that additional active transporters are involved. However, the efflux talinolol seems to be low and obviously not of clinical relevance.

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Footnotes:

a) All authors have no conflict of interest. The data were presented in a poster during the 8th Annual Congress of the Association of Clincal Pharmacology in Würzburg, Germany by Minarikova et al., Abstract in Int. J. of Clin. Pharmacology and Therapeutics 44/10, 2006 and in a oral presentation during the Annual Meeting of the ASCPT in Anaheim, California by Minarikova et al., Abstract in CPT 81, Suppl 1, OI-C-I, 2007.

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Legend to the figures:

Figure 1: Permeability of talinolol normalized to creatinine permeability in the materno-fetal versus feto-maternal direction (n=8). Wilcoxon´s signed rank test was used to evaluate differences.

Figure 2: Permeability of talinolol normalized to creatinine permeability in materno-fetal direction in the presence of PSC833, probenecid and verapamil (n=6). Wilcoxon´s signed rank test was used to evaluate differences.

Table 1: Permeability of antipyrine and creatinine and viability characteristics during in-vitro perfusions. Arithmetic means and 95% confidence intervals (in parentheses) are given.

protocol	N	glucose consumption ($\mu mol \times min^{-1} \times g^{-1}$)	lactate production ($\mu mol \times min^{-1} \times g^{-1}$)	antipyrine permeability ($ml \times min^{-1} \times g^{-1}$)	creatinine permeability ($ml \times min^{-1} \times g^{-1}$)
1. study (materno-fetal versus feto-maternal permability)					
materno-fetal	8	0.21 (0.14, 0.28)	0.45 (0.30, 0.60)	0.10 (0.07, 0.12)	0.015 (0.011, 0.018)
feto-maternal	8	0.21 (0.13, 0.28)	0.39 (0.29, 0.49)	0.07 (0.04, 0.10)	0.020 (0.014, 0.026)
2. study (materno-fetal permeability in presence of inhibitors)					
control	6	0.32 (0.03, 0.62)	0.59 (0.05, 1.12)	0.09 (0.003, 0.18)	0.038 (0.001, 0.075)
PSC833	6	0.32 (0.10, 0.54)	0.66 (0.04, 1.27)	0.11 (0.05, 0.17)	0.042 (0.010, 0.075)
control	6	0.29 (0.15, 0.43)	0.55 (0.30, 0.80)	0.09 (0.04, 0.15)	0.021 (0.016, 0.026)
probenecid	6	0.36 (0.12, 0.61)	0.79 (0.32, 1.27)	0.07 (0.04, 0.11)	0.019 (0.014, 0.024)
control	6	0.27 (0.17, 0.37)	0.54 (0.20, 0.87)	0.10 (0.05, 0.15)	0.025 (0.018, 0.031)
verapamil	6	0.26 (0.18, 0.34)	0.48 (0.28, 0.68)	0.10 (0.06, 0.13)	0.025 (0.021, 0.028)

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Figure 1

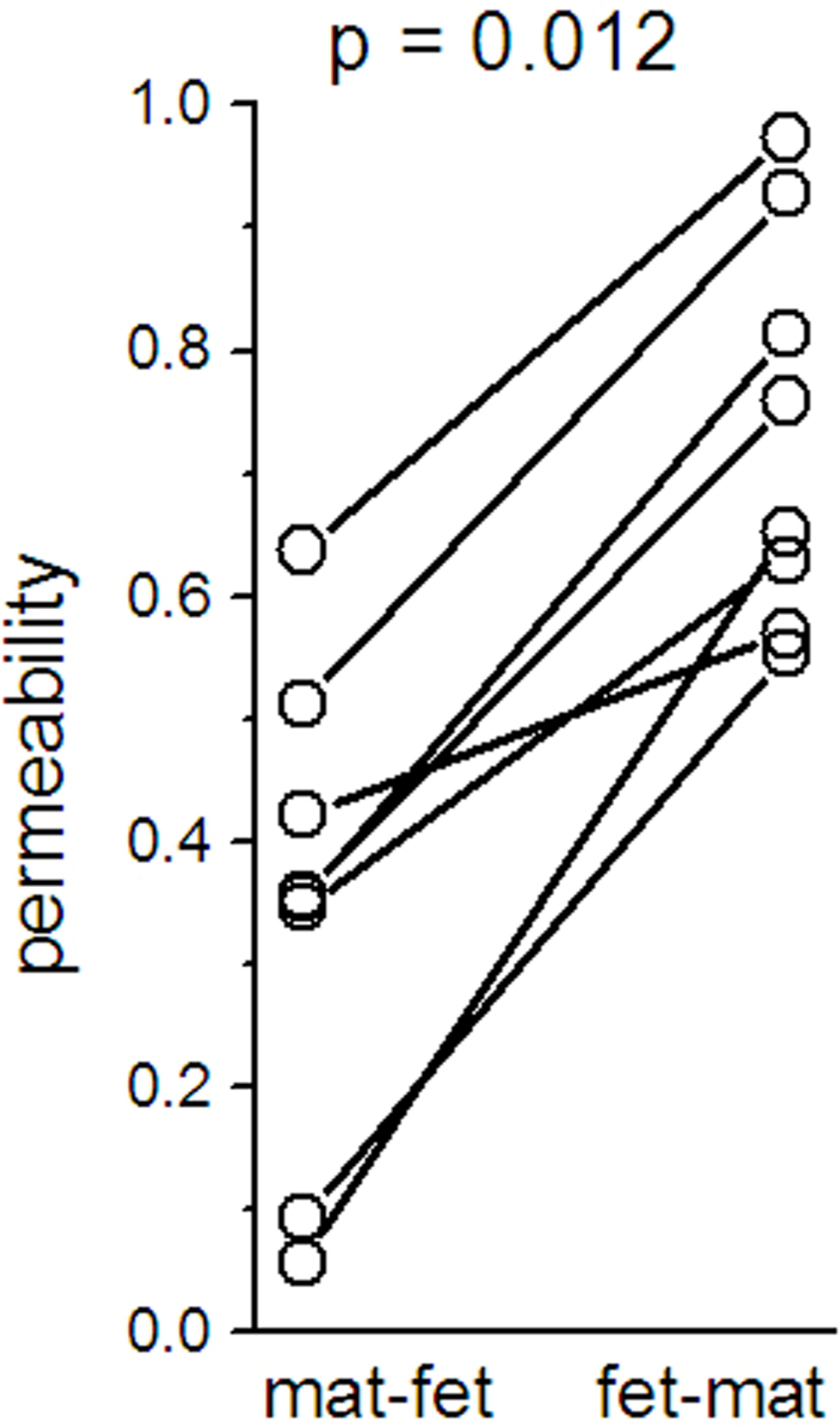

Figure 2