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ORAL ABSORPTION OF CLARITHROMYCIN IS NEARLY ABOLISHED BY CHRONIC COMEDICATION OF RIFAMPICIN IN FOALS

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Running title: Interaction of clarithromycin with rifampicin in foals

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List of abbreviations:

ABCB1, P-glycoprotein; ABCC2, multidrug resistance associated protein 2 (MRP2); CLR, clarithromycin; BAL, bronchoalveolar lavage; BALC, bronchoalveolar lavage cells; CYP, cytochrome P450; EC, bronchial/alveolar epithelial cells; ELF, epithelial lining fluid; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; PEPT, peptide transporter; PXR, pregnane-X receptor; RIF, rifampicin

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Abstract

The delivery of clarithromycin (CRL) to its site of action in bronchial/alveolar epithelial cells (EC), bronchial epithelial lining fluid (ELF) and bronchoalveolar lavage cells (BALC) may be influenced by CYP3A4 and the drug transporters ABCB1, ABCC2 and OATPs which can be modulated and/or upregulated via the nuclear pregnane X receptor (PXR) by rifampicin (RIF). Therefore, we evaluated disposition and pulmonary distribution of CLR (7.5 mg/kg b.i.d., 21 days) and expression of ABCB1, ABCC2, OATP1A2 and OATP2B1 in EC and BALC before and after comedication of RIF (10 mg/kg b.i.d., 11 days) in 9 healthy foals (41-61 days, 115-159 kg) in which the genetic homology of drug transporters is close to their human analogs. After RIF comedication, relative bioavailability of CLR decreased by more than 90%. Concentrations in plasma (29.8±26.3 ng/ml vs. 462±368 ng/ml), ELF (0.69±0.66 µg/ml vs. 9.49±6.12 µg/ml) and BALC (10.2±10.2 µg/ml vs. 264±375 µg/ml, all $p<0.05$) were lowered drastically whereas levels of the metabolite 14-hydroxyclarithromycin (14OH-CLR) were not elevated despite higher 4β-OH-cholesterol/cholesterol plasma concentration ratio, a surrogate for CYP3A4 induction. In presence of CLR, *ABCC2* and *PXR* mRNA content were significantly and coordinately ($r^2=0.664$, $p<0.001$) reduced in BALC after RIF. In EC, mRNA expression of *OATP1A2* increased but of *OATP2B1* decreased (both $p<0.05$).

RIF interrupts oral absorption and decreases CRL plasma levels below the minimal inhibitory concentration for eradication of *Rhodococcus equi*. Evidence exists for RIF to influence the cellular uptake of CLR in bronchial cells and the PXR expression in BALC in presence of high CLR concentrations.

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Introduction

Efficacy of drugs is dependent on the availability of active concentrations at the site(s) of the desired pharmacodynamic effect. In the case of infectious lung diseases, for instance, the minimum inhibition concentrations (MIC) for antimicrobial agents must be exceeded in the environment of the respective bacteria; i.e. in the bronchial and alveolar epithelial cells (EC), in the bronchial epithelial lining fluid (ELF) and in bronchoalveolar lavage cells (BALC) of which about 80% are alveolar macrophages. Thus, the frequently prescribed macrolide antibiotics penetrate into these pulmonary spaces to reach drug levels many times above the concurrent plasma concentrations at steady-state (BALC>ELF>plasma) (Conte, Jr. et al., 1995). The mechanisms by which macrolides accumulate in pulmonary cells are poorly understood. So far, trapping of the basic compounds in acidic compartment of alveolar macrophages (i.e. lysosomes, endosomes) is the only plausible rationale. However, BALC/plasma gradients of 50-100 : 1 and steeper and accumulation in the alveolar/bronchial ELF are not solely explainable by base trapping. There is ample evidence that unidirectional penetration of drugs from the blood throughout the vascular and the alveolar/bronchial epithelium into the alveolar/bronchial ELF and from there into the BALC is achieved by the coordinate interplay of multidrug transporters. To the current knowledge, the cell membranes along the pulmonary penetration route are equipped with uptake carriers of the organic anion transporting polypeptide (OATP), organic cation transporter (OCT) and peptide transporter (PEPT) families and with efflux carriers of the ATP-binding cassette (ABC) family which are also expressed along the intestinal/hepatic absorption route of the drugs (Bosquillon, 2010; Chan et al., 2004).

To evaluate whether and how multidrug transport proteins influence absorption and pulmonary distribution of macrolides, we initiated a multiple-dose drug interaction

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study with clarithromycin (CLR) and rifampicin (RIF) in healthy foals. CLR is a substrate of cytochrome P450 (CYP) 3A4, ABCB1 (P-glycoprotein) and probably of ABCC2 (multidrug resistance associated protein 2) and of OATPs (Garver et al., 2008; Seithel et al., 2007; Suzuki et al., 2003; Lan et al., 2009; Munic et al., 2010). The efflux carriers ABCB1 and ABCC2 and several OATPs are modulated in the presence of RIF (Geick et al., 2001; Vavricka et al., 2002) After chronic treatment, however, RIF can up-regulate gene expression of intestinal and hepatic CYP3A4, ABCB1, ABCC2 and OAP1A2 via the nuclear pregnane X receptor (PXR) pathway (Lau et al., 2006; Tirona, 2011; Vavricka et al., 2002; Zong and Pollack, 2003). We have recently shown, that RIF may also regulate pulmonary ABCB1 and ABCC2 of healthy foals (Venner et al., 2010). Therefore, the overall changes in absorption and pulmonary distribution of CRL may be caused by competitive effects in presence of RIF and by the chronic effects as caused by PXR-type transporter induction.

We have chosen foals for our mechanistic drug interaction study because of the clinical challenge in horse-breeding to eradicate *Rhodococcus equi* which resists innate macrophage defense in adult horses but causes severe caseous, necrotisizing lung infection in the foals with high mortality rate of up to 80% (Hillidge, 1987). Combined antimicrobial therapy with macrolides and RIF has become the most effective treatment protocol to increase the survival rate from 20% to nearly 90% (Hillidge, 1987). In a retrospective study it was shown that the combination of RIF with CLR is superior to combinations with erythromycin or azithromycin (Giguère et al., 2004). Secondly, we have chosen the animal model because bronchoscopy and bronchoalveolar lavage are accepted diagnostic techniques in foals for sampling of biomaterial. Finally, the sequence homology of the equine drug transporters is distinctly closer to the human analogs (ABCB1, 92%; ABCC2, 82%; OATP1A2, 85%; OATP2B1 89% on protein basis) than, for instance, the homology of the transporter

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of rats which are often used to predict the situations in human beings (Abcb1a, 86%; Abcb1b, 79%; Abcc2, 77%; Oatp1a5, 72%; Oatp2b1, 76% on protein basis, www.ncbi.nih.gov, www.ebi.ac.uk) (Hagenbuch and Meier, 2004).

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Material and methods

Study protocol

Animals: The drug interaction study was performed after approval by the State Authority of Mecklenburg/Vorpommern (reference code: LALLF M-V/TSD/7221.3-1.1-066/08). Nine foals (4 females, 5 males, age 41-61 days, body weight 115-159 kg) of warm-blooded horses of the Oldenburger trait were included after confirmation of good health by physical examinations including sonography of the lung and routine clinical-chemical and hematological screenings. The animals were kept at natural light rhythm on paddocks together with their mares and had free access to equine milk, hay, oats and tap water. All clinical examinations were done in individual stables which were covered with straw. Prior to the study, the foals did not receive any other medication.

Study design: The drug interaction study was performed under steady-state condition within a study period of 21 days. Initially, the foals were treated orally with 7.5 mg/kg CLR (Abbott, Wiesbaden, Germany) twice daily for 6 days. A last dose was given in the morning of the 7th study day. On treatment days 9-19, the animals received 7.5 mg/kg CLR and 10 mg/kg RIF (Gruenenthal GmbH, Aachen, Germany) twice daily. Administration of CLR was continued until the morning of the 21st day while RIF was not given on the 20th day or in the morning of the 21st day to avoid competitive interferences with the pharmacokinetics of CLR after last administration. RIF tablets suspended in 30 ml water and the commercial CLR suspension were sprinkled in the mouth using a syringe to be completely swallowed by the foals.

To evaluate pharmacokinetics of CLR on treatment days 7 and 21, venous blood was collected via an indwelling jugular vein cannula (Vygon, Aachen, Germany) before and 0.33, 0.66, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 16, 24, 36 and 48 h after administration.

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Plasma was separated by centrifugation at 2.000 x g for 10 min and stored at least at -80°C until further analysis.

To measure drug distribution in the ELF and BALC, a bronchoalveolar lavage (BAL) was performed 12 h after the last administrations of CLR of each study period as described recently (Venner et al., 2010). In brief, after anesthesia with ketamine (Serumwerke, Bernburg, Germany) and diazepam (ct-Arzneimittel, Berlin, Germany), a flexible fiberscope (Karl Storz, Tuttlingen, Germany) was advanced through the nose into the trachea to take two biopsy specimen from the bronchial epithelium behind the carina. Then, the endoscope was advanced and wedged in a second-generation bronchus. The lavage was performed by repeated instillation of 100 ml phosphate buffered saline (pH 7.4, temperature about 37 °C). The first aspirate was discarded to remove the excess of epithelial cells. Three additional aliquots were combined, filtered through a layer of gauze (Lohmann and Rauscher, Neuwied, Germany) and centrifuged at $400 \times g$ for 10 min. The BALC pellet consisted of 77-84.5% alveolar macrophages, 11.5-18.5% lymphocytes and 2.5-8.5% mast cells as confirmed by May-Gruenwald staining. For mRNA analysis of *ABCB1, ABCC2* and *PXR,* the biopsy specimens and aliquots of 5×10^6 BALC were incubated overnight with RNAlater buffer (4 °C) (Qiagen, Hilden, Germany) before freezing. For drug analysis, aliquots of BALC and of the supernatant were shock frozen using dry ice and stored at -80°C until further use.

Quantitative assays for clarithromycin and 4-β-hydroxycholesterol

CLR and 14-hydroxyclarithromycin (14OH-CLR) were quantified in plasma, lavage fluid and BALC using a validated LC-MS/MS method as recently described (Oswald et al., 2011a). The limit of quantification for all matrices was 2.5 ng/ml. The within-day accuracy of the assay ranged from -11.8 to 9.3% of the nominal concentrations and precision was 1.6 to 11.2% of means (coefficient of variation). Between-day accuracy

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was -11.7 to 8.6% of the nominal concentrations and precision was 3.7 to 10.0% of the respective mean control values. CLR and 14OH-CLR concentrations in ELF were assessed by normalizing to the concentration ratio of urea in plasma over bronchoalveolar fluid and in BALC to a mean macrophage cell volume of 1.2 µl/10^6 cells in foals (Jacks et al., 2001; Rennard et al., 1986). Urea was quantified using the kit LT-UR 0010 (Labortechnick Eberhard Lehmann, Berlin, Germany).

Plasma concentrations of 4-β-hydroxycholesterol (4β-OH-C) were assayed using GC-MS for a isotope dilution method with [26.26.26.27.27.27-$^2H_6$] 4β-OH-C as an internal standard as described previously (Tomalik-Scharte et al., 2009). The lower limit of quantification was 3.0 ng/ml for plasma. Between-day and within-day precision was 2.1 and 2.7%, respectively, of the mean values and between-day and within-day accuracy was between 2.9 and 3.3% of the nominal values.

Quantitative mRNA expression of drug transporters and PXR

The bronchial biopsy specimens and BALC were homogenized using a dismembrator (Braun, Melsungen, Germany), total RNA was prepared applying the NucleoSpin® RNAII Kit (Macherey-Nagel, Dueren, Germany) and reverse transcription of 100 ng RNA was performed using the SuperScript®VILO™ cDNA Kit (Invitrogen, Karlsruhe, Germany) according to the protocols of the manufacturers. The quantitative real-time RT-PCR analysis for *ABCB1, ABCC2* and *PCR* was conducted using primers assembled for equine mRNA (PrimerDesign, Southampton, United Kingdom). The sequences were as follows, ABCB1_SE 5`-AGGATGTTCTGTTGGTATTCTCA-3`; ABCB1_AS 5`-GACACTTTGGCTTTGGCATAG-3`; ABCC2_SE 5`-ACTTCAATG-CCACCAACTATCC-3`; ABCC2_AS 5`-CACCTTGTGCTAATCCCAGAG-3`; PXR_SE 5`-CGATTGTCCAAAGTTGATAATTTACG-3` and PXR_AS 5`-CGGAGCCATTAG-GAATAGTAGAAT-3`. Sequences for the probes for *ABCB1*, *ABCC2* and *PXR* were 5`-(FAM)-CAAATGAACTGACCTGCCCCACTGCC, 5`-(FAM)-

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CCATAGACGCCAACTCTCAAGTCCCTCT and 5`-(FAM)-CTCTCTGCCAACCATCTCCATAGCCT, respectively. For mRNA quantification of OATP1A2 and OATP2B1, we used equine primers according to the instruction of the manufacturer (Applied Biosystems, Foster City, USA). The RT-PCR was performed using the TaqMan® method with human *18s* rRNA (Applied Biosystems, Foster City, USA) as reference due to the high homology between the equine and human gene. Each experiment was conducted in duplicates. Gene expression was quantified using the ΔΔct method.

Biometrical evaluation

Maximum (C_{max}) and minimum (C_{min}) plasma concentrations and the time of C_{max} (t_{max}) at steady-state were taken from the plasma concentration-time curves. The area under the plasma concentration-time curve (AUC_{0-12h}) was calculated using the trapezoidal rule and the average plasma concentration (C_{av}) was derived (AUC/dosing interval). Terminal elimination half-life ($t_{1/2}$) was estimated by log-linear regression analysis. For group result, arithmetic means±standard deviations (mean±SD) are given. Differences between two groups were evaluated using the nonparametric Wilcoxon test and correlations were assessed using Spearman´s rank test, both with $p<0.05$ as level of significance.

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Results

CLR was slowly absorbed during the chronic oral treatment to generate average plasma concentrations between 133 ng/ml and 1300 ng/ml at steady-state (Figure and Table 1). The plasma exposure ($AUC_{0\text{-}12h}$) of the metabolite 14OH-CLR amounted to 33±9% of the CLR exposure (metabolic ratio). CLR accumulated along the pulmonary distribution route to reach more than 30-fold higher levels in the ELF and 700-fold higher concentrations in BALC than in plasma at trough 12 hours after administration. 14OH-CLR underwent similar accumulation even though to a lower extent (ELF, about 5-fold; BALC, about 44-fold; Table 2). Please note in Table 1, that the concentrations for CLR and 14OH-CLR in plasma are given in ng/ml and in ELF and BALC in µg/ml. CLR was eliminated in foals at steady-state with terminal half-lives between 4.7 and 7.3 hours.

After comedication of RIF for 11 days, relative bioavailability of CLR decreased by more than 90%; accordingly, the average plasma concentrations (C_{av}) were significantly reduced and fell even below the minimum inhibition concentration (MIC) for *Rodoccocus equi* (Jacks et al., 2003). The elimination rate was not significantly influenced. The drastic decrease of CLR bioavailability did not result in a proportional increase of metabolite exposure, although relative 14OH-CLR exposure increased more than 7-fold compared to the situation before RIF comedication (metabolic ratio: 2.56±0.97 versus 0.33±0.09, $p<0.05$). Lower bioavailability of CLR after RIF was also associated with parallel lowering of CLR and 14OH-CLR in ELF and BALC by more than 90% and 60%, respectively, as compared to the levels reached by CLR mono-therapy. Remarkably, CLR penetrated significantly better into the ELF after RIF comedication as confirmed by the increase of the ELF/plasma concentration ratio (Figure 2). RIF treatment was also associated with induction of systemic metabolic activity. The 4β-OH-C/cholesterol plasma concentration ratio significantly increased

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from 0.68±0.34 to 1.05±0.40 (p=0.004) (Figure 3). However, the pulmonary mRNA expression of the efflux transporters ABCB1 and ABCC2 and of the nuclear PXR receptor was not significantly up-regulated by RIF; the bronchoalveolar mRNA content of *ABCC2* and *PXR* was even significantly reduced (Figure 4). Nevertheless, we found a significant correlation between the decrease in *PXR*mRNA and the decrease in *ABCC2*mRNA expression in BALC (Figure 5). *OATP1A2*mRNA and *OATP2B1*mRNA were expressed in EC. In BALC, only OATP2B1 was markedly expressed. RIF comedication significantly decreased the mRNA content of OATP1A2 in EC but increased that of OATP2B1 (Figure 6). Content of both *OATP*mRNAs was not significantly correlated to the content of *PXR*mRNA

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Discussion

By this drug interaction study it was clearly shown, that chronic comedication of RIF leads to a dramatic lowering of the average steady-state plasma concentrations of CLR by more than 90% and in turn to a similarly limited distribution into the bronchial ELF and BALC. RIF comedication was not associated with up-regulation of the pulmonary efflux transporters ABCB1 and ABCC2. Nevertheless, the distribution of CLR into the ELF in absolute measures was decreased while it was nearly twofold increased relative to the (lowered) plasma levels after RIF. Interestingly, RIF comedication was associated with decreased *OATP1A2*mRNA but increased *OATP2B1*mRNA expression levels in EC.

The dramatic decrease of the steady-state levels is doubtlessly the consequence of nearly complete abolition of CLR bioavailability because the plasma half-lives remained unchanged after comedication of RIF. In healthy foals, bioavailability of CLR after oral administration is incomplete (60%) as caused at least in part by presystemic “first pass” metabolism by which the active 14OH-CLR is generated (Womble et al., 2006). The results of in-vitro studies using human liver microsomes and recombinant CYPs suggested that CYP3A4 plays a major role in the overall metabolic clearance of CLR (Suzuki et al., 2003). Another reason for deficit bioavailability could be intestinal efflux transport. Macrolides are substrates of ABCB1 as confirmed by competitions assays using Caco2, MDCK and ABCB1-overexpressing MES-SA/Dx5 (ATCC, CRL-1976) human uterine sarcoma cells. From these in-vitro studies, the overall impression can be derived, that CLR has moderate affinity to ABCB1 (Munic et al., 2010; Pachot et al., 2003; Hughes and Crowe, 2010). There is also evidence for macrolides to be substrates of ABCC2 as shown for azithromycin by pharmacokinetic studies in Abcc2-deficient rats and in a drug interaction study with probenecid, an inhibitor of ABCC2 (Sugie et al., 2004).

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Therefore, the drastic lowering of the average CLR plasma concentrations by more than 90% may have resulted from induction of hepatic and intestinal CYP3A4 and intestinal ABCB1, and probably ABCC2.

RIF is a prototype ligand for the nuclear PXR receptor that regulates many drug metabolizing enzymes and multidrug transport proteins in the small intestine and the liver (Handschin and Meyer, 2003; Glaeser, 2011; Oswald et al., 2011b; Tirona, 2011). Induction of hepatic CYP3A4 in our study was confirmed by significant increase of the 4ß-hydroxycholesterol/cholesterol plasma concentration ratio, an accepted endogenous metrics for hepatic CYP3A4 activity in-vivo (Yang and Rodrigues, 2010). Nevertheless, the plasma levels of 14OH-CLR increased only to a limited extent and did not agree with the large loss in CLR bioavailability. Therefore, induction of intestinal efflux transport seems to be the major reason for lower bioavailability which is also supported by the results of clinical studies in man. According to the summary of product characteristics (SMPC) of the manufacturers for CLR, strong enzyme inducers of the CYP450 system may accelerate the metabolism of CLR and, by this, decrease the plasma levels of the parent drug in human patients by about 30-40% and increase the concentrations of the microbiologically active 14OH-CLR by the same extent (e.g. Klacid®, Abbott, Wiesbaden, Germany). In contrary to the product information, the results of two clinical studies in patients with pulmonary *Mycobacterium avium* complex infections showed a somewhat different feature. In all patients, the trough plasma concentrations of CLR were markedly lower when administered together with RIF (<20% of the levels after monotherapy); the 14OH-CLR levels, however, were not different (Wallace, Jr. et al., 1995; Taki et al., 2007). Interestingly, the decrease of CLR exposure could be avoided if RIF and CLR were administered at different times, i.e., CLR seems to be absorbed by a

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mechanism which is susceptible to direct competition with RIF (Taki et al., 2007) but this mechanism has not been identified yet.

Therefore, we are encouraged to provide an alternative hypothesis to which inhibition of a so far unknown intestinal uptake transporter in the presence of RIF may have caused lower bioavailability of CLR; instead of or additionally to the absorption deficit as caused by RIF-type induction of ABCB1. Candidates might by members of the OATP-family which are known to be modulated by RIF in-vitro (Vavricka et al., 2002). Firstly, CLR is a potent inhibitor of the taurocholate uptake in rat Oatp1a5-transfected MDCK cells, the nearest analog to human OATP1A2. Both rat Oatp2b1 and human OATP2B1 were not inhibited by clarithromycin (Garver et al., 2008; Lan et al., 2009). Secondly, oral bioavailability of CLR in rats was reduced by 45% in the presence of RIF. Thirdly, RIF had no effect on body clearance of CLR and did most likely not cause induction of metabolic enzymes and/or transporters after the short-time comedication in this study (120-180 min) (Garver et al., 2008). Therefore, a member of the OATP-family might be involved in intestinal absorption of CLR although there is little evidence that rat Oatp1a5/human OATP1A2 or rat Oatp2b1/human OATP2B1 really are the candidates (Lan et al., 2009). Hence, alternative intestinal absorption pathways must exist which are susceptible to inhibition by RIF. Anyway, in assuming competition of RIF with an intestinal uptake transporter for CLR, it must be considered that RIF also modulates ABCB1 and ABCC2 (Zong and Pollack, 2003; Lau et al., 2006) which are also expressed in the horse intestine (Tyden et al., 2009). Thus, the net effect resulting from modulation of intestinal OATP(s) and/or induction of ABCB1/ABCC2 must have overshadowed in extent the modulating effects of RIF on the ABCB1/ABCC2-mediated efflux of CLR.

Whatsoever mechanism has influenced the “first-pass” route of CLR, the average steady-state plasma concentrations in our foals dropped down to levels below the

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MIC (90%) of 0.12 µg/ml for *Rhodoccocus equi* (Jacks et al., 2003). Although manifold decreased, the concentrations in the ELF and BALC were still above the desired MIC. Nevertheless, there are many doubts from a pharmacokinetic point of view that combination therapy of CLR with RIF might really be superior to other eradication protocols as suggested by the results of a retrospective clinical study in foals (Giguère et al., 2004). Absence of major drug interactions as shown in our recent pharmacokinetic study with tulathromycin and RIF should be confirmed before launching a combination treatment in clinical practice (Venner et al., 2010).

A second major finding in our study was the more intensive distribution of CLR into the bronchial ELF despite markedly lower plasma concentrations after RIF comedication. We have hypothesized that finding in advance because, as discussed above, CLR is a substrate of ABCB1 (and probably of ABCC2) and RIF is a strong inducer of both. Both ABCB1 and ABCC2 are expressed in the apical membrane of the EC and in the cell membrane of BALC and both may mediate active efflux of their substrates into the ELF and the environment of macrophages, respectively (Bosquillon, 2010; Seral et al., 2003a; Seral et al., 2003b). According to our recent preliminary data, mRNA expression of ABCB1 and ABCC2 (semi-quantitative RT-PCR in pooled samples) can be up-regulated by RIF in foals (Venner et al., 2010). We also found, that the trough BALC concentrations of RIF after administration of 10 mg/kg given twice daily (6.0±1.3 µmol/l, N=9, Oldenburger trait, unpublished own result) are in the same order of magnitude as the in-vitro concentrations necessary to activate the nuclear PXR and to increase CYP3A4, ABCB1 and ABCC2 activity (2-10 µM) (Geick et al., 2001; Kast et al., 2002). In fact, our results did not suit our working hypothesis; ABCB1 expression remained unchanged and ABCC2 was even significantly down-regulated by RIF comedication. However, we must consider some methodological limitations because quantitative PCR was performed 48 h after RIF

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last administration. We cannot exclude that mRNA levels returned to their pre-treatment levels while expression of the transporter protein in the cell membrane was still high, causing an increase in CLR accumulation in the ELF.

Pulmonary penetration of drugs is extremely complex, not well understood and not only mediated by efflux transporters. In man, the uptake carriers PEPT2 and OCTN2 are expressed in the apical membrane of the EC. In alveolar macrophages, OCTN1 and OATP2B1 are expressed. In the lung, mRNA transcripts were also identified for OCT3, OCTN1, OATP1A2 and OATP2B1 (Bosquillon, 2010; Endter et al., 2009; Moreau et al., 2011). OATP1A2 and 2B1 are highly conserved in Equus caballus (mRNA homology compared to human genes: OATB1A2 88%, OATP2B1 84%, ww.ncbi.nih.gov, www.ebi.ac.uk). To our data in foals, OATP1A2 is expressed in EC and OATP2B1 in EC and BALC; the exact cellular localization is unknown so far.

It is rather speculative to find a conclusive rationale for higher ELF/plasma ratios of CLR after RIF comedication because of the complexity of parallel processes. In our study, equine OATP2B1 in EC was regulated by RIF. Others also found OATP1A2 to be induced by RIF via a PXR response element (Meyer zu Schwabedissen et al., 2008). On the other hand, OATPs are inhibited in the presence of RIF (Vavricka et al., 2002). If the pulmonary OATPs are localized to the basolateral site of EC, higher ELF/plasma ratios are explained by upregulation of OATP2B1 by chronic RIF comedication. In case of apical localization of the uptake carriers, the pharmacokinetic phenomenon in presence of RIF (downregulation and or inhibition) is in line with delayed CLA reuptake from the bronchial ELF back to EC during systemic elimination of the drug. Therefore, information on the pulmonary localization of OATPs and quantitative data on affinity of CLA and RIF to the uptake transporters and their regulatory elements is needed before the results of the drug interaction study can be rationally discussed.

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A third interesting and, to our knowledge, new finding in our study was the coordinate down-regulation of *ABCC2*mRNA expression in significant correlation to the *PXR*mRNA content (r=0.815) in the BALC after comedication of RIF; a tendency for lower *PXR*mRNA levels was also observed in EC. Please notice, that the down-regulation of PXR occurred in the presence of high cellular CLR concentrations (about 350 mM !). PXR-activating concentrations of RIF in presence of high CLR concentrations obviously inhibit mRNA expression of PXR whereas the interaction between PXR with other nuclear receptors and transcriptional factors that regulate the ABCC2 target gene seemed not to be disrupted by RIF/CLR as shown for ketoconazole and CYP3A4 (Lim et al., 2009). Future research is required to elucidate the mechanism behind our finding in BALC of foals.

Conclusions: Chronic comedication of RIF in foals leads to an unexpectedly strong decrease of the bioavailability of CLR. By this undesired drug interaction, the plasma levels fall below the MIC for eradication of *Rhodococcus equi*. Evidence exists for RIF to influence the cellular uptake of CLR in bronchial cells and the PXR expression in BALC in presence of high CLR concentrations.

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Authorship Contributions

Participated in research design: Peters, Grube, Kroemer, Venner and Siegmund.

Conducted experiments: Peters, Block, Freyer, Lämmer and Venner.

Contributed new reagents or analytic tools: Oswald and Lütjohann.

Performed data analysis: Peters, Block and Oswald.

Wrote or contributed to the writing of the manuscript: Peters, Oswald, Grube, Kroemer, Venner and Siegmund.

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Footnotes

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Conflict of interest: The authors declare that they have no conflict of interest.

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Legends for Figures

Figure 1: Plasma-concentration time curves of clarithromycin (above) and 14-hydroxyclarithromycin (below) after chronic treatment with 7.5 mg/kg twice daily before (-RIF) and after (+RIF) comedication of rifampicin (10 mg/kg b.i.d., 11 days) in 9 healthy foals.

Figure 2: Accumulation of clarithromycin in bronchial epithelial lining fluid (ELF) and bronchoalveolar lavage cells (BALC) after chronic treatment with 7.5 mg/kg twice daily before (-RIF) and after (+RIF) comedication of rifampicin (10.0 mg/kg b.i.d., 11 days). The cell/plasma ratios are given for 8 healthy foals (the plasma level was in one animal <LOQ).

Figure 3: 4β-OH-cholesterol/cholesterol plasma concentration ratios before (-RIF) and after (+RIF) treatment with rifampicin (10.0 mg/kg b.i.d., 11 days) in 9 healthy foals.

Figure 4: Expression of *ABCB1*mRNA, *ABCC2*mRNA and *PXR*mRNA (relative to *18S*rRNA) in bronchoepithelial cells (upper figures) and bronchoalveolar lavage cells (lower figures) before (-RIF) and after (+RIF) treatment with rifampicin (10.0 mg/kg b.i.d., 11 days) in 9 healthy foals.

Figure 5: Correlation between the mRNA content of PXR and ABCC2 bronchoepithelial cells (left) and brochoalveolar lavage cells (right) in 9 healthy foals.

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Figure 6: Expression of *OATP1A2*mRNA and *OATP2B1*mRNA (relative to *18S*rRNA) in bronchoepithelial cells (EC) and bronchoalveolar lavage cells (BALC) before (-RIF) and after (+RIF) treatment with rifampicin (10.0 mg/kg b.i.d., 11 days) in 9 healthy foals.

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Table 1: Pharmacokinetic characteristics of clarithromycin and 14-hydroxyclarithromycin in nine healthy foals at steady state after monotherapy of clarithromycin (7.5 mg/kg body weight, bid for 7 days) followed by comedication with rifampicin (10 mg/kg body weight, bid for 11 days). Furthermore concentrations of clarithromycin and 14-hydroxyclarithromycin in plasma

		$AUC_{0\text{-}12h}$ (µg×h/ml)	C_{max} (ng/ml)	C_{min} (ng/ml)	C_{av} (ng/ml)	t_{max} (h)	$t_{½}$ (h)	$plasma_{12h}$ (ng/ml)	ELF_{12h} (µg/ml)	$BALC_{12h}$ (µg/ml)
clari-thromycin	without RIF	5.54 ± 4.42	614 ± 365	262 ± 254	462 ± 368	3.73 ± 1.79	6.11 ± 0.83	301 ± 270	9.49 ± 6.12	264 ± 375
	RIF	0.35 ± 0.31*	65.0 ± 51.5*	9.26 ± 9.63*	29.8 ± 26.3*	2.84 ± 1.79	6.88 ± 3.44	13.9 ± 15.8	0.69 ± 0.66*	10.2 ± 10.2*
14-hydroxy-clari-thromycin	without RIF	1.58 ± 1.03	161 ± 69.9	84.7 ± 68.1	132 ± 86.0	3.56 ± 2.59	8.11 ± 1.61	92.0 ± 64.3	0.41 ± 0.20	4.73 ± 5.49
	RIF	0.72 ± 0.44*	109 ± 54.3*	21.0 ± 21.3*	60.4 ± 36.8*	2.89 ± 1.76	5.10 ± 0.88*	29.6 ± 26.7	0.13 ± 0.08*	1.71 ± 1.22*

*p<0.05 (Wilcoxon test)

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Table 2: Ratios of clarithromycin and 14-hydroxyclarithromycin 12 h after respective last administration of clarithromycin in epithelial lining fluid (ELF) to plasma, bronchoalveolar lavage cells (BALC) to plasma and BALC to ELF after monotherapy (without RIF) of clarithromycin (7.5 mg/kg body weight, bid for 7 days) followed by combination treatment (with RIF) of clarithromycin (7.5 mg/kg body weight, bid for 13 days) and rifampicin (10 mg/kg body weight, bid for 11 days) are given.

		ELF/plasma	BALC/plasma	BALC/ELF
clarithromycin	without RIF	36.1 ± 14.8	757 ± 478	25.8 ± 23.0
	RIF	62.0 ± 48.6*	838 ± 449	14.3 ± 6.79
14-hydroxyclarithromycin	without RIF	4.82 ± 1.86	44.2 ± 17.1	11.2 ± 9.14
	RIF	4.88 ± 2.02	63.0 ± 20.0	14.2 ± 4.90

*p<0.05 (Wilcoxon test)

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Figure 1

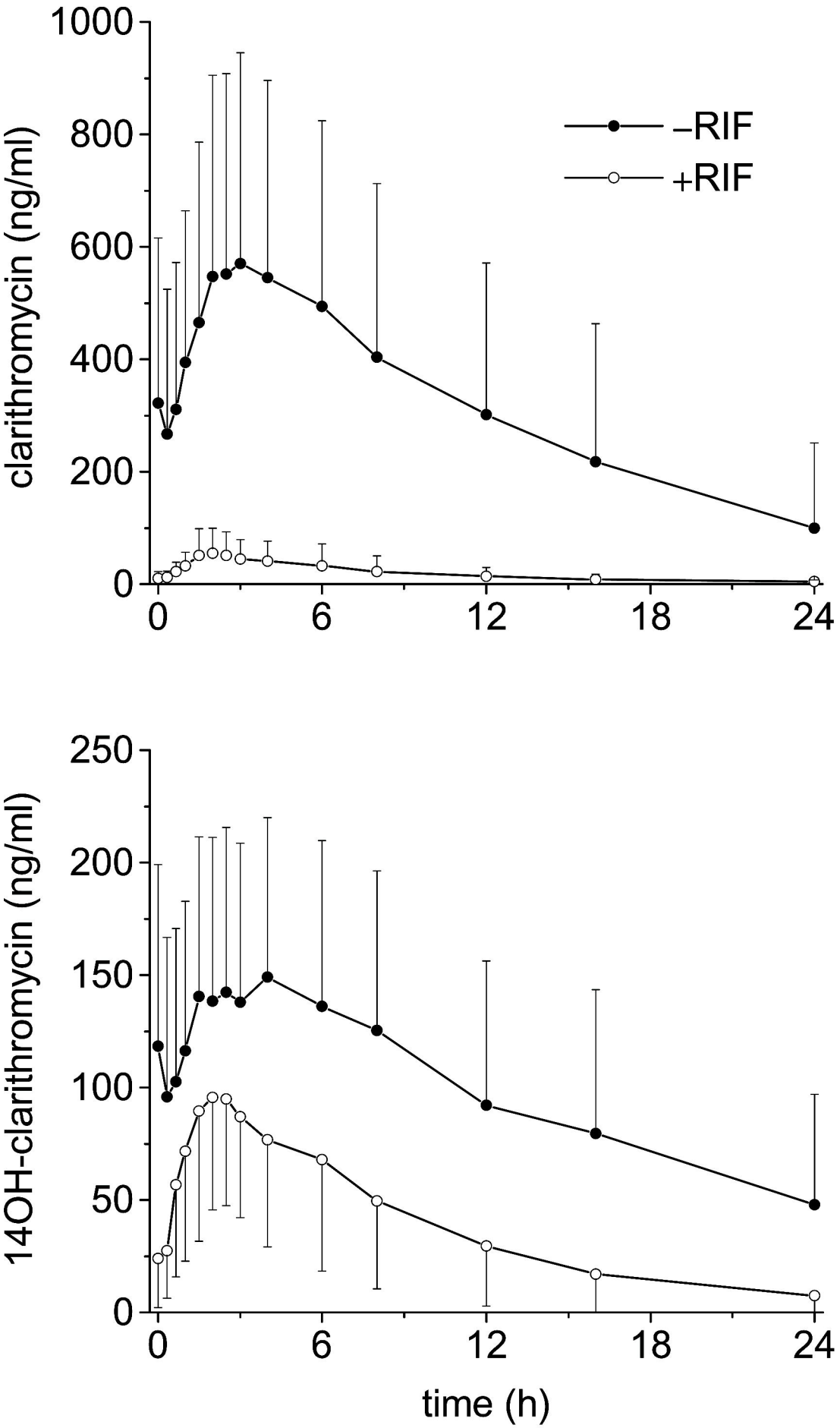

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Figure 2

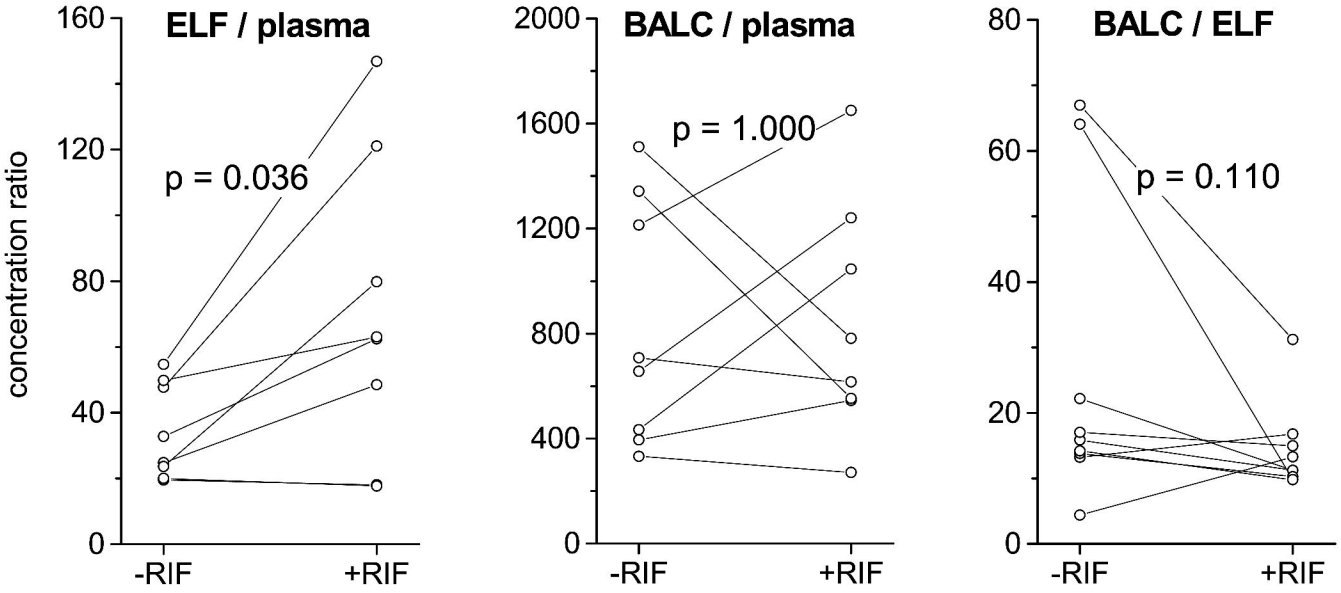

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Figure 3

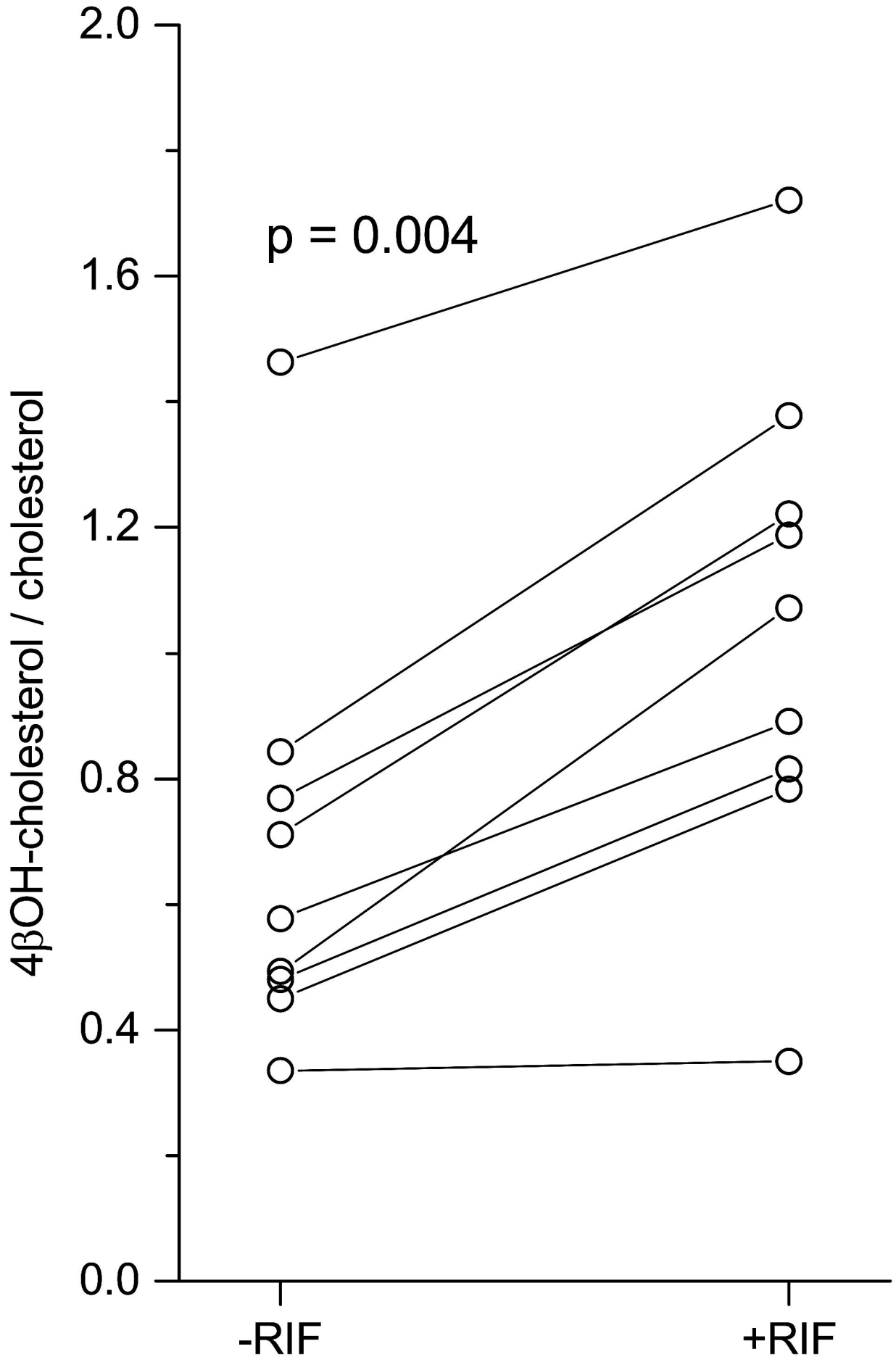

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Figure 4

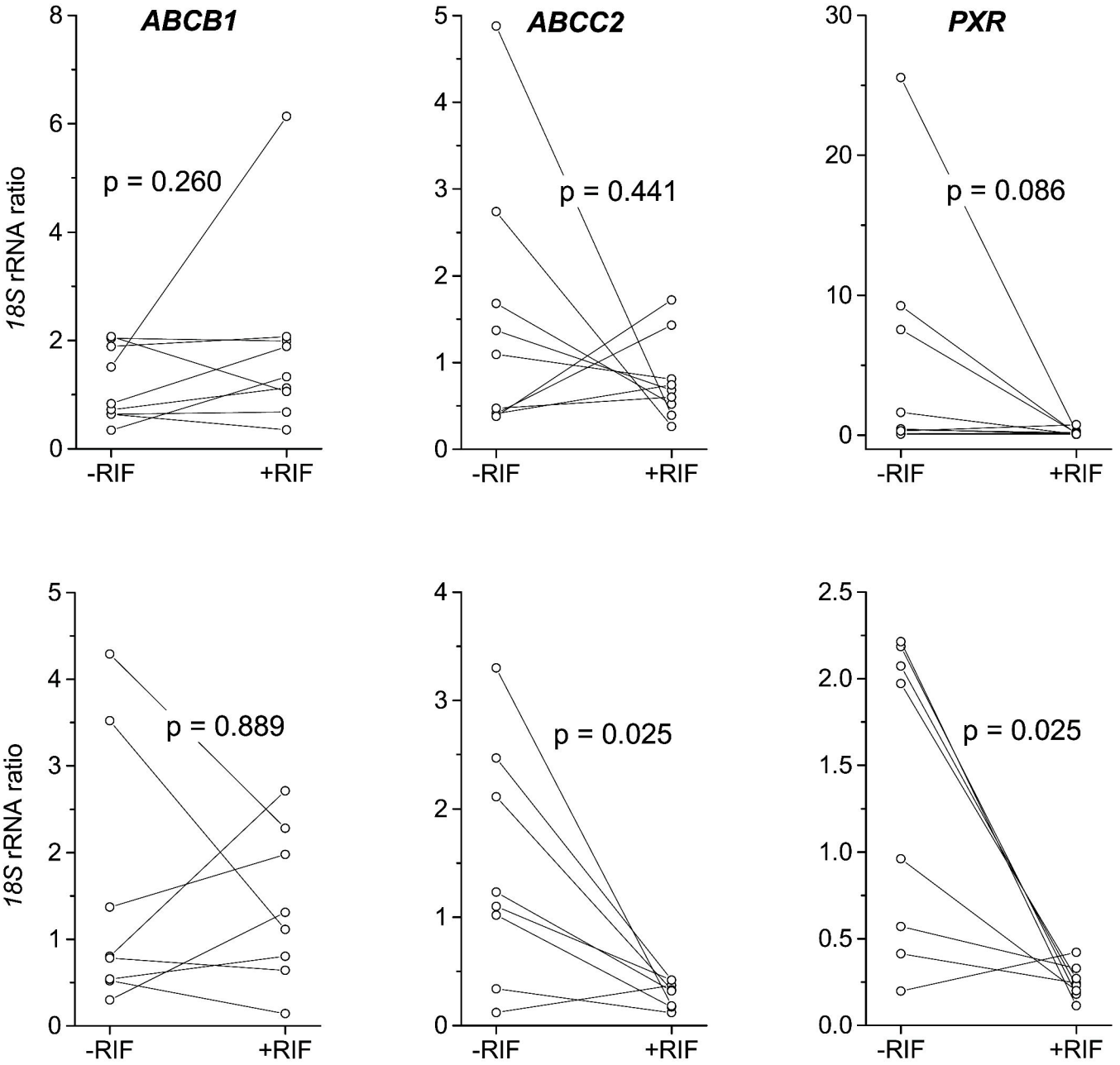

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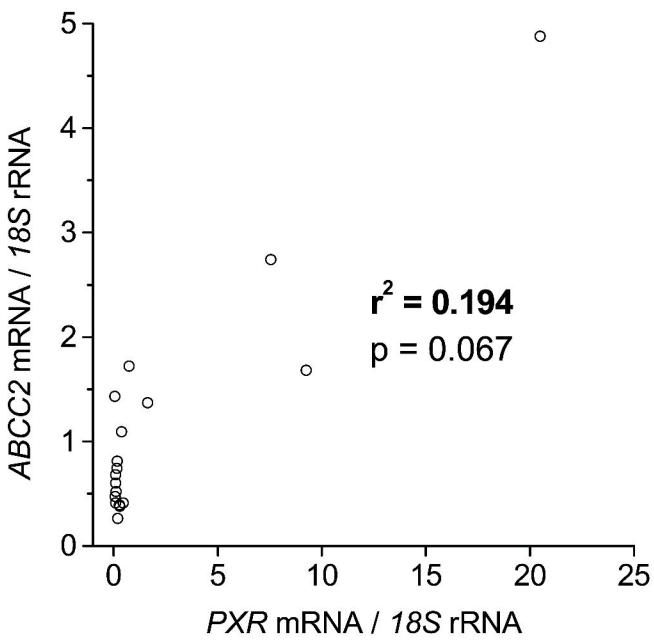
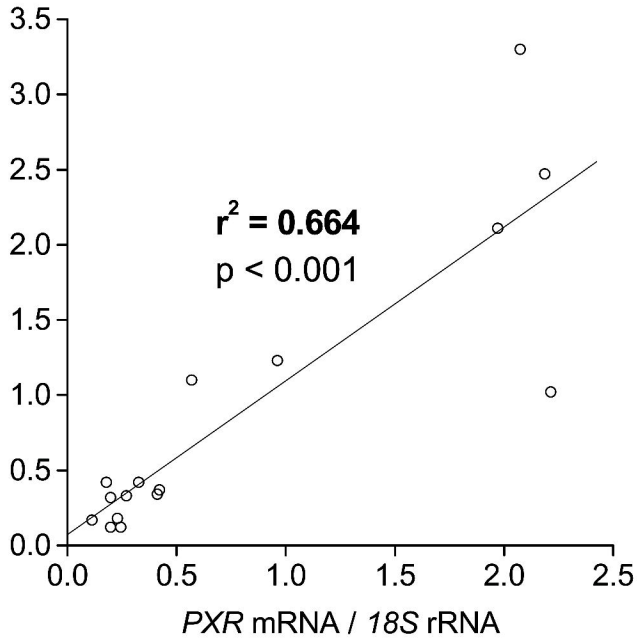
Figure 5





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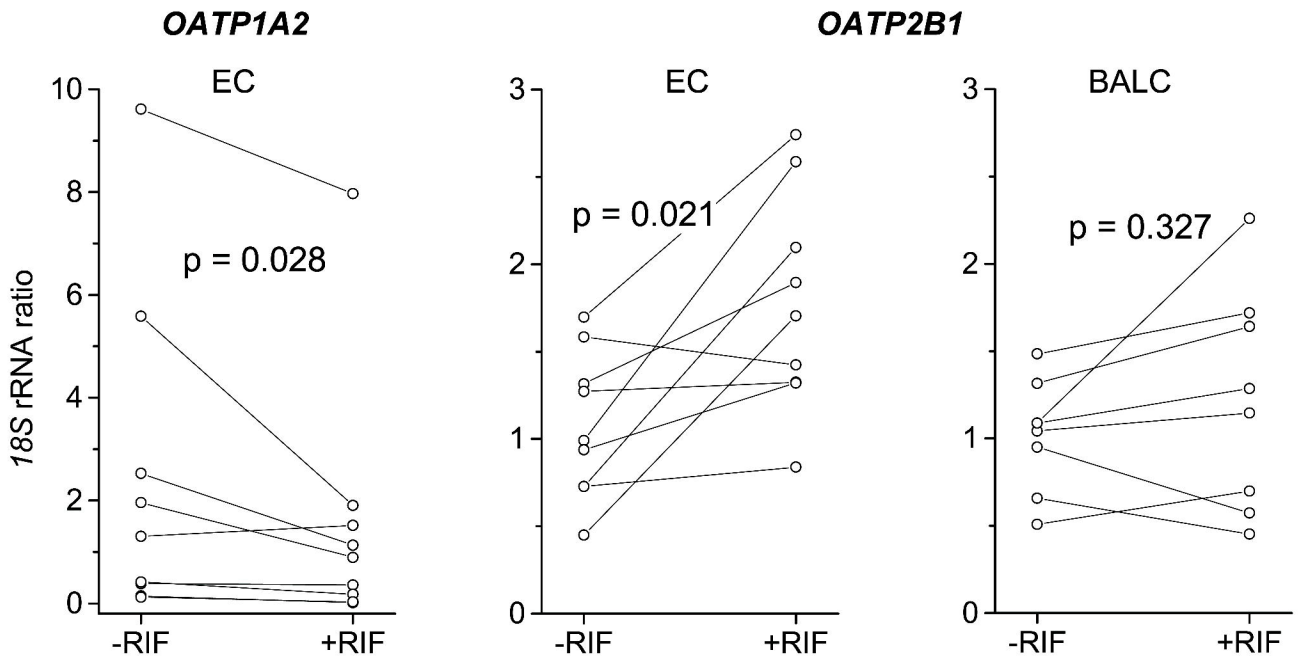
Figure 6