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Higher clearance of micafungin in neonates compared to adults: role of age-dependent micafungin serum binding

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Abstract:

Micafungin, a new echinocandin antifungal agent, has been widely used for the treatment of various fungal infections in human populations. Micafungin is predominantly cleared by biliary excretion and it binds extensively to plasma proteins (>99.5%). Micafungin body weight-adjusted clearance is higher in neonates than in adults, but the mechanisms underlying this difference are not understood. Previous work had revealed the roles of sinusoidal uptake (Na⁺-taurocholate co-transporting peptide, NTCP; organic anion transporting polypeptide, OATP) as well as canalicular efflux (bile salt export pump, BSEP; breast cancer resistance protein, BCRP) transporters in micafungin hepatobiliary elimination. In the present study, the relative protein expression of hepatic transporters was compared between liver homogenates from neonates and adults. Also, the extent of micafungin binding to serum from neonates and adults was measured in vitro. The results indicate that relative expression levels of NTCP, OATP1B1/3, BSEP, BCRP, and MRP3 were similar in neonates and in adults. However, micafungin fraction unbound (fu) in neonatal serum was about 8-fold higher than in adult serum (0.033 ± 0.012 versus 0.004 ± 0.001, respectively). While there was no evidence for different intrinsic hepatobiliary clearance of micafungin between neonates and adults, our data suggest that age-dependent serum protein binding of micafungin is responsible for its higher clearance in neonates compared to adults.

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4 **Higher clearance of micafungin in neonates compared to adults: role of**
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13 Souzan B. Yanni, Philip B. Smith, Daniel K. Benjamin Jr., Patrick F. Augustijns, Dhiren R.
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4 **Running title:** Mechanisms underlying micafungin clearance in neonates
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6 **Abbreviations:** BCRP, Breast Cancer Resistance Protein; BSEP, Bile Salt Export Pump;
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8 Cl, Clearance; f_u , fraction of drug unbound; HPLC, High Performance Liquid
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10 Chromatography; MRP2 and MRP3, Multidrug Resistance-associated Protein 2 and 3;
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12 NTCP, Na⁺-Taurocholate Co-transporting Peptide; OATP, Organic Anion Transporting
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14 Polypeptide; P-gp, P-glycoprotein; Q_H , hepatic blood flow; SCHH, Sandwich-Cultured
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For Peer Review

Abstract

Micafungin, a new echinocandin antifungal agent, has been widely used for the treatment of various fungal infections in human populations. Micafungin is predominantly cleared by biliary excretion and it binds extensively to plasma proteins (>99.5%). Micafungin body weight-adjusted clearance is higher in neonates than in adults, but the mechanisms underlying this difference are not understood. Previous work had revealed the roles of sinusoidal uptake (Na^+ -taurocholate co-transporting peptide, NTCP; organic anion transporting polypeptide, OATP) as well as canalicular efflux (bile salt export pump, BSEP; breast cancer resistance protein, BCRP) transporters in micafungin hepatobiliary elimination. In the present study, the relative protein expression of hepatic transporters was compared between liver homogenates from neonates and adults. Also, the extent of micafungin binding to serum from neonates and adults was measured *in vitro*. The results indicate that relative expression levels of NTCP, OATP1B1/3, BSEP, BCRP, and MRP3 were similar in neonates and in adults. However, micafungin fraction unbound (f_u) in neonatal serum was about 8-fold higher than in adult serum (0.033 ± 0.012 versus 0.004 ± 0.001 , respectively). While there was no evidence for different intrinsic hepatobiliary clearance of micafungin between neonates and adults, our data suggest that age-dependent serum protein binding of micafungin is responsible for its higher clearance in neonates compared to adults.

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Keywords: micafungin, pediatric drug disposition, serum binding, clearance prediction, antifungal

Introduction

Micafungin is a new semi-synthetic echinocandin-type antifungal drug that has been widely used for the treatment of various fungal infections in human populations. Pharmacokinetic studies with ^{14}C -micafungin determined that approximately 90% of the plasma clearance occurs via biliary elimination of the parent drug [1], whereas urinary excretion is a minor elimination route in human and rat [2-4]. Like caspofungin and anidulafungin, micafungin is a peptide-like compound. Due to the presence of a sulfate group, micafungin is negatively charged at physiological pH (see Figure 1). Micafungin displays high plasma protein binding (>99%) in humans and animals [4].

Pharmacokinetic parameters of micafungin were determined in several human age groups: neonates < 1 month old, children <8 years of age, adolescents, and adults [5, 6]. Micafungin was found to have a higher body weight-adjusted clearance in neonates than older groups. The necessity to use higher body-weight adjusted doses in neonates in order to achieve therapeutic plasma concentrations was recently confirmed by a population pharmacokinetic analysis [7]. As of today, no studies have been conducted to elucidate the cause of this age-difference in the disposition of micafungin in humans.

In order to obtain better insight in the age-dependent disposition of micafungin, it was crucial to first understand the clearance mechanisms in the adult population. In this context, we have recently conducted *in vitro* transport studies with micafungin in sandwich-cultured human hepatocytes (SCHH) [8], which revealed the following: a) the sinusoidal uptake transporter Na^+ -taurocholate co-transporting peptide (NTCP, *SLC10A1*) and the canalicular efflux transporter Bile Salt Export Pump (BSEP, *ABCB11*) were found to mediate the hepatobiliary disposition of micafungin; b) Breast Cancer Resistance Protein (BCRP, *ABCG2*), Multidrug Resistance Associated Protein 3 (MRP3, *ABCC3*), and Organic Anion Transporting

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2 Polypeptide (OATP, *SLCO*) were found to modestly associate with the disposition of
3 micafungin in SCHH; c) by using B-CLEAR[®] technology (Qualyst Inc., Research Triangle
4 Park, NC) [9-11], *in vivo* plasma and biliary clearance of micafungin were calculated based
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6 on the *in vitro* data obtained in SCHH and found to be in agreement with the clinical data.
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10 Because of ethical considerations, it is almost impossible to obtain fresh liver tissue from
11 neonates suitable for establishing SCHH, which would support investigating transporter-
12 mediated disposition of micafungin and predicting *in vivo* hepatobiliary clearance like in
13 adults. However, it was possible to obtain frozen liver tissue samples from neonates (and
14 adults) to support determining the protein expression of hepatic drug transporters. In addition,
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16 because protein binding is known to influence drug distribution and drug clearance, and
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18 because it is variable as a function of age [12-15], the extent of micafungin serum binding in
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20 neonates was determined *in vitro*.
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28 In this study, we calculated *in vivo* hepatobiliary clearance of micafungin in neonates based
29 on: a) the *in vitro* intrinsic clearance in adults as determined previously [8]; b) scaling factors
30 applicable for human neonatal liver; and c) micafungin fraction unbound in serum of
31 neonates. We showed that the calculated *in vivo* plasma and biliary clearance in neonates are
32 in agreement to those observed in clinic. Our data provide evidence for the mechanism
33 underlying higher clearance of micafungin in neonates compared to adults.
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Materials and Methods

Chemicals and Reagents. Micafungin–sodium was a gift from Dr. John Perfect at Duke University. Serum samples from neonates or adult (n=6/group) were provided by Dr. Daniel Benjamin at Duke Center Research Institute. Frozen liver from neonates and adults (5 samples per group) were obtained from the Comparative Human Tissue Network (CHTN) (Columbus, OH) under an approved UNC-Chapel Hill Institutional Review Board protocol. NuPAGE 4-20% Bis-Tris gel and all other gel electrophoresis and Western blot reagents and supplies were purchased from Invitrogen (Carlsbad, CA). Primary antibodies against human BSEP [*ABCB11*] (F-6), NTCP [*SLC10A1*] (M-130) and OATP1B1 [*SLCO1B1*] (and OATP1B3 [*SLCO1B3*] to a lesser extent) (N-16) were purchased from Santa Cruz (Santa Cruz, CA), while those against MRP3 [*ABCC3*] (M3II-9), BCRP [*ABCG2*] (BXP-53), and GAPDH were purchased from Alexis (San Diego, CA). Horseradish peroxidase-conjugated secondary antibody suitable for each primary antibody and the chemi-luminescent substrate SuperSignal® West Dura were purchased from Pierce Chemical (Rockford, IL).

Protein Expression of Drug Transporters in Liver Tissues from Neonates and Adults.

Frozen liver tissues from neonates (postnatal age 0-1 month), and from adults (older than 18 years) were homogenized in 1.15% KCL buffer, pH 7.4 (1:3, w/v) and crude homogenates were prepared after centrifugation at 800 rpm for 3 min. Protein concentration in crude liver homogenates was determined by the bicinchoninic acid method against bovine serum albumin standard, and homogenates at 1mg/ml were prepared in sample buffer containing a protease inhibitors cocktail (Sigma-Aldrich, St. Louis, MO). Western blot analysis for the expression of transport proteins was performed by separating the transport proteins in liver homogenate samples loaded on SDS page gel (20 µl/well) with gel electrophoresis and the proteins were then transferred to nitrocellulose membranes according to the vendor's

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2 instructions (Invitrogen, Carlsbad, CA). Primary antibodies against human NTCP, BSEP,
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4 OATP1B1/3, MRP3, BCRP, GAPDH were appropriately diluted (1:200, 1:200, 1:200, 1:200,
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6 1:500, and 1:4000, respectively) and incubated with the membrane for 2 hr at 37°C.
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8 Horseradish peroxidase-conjugated secondary antibody suitable for each primary antibody
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10 was cross-reacted with the transporter protein-primary antibody complex; immunoreactive
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12 protein bands were detected using a Bio-Rad VersaDoc imaging system (Bio-Rad, Hercules,
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14 CA). For quantitative determination of protein expression, three density measurements of
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16 each band corresponding to BSEP, OATP1B1/3, MRP3, BCRP, GAPDH (molecular weight
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18 190 kDa, 90 kDa, 170 kDa, 70 kDa, 35 kDa, respectively) were determined and the mean
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20 density was normalized to the mean density of the GAPDH band (used to adjust expression
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22 based on actual loaded protein samples). Relative expression of the four transporters to
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24 GAPDH in each liver homogenate sample in the neonatal or adult group was determined for
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26 each of the five liver homogenate samples per group. The mean was expressed per kg body
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28 weight based on the following scaling parameters: 35 and 70 mg protein/gram liver in
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30 neonates and adult (experimentally determined), respectively; 120 g and 1500 g average liver
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32 weight in neonates or adult (data listed by Björkman [16]), respectively; 3 kg and 70 kg
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34 average body weight in neonates and adult (data listed by Björkman [16]), respectively. The
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36 relative protein expression in neonates was expressed as percent of the mean expression in
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38 adult samples.

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40 **Micafungin Serum Fraction Unbound in Neonates and Adults.** Micafungin fraction
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42 unbound in serum from human neonates or adults was determined by equilibrium dialysis
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44 according to a modified method described by Kalvass and Maurer [17]. Briefly,
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46 Spectra/Por[®] 2 dialysis membranes (Spectrum Laboratories Inc., Rancho Dominguez, CA)
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48 were conditioned in water for 15 min, followed by 30% ethanol for 15 min, and sodium
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50 phosphate buffer (100 mM, pH 7.4) for 15 min. Serum samples from neonates (prenatal age
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2 23-40 weeks of gestation; postnatal age 1-52 days; body weight 0.6-3.5 kg) (n=6) or adults
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4 (n=6) were spiked with micafungin (10 μM) and 70 μl aliquots of each sample were loaded
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6 into a 96-well equilibrium dialysis apparatus (HTDialysis, Gales Ferry, CT) and dialyzed
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8 against an equal volume of sodium phosphate buffer (100 mM, pH 7.4). The 96-well
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10 equilibrium dialysis apparatus was incubated for 4.5 hr with orbital shaking (100 rpm) at
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12 37°C. These equilibrium dialysis conditions were found to be sufficient for micafungin to
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14 reach equilibrium. Aliquots from dialyzed samples (serum side and buffer side) were
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16 processed and the concentrations of extracted micafungin in both serum and buffer matrices
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18 were determined based on calibration curves of micafungin prepared in the corresponding
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20 matrix. Micafungin f_u was determined according to Equation 1.
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22 **Analytical Method.** An HPLC method was developed to separate micafungin and diclofenac
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24 (internal standard) using a C8 XTerra® column (3 x 100 mm, 3.5 μm Waters); a linear gradient
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26 of mobile phase A:B (v/v) was used starting from 70:30 at 0 min to 5:95 in 9 minutes at a
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28 flow rate of 0.5 ml/min, where (A) was 5 mM ammonium acetate with 0.1% formic acid, pH
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30 4.0, and (B) was acetonitrile with 0.1% formic acid. Micafungin was detected by
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32 fluorescence emission at 464 nm (excitation at 273 nm), while diclofenac was detected by
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34 UV at 280 nm; the retention times were 5.5 min for micafungin and 6.3 min for diclofenac.
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36 The method was linear for a micafungin concentration range of 0.1-5 μM . Accuracy and
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38 precision of the method were 12% and 3.3% for 0.3 μM and 0.2% and 2.8% for 2.5 μM ,
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40 respectively.
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42 **Data Analysis.** Micafungin f_u was determined from the following equation:

$$f_u = \frac{[\text{micafungin}_{\text{buffer}}]}{[\text{micafungin}_{\text{plasma}}]} \quad (\text{Equation 1})$$

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47 The f_u values in six neonates were plotted against the age of gestation at birth (in weeks) or
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49 against postnatal age at birth (in days) of each subject.
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In vivo micafungin plasma and biliary clearance values were predicted based on previously obtained *in vitro* data [8], and the serum binding data obtained in the present study. The *in vitro* intrinsic biliary clearance (ml/min/mg protein) and *in vitro* intrinsic accumulation clearance were first scaled to kg body weight using the parameters listed in Table 1 as published by Björkman *et al.* [16]. In the absence of albumin during *in vitro* incubations [8], unbound intrinsic biliary clearance (intrinsic $Cl'_{biliary}$) and unbound intrinsic plasma clearance (intrinsic Cl'_{plasma}) were assumed to correspond to *in vitro* intrinsic biliary clearance and *in vitro* intrinsic accumulation clearance, respectively. The calculated *in vivo* biliary clearance ($Cl_{biliary}$) and *in vivo* plasma (accumulation) clearance (Cl_{plasma}) values were calculated according to the well-stirred model of hepatic disposition, assuming a micafungin blood/plasma concentration ratio of 1:

$$\text{Calculated } in \text{ vivo } Cl_{biliary} = \frac{Q_H \times f_u \times \text{intrinsic } Cl'_{biliary}}{Q_H + f_u \times \text{intrinsic } Cl'_{biliary}} \quad (\text{Equation 2})$$

$$\text{Calculated } in \text{ vivo } Cl_{plasma} = \frac{Q_H \times f_u \times \text{intrinsic } Cl'_{plasma}}{Q_H + f_u \times \text{intrinsic } Cl'_{plasma}} \quad (\text{Equation 3})$$

where Q_H represents the hepatic plasma flow rate. The Q_H values in neonate and adult are listed in Table 1.

Statistical Analysis. An unpaired *t*-test (assuming unequal variance) was performed to determine the significance of the difference in micafungin f_u between neonates and adults.

Results

Protein Expression of Drug Transporters in Liver Tissues from Neonates and Adults.

The immunostaining bands corresponding to NTCP, BSEP, BCRP, MRP3, and the hepatic isoforms belonging to the OATP1B subfamily in five neonatal liver homogenate samples (N1-N5) and in five adult liver homogenate samples (A1-A5) are shown in the upper part of

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Figure 2. As illustrated in the lower panel of Figure 2, the protein expression levels of these transporters in neonates, relative to GAPDH and expressed per kg body weight, were between 84 and 115% of adult levels. No statistically significant differences were observed in transporter expression levels between neonates and adults.

Serum Fraction Unbound of Micafungin in Neonates and Adults. Using equilibrium dialysis, micafungin f_u was determined as function of time and drug concentration (data not shown). In neonates, the mean f_u from six different neonatal samples conducted at three different days was found to be 0.033 ± 0.017 (96.7% bound) compared to a mean f_u value of 0.004 ± 0.003 (99.6% bound) in adult (Figure 3). The difference of f_u between neonates and adults is statistically significant ($P < 0.001$). Figure 4 illustrates the individual f_u values in neonates along with the postgestational age at birth as well as the postnatal age. The highest f_u was observed for the full term subjects 1 and 3 (40 weeks postgestation), while lower f_u values were found for premature subjects 2 and 4 (24 and 31 weeks of gestation, respectively). The lowest micafungin f_u was observed for subject 2, which is not only the most premature, but also the oldest (52 days after birth) baby.

These results indicate that micafungin f_u in neonate serum is higher than in adult serum. These results also indicated that, while there appears to be a weak relationship between postgestational age at birth and f_u , there is no evidence for a relationship between postnatal age in neonates and f_u .

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2 **Prediction of *in vivo* Plasma and Biliary Clearance of Micafungin in Neonates.** *In vitro*
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4 intrinsic accumulation CI and *in vitro* intrinsic biliary CI of micafungin have recently been
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6 determined using sandwich-cultured human hepatocytes from adult liver donors [8], and were
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8 23.5 ± 1.6 and 14.0 ± 9.0 $\mu\text{l}/\text{min}/\text{mg}$, respectively. To estimate *in vitro* intrinsic clearance
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10 values for micafungin in neonates, *in vitro* intrinsic clearance values of adults were used,
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12 given that expressions of drug transporters associated with the clearance of micafungin in
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14 neonates and adults were comparable (Figure 2). As illustrated in Table 1, intrinsic clearance
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16 values were scaled to *in vivo* intrinsic clearance using neonate-based scaling factors as
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18 reported [16, 18]. *In vivo* intrinsic clearance values were then used to calculate *in vivo*
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20 clearance values based on the well-stirred model (Equation 2 and Equation 3), taking into
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22 account micafungin f_u (as determined in the present study) as well as adult/neonatal values
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24 for Q_H . The results shown in Table 1 illustrate a good agreement between the observed and
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26 calculated plasma clearance values. In addition, the calculated clearance values in neonates
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28 were 3-fold higher than in adults as it has been observed in clinic.
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Discussion

Micafungin, which is used to treat life-threatening fungal CNS infection in neonates and young infants, is mainly cleared into the bile [1]. Surprisingly, the clearance of micafungin in neonates, the human population generally displaying immature drug metabolizing enzymes and drug transporter function, is reported to be three times that of adults. Because it undergoes biliary clearance, we hypothesized that the higher clearance of micafungin in neonates might be due to the higher drug uptake and/or secretion by sinusoidal and canalicular liver transporters. The transporters that mediate the uptake and secretion of micafungin were identified in our previous *in vitro* mechanistic study using human sandwich-cultured hepatocytes prepared from fresh liver of adult donors [8]. These *in vitro* data confirmed the unique utility of this *in vitro* model system in exploring the mechanisms of hepatic uptake and biliary excretion of endogenous and exogenous compounds and in predicting *in vivo* biliary clearance of drugs [9-11, 19-22]. In our previous study, we have indeed reported that in *in vitro* hepatic accumulation of micafungin in adult human hepatocytes occurred mainly by transporter-mediated mechanisms. The uptake of micafungin into the hepatocytes was mediated by NTCP >> OATP, whereas the biliary excretion involved the canalicular transporters BSEP > BCRP. The results also suggested a role for the sinusoidal efflux transporter MRP3, in the hepatic disposition of micafungin.

Unfortunately, because of ethical considerations, it is nearly impossible to obtain human neonatal liver tissue suitable for establishing hepatocyte cultures. Therefore, we considered another approach to obtain better insight into the age-dependent clearance of micafungin. By measuring the protein expression of NTCP, BSEP, BCRP, MRP3 and both hepatic OATP1B isoforms in neonatal versus adult liver homogenates, we pursued to make a semi-quantitative assessment regarding the possible age-dependent activity of these

Deleted: The biliary excretion of micafungin, rather than its hepatic accumulation, appeared to be **rate-limiting** for its hepatic elimination in adult liver.

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transporters. A few studies on the ontogeny of transporter expression and function have indeed demonstrated that transporter protein expression levels correlate with function. For example, in a developmental mouse model, Pinto *et al.* [23] showed that digoxin accumulation in brain, kidney, and liver agreed with the expression level of the Mdr1a gene encoding for the P-gp efflux transporter in an age-dependent manner. Goralski *et al.* [24] have shown an agreement between the expression and function of transporters by demonstrating that deficient Mdr1a/b expression in neonatal mouse brain resulted in enhanced accumulation of digoxin and cyclosporin in neonatal mice compared to adult mice.

As mentioned earlier, we have previously shown that micafungin is predominantly relying on

the bile salt transporters NTCP and BSEP for its hepatobiliary elimination. At the uptake level, our data support comparable expression of the main micafungin transporter NTCP

between neonates and adults. Previous work by Hardikar *et al.* [25] in rats, indicated that

Ntcp protein was expressed as early as postnatal day 1, and the expression levels on day 1

and day 7 (equivalent to 1 month human neonate) postnatal liver were also comparable to

those in adults. Furthermore, as shown in this study (see Figure 2), also BSEP exhibits

comparable expression in neonates and in adults. This result was consistent with previous

data on quantitative expression of BSEP and other canalicular transport proteins in human

fetal, pediatric and adult livers [26, 27]. In these studies, the authors reported that BSEP

protein was expressed in fetus and pediatric human liver at the same level as adult, however,

in fetus, the localization of BSEP was found to be at both the canalicular and cytosolic

locations. In general, very limited information on the ontogeny of drug transporters in human,

not only at the functional level, but also at the expression level has been obtained. Studies on

the age-dependent expression and localization of MDR1/P-gp in human brain [28] and small

intestine [29] are exceptions. In the rat, Gao *et al.* observed that the canalicular transporter

Bsep was detected at the canalicular membrane before birth but reached adult expression

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2 levels, by postnatal day 12 (day 7 in neonatal rat is equivalent to neonate in human) [30]. The
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4 authors indicated that by day 12, hepatocytes were fully polarized and localization of
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6 transporters (at either canalicular or sinusoidal membranes) was comparable to what was
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8 observed in adult hepatocytes. They also reported that the development of polarized
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10 hepatocyte membranes and expression of major bile salt transporters Bsep and Ntcp occurs
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12 shortly before birth and that correlated with the development of the enterohepatic circulation
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14 of bile salts. Therefore, full development of this excretion route early in neonatal life
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16 indicates that age-dependent expression (and thus function) of these transporters may not
17
18 explain the age-dependent clearance of micafungin. It follows that the similarity in the
19
20 expression of these transporters between neonates and adults can confidently be used to
21
22 quantitatively estimate micafungin hepatic clearance in neonates, based on the *in vitro*
23
24 intrinsic uptake and biliary clearance that we previously measured for adults.

25
26 Because protein binding is known to affect drug clearance, especially of those drugs that
27
28 possess high protein binding like micafungin, we measured f_u of micafungin *in vitro* in serum
29
30 from neonates and adults; as mentioned in the results section, we observed a significantly
31
32 higher unbound fraction in neonates. McNamara and Alcorn [12] have shown that for
33
34 lipophilic drugs, ~ 2-fold higher f_u values can be expected in pediatric plasma compared to
35
36 adults based on lower plasma protein concentrations in neonates than in adults. However, the
37
38 model that was developed by McNamara and Alcorn [12] under-predicted the f_u value of
39
40 micafungin in neonates compared to the value we have experimentally measured in the
41
42 present study using *in vitro* equilibrium dialysis in neonate serum. One explanation for the 8-
43
44 fold higher f_u in neonates compared to adult could be that micafungin binds not only to
45
46 plasma proteins [more likely to albumin than gamma-globulin (31)], but also to lipoproteins,
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48 cholesterol, or other serum components that were found to be higher in adult serum than in
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50 neonate serum [12,15]. Furthermore, the physicochemical properties of a peptide like
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2 micafungin differ substantially from those of the model drugs used to establish the model
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4 obtained by McNamara and Alcorn [12]. Due to limited availability of clinical
5
6 pharmacokinetic data in neonates, it is presently unclear to what extent lower protein binding
7
8 (higher free fraction in the blood) in neonates versus adults is a more common reason for
9
10 enhanced drug clearance in neonates. In addition, in contrast to what we have presently
11
12 observed for micafungin, the enzyme or transporter-mediated elimination pathways of many
13
14 drugs show pronounced age-dependency [32-34], thus complicating the interpretation of
15
16 possible effects of altered protein binding on overall *in vivo* drug elimination. Caspofungin,
17
18 which is another echinocandin antimycotic, also shows a small unbound fraction in blood
19
20 (about 3.5%) [35]. However, in contrast to micafungin, caspofungin clearance in neonates (<
21
22 3 months) is significantly lower as compared to older children (2-11y) and, to a lesser extent,
23
24 as compared to adults [36]. For this reason, the caspofungin recommended dose (normalized
25
26 for body surface area) in neonates is about half the dose used in adults. While the
27
28 caspofungin free fraction in neonate serum has not been measured, there is clearly no
29
30 evidence that reduced caspofungin serum binding in neonates plays an important role in
31
32 altered caspofungin disposition in this age group. The immunosuppressant cyclosporin A is
33
34 another cyclic peptide exhibiting low unbound fraction in blood as well as age-dependent
35
36 pharmacokinetics, with 2-fold lower oral clearance in neonates compared to older children
37
38 and adults [37]. However, cyclosporin A shows a complex binding profile in plasma (mainly
39
40 to lipoproteins) as well as in blood (distribution to erythrocytes) [38], while the ontogeny of
41
42 this behavior has not been investigated. From a more general point of view, the clinical
43
44 relevance of plasma protein binding alterations (most often considered from a binding
45
46 competition perspective) as a mechanism underlying drug clearance differences should not be
47
48 overestimated [39-40]. Nevertheless, the 8-fold increase in unbound micafungin in neonates
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50 as compared to adults is substantial, especially in light of the less pronounced effects reported
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2 previously for a list of more than 25 drugs [12]. In this list, the largest differences were
3 reported for propranolol, quinidine and verapamil, which all exhibited 3-4 fold increased
4 unbound fractions in neonates compared to adults. Unfortunately, clinical PK data in
5 children, especially neonates, are very limited or lacking. In the case of verapamil, Piovan et
6 al. [41] reported plasma levels of verapamil and its metabolite norverapamil in children
7 between 15 days and 17 years. Their data demonstrated gradually increasing
8 norverapamil/verapamil levels from birth onwards to reach a plateau by the age of 1 year,
9 which then remained constant until adulthood. There was no evidence for any effect of an
10 increased free verapamil fraction on verapamil clearance in neonates as compared to older
11 children. This further suggests that for many drugs, altered plasma protein binding in
12 neonates does not have a substantial impact on clearance. In addition, even when some effect
13 would exist, it may not be easily observed due to concomitant changes in activity of drug
14 metabolizing enzymes and transporters [32-34].
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28 As we have indicated in the results section (Table 1), the *in vivo* plasma clearance and biliary
29 clearance values we calculated for micafungin in neonates were found to be three times those
30 in adults, thus mirroring the difference in clearance between neonates and adults observed in
31 the clinic. Since the calculated *in vivo* clearance was based on the adult intrinsic clearance
32 values, the higher f_u value of micafungin in neonates, is the factor that explains the higher *in*
33 *in vivo* clearance of micafungin in neonates. According to the well-stirred model applied here,
34 neither the different relative liver protein content (mg/kg body weight) nor the higher Q_H in
35 neonates, contributed to the higher *in vivo* clearance of micafungin in neonates.
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44 To summarize, similar protein expression of liver drug transporters, but higher f_u of
45 micafungin in neonates compared to adults have been determined by using *in vitro* methods
46 and biological samples from both age groups. The *in vivo* plasma and biliary clearance in
47 neonates calculated based on *in vitro* transport data of adults generated by SCHH indicated
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2 that f_u may be the key contributor to the higher clearance of micafungin observed in neonates,
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4 however a definitive conclusion could not be made with the results that have been presented
5
6 in this study. For instance, a possible role of one or more transporters that differ from those
7
8 identified in adults may still be involved in micafungin disposition in neonatal liver. In
9
10 addition, we assumed that neonates display no additional enzymatic clearance mechanisms
11
12 for micafungin as compared to adults. However, Hope *et al.* [7] recently reported that
13
14 substantially higher levels of the metabolite M5 were measured in neonates as compared to
15
16 adults. It is clear that the existence of a neonate-specific metabolic pathway for micafungin
17
18 could also contribute to its higher clearance, but further research is required in this respect.

19
20 The extent of renal excretion of micafungin (and/or) metabolites in neonates has not been
21
22 measured. However, based on mass balance data obtained in adults, and based on the fact that
23
24 no dose adjustments are needed in patients with renal impairment [7], renal micafungin
25
26 elimination was demonstrated to play a very minor role as opposed to fecal elimination. This
27
28 suggests that the several fold higher clearance in neonates compared to adults is unlikely to
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30 be due to (much) enhanced renal clearance in neonates compared to adults. Future non-

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32 clinical work in sandwich-cultured hepatocytes from juvenile animals or using apical and
33
34 basolateral membrane vesicles prepared from human neonatal frozen liver should be
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36 conducted in order to ultimately understand all mechanisms underlying altered micafungin
37
38 disposition in neonates.

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Figure Legends

Figure 1. Micafungin chemical structure

Figure 2. Expression of Transporter Proteins in Liver from Human Neonates and Adults.

Western blot analysis on liver homogenates from neonates and adults was used to determine the relative expression of hepatic transporters involved in micafungin uptake and biliary excretion. The top parts of panel A and panel B depict the protein expression bands in five neonate samples (N1-N5) and in five adult samples (A1-A5) for uptake and efflux transport proteins, respectively. GAPDH protein expression was measured for normalization. The bottom parts of both panels represent the mean (\pm SD) of GAPDH-normalized expression of the transporters in neonates (per kg body weight) relative to protein expression (per kg body weight) in adults where adult expression is 100%. Samples N3 and A5 were excluded from densitometric analysis for NTCP due to irregular band shape.

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Figure 3. Micafungin Fraction Unbound in Serum from Human Neonates Compared to Adults. Fraction unbound in serum from neonates and adults was determined by equilibrium dialysis as discussed in the methods section. Bars represent the mean (\pm SD) of values from 6 subjects /group. Statistical significance indicated by ***, $P < 0.001$ based on the unpaired t -test (unequal variance).

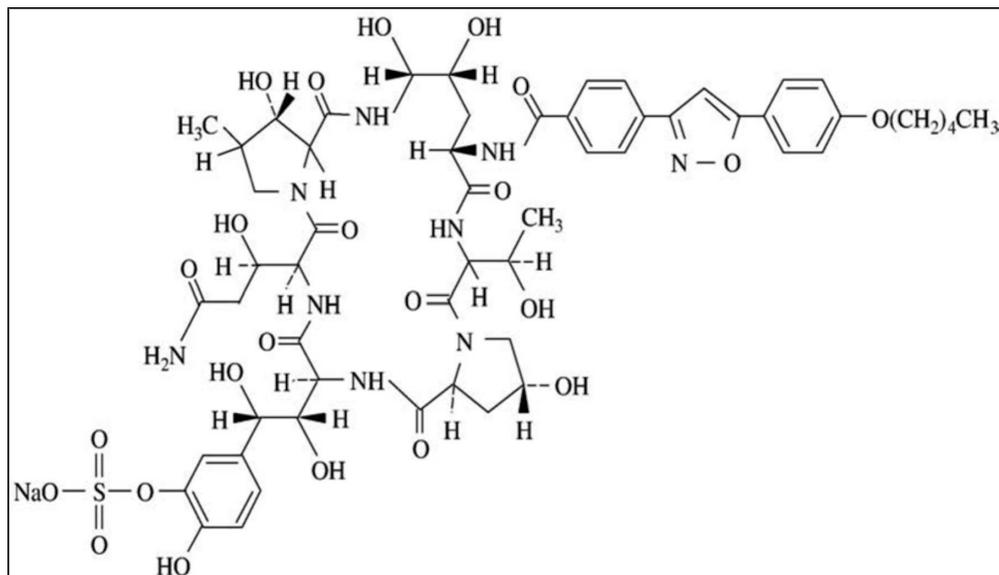
Figure 4. Micafungin Fraction Unbound in Serum from Neonates. Grey triangles (\blacktriangle) represent the f_u (mean \pm SD for three determinations) of micafungin in each subject. Each subject's postnatal age (\blacklozenge) or postgestational age at birth (\blacksquare) are also plotted. The mean value of f_u for the six subjects is represented by a dashed line (--).

Table 1. Calculation of *in vivo* Plasma and Biliary Clearance of Micafungin in Adults and Neonates

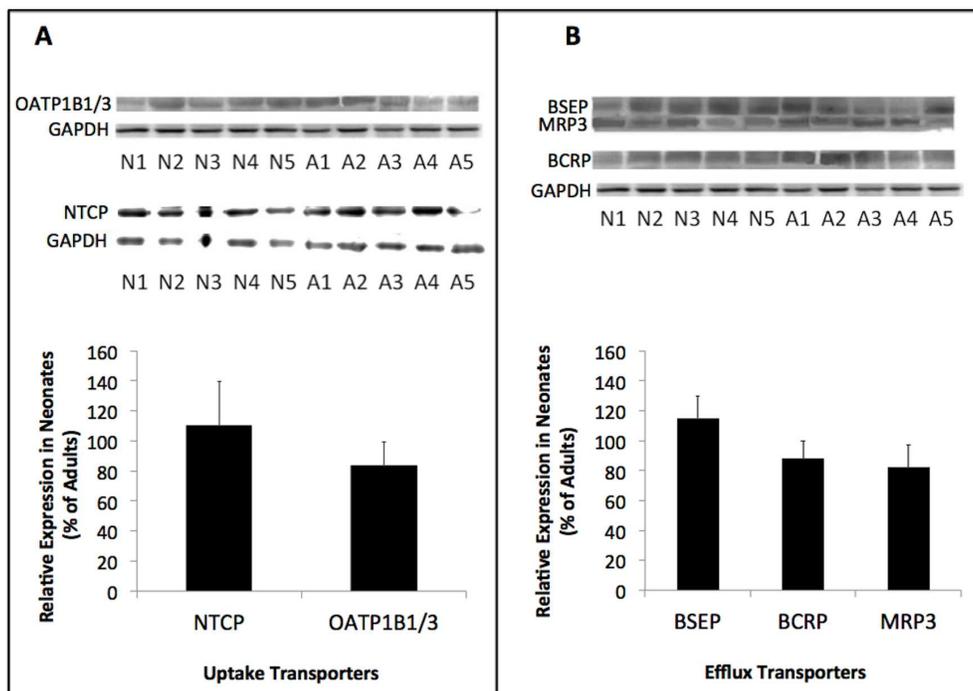
| Physiological Parameters & Scaling Factors | Adult* | Neonate |
|---|----------------------|--------------------------|
| f_u | 0.004 | 0.033 |
| mg protein/g liver | 71 | 24** |
| g liver/kg body weight | 26 | 43*** |
| QH, ml/min/kg | 21 | 73*** |
| Observed Plasma Cl (ml/min/kg) | 0.229 | 0.622[#] |
| Intrinsic Accumulation Cl (ml/min/mg) | 0.024 ± 0.002 | 0.024 ± 0.002 |
| Intrinsic Plasma Cl (ml/min/kg) | 43.4 ± 3.0 | 24.3 ± 2.6 |
| Calculated Plasma Cl (ml/min/kg) | 0.175 ± 0.012 | 0.793 ± 0.085 |
| Observed Biliary Cl (ml/min/kg) | 0.160 | Unknown |
| Intrinsic Biliary Cl (ml/min/mg) | 0.014 ± 0.009 | 0.014 ± 0.012 |
| Intrinsic Biliary Cl (ml/min/kg) | 25.5 ± 15.7 | 14.3 ± 8.8 |
| Calculated Biliary Cl (ml/min/kg) | 0.101 ± 0.062 | 0.467 ± 0.287 |

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2 Intrinsic clearance values were generated as described in the methods section. Intrinsic biliary
3
4 clearance was determined using B-CLEAR[®] technology (Qualyst, Inc.). *, Yanni *et al.* [8]; **,
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6 Barter *et al.* [18]; ***, Björkman [16]; #, Heresi *et al.* [5].
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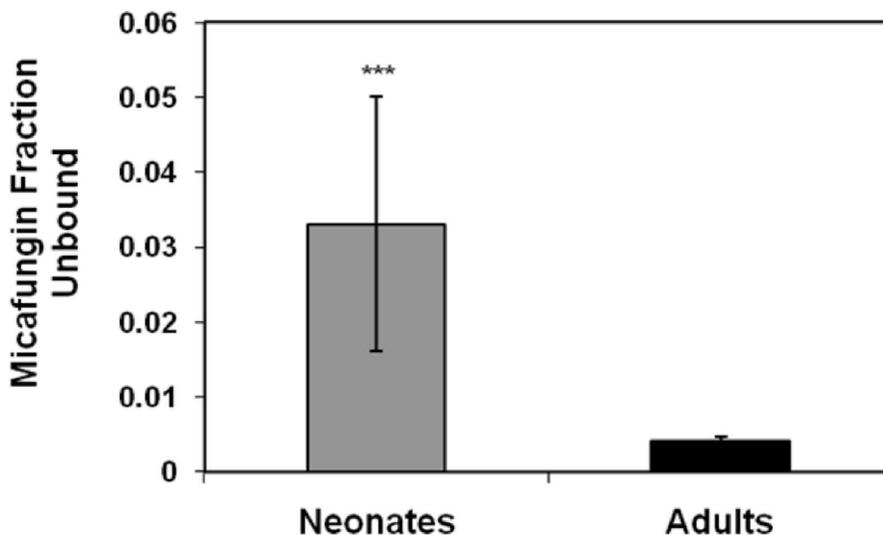
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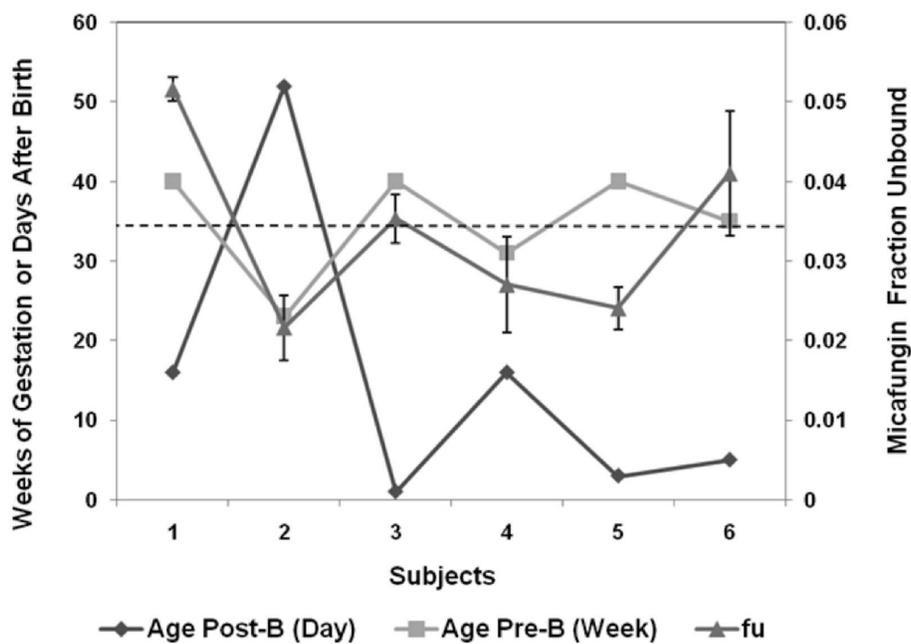
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