Methadone N-Demethylation by the Common CYP2B6 Allelic Variant CYP2B6*6

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ABSTRACT

The long-acting opioid methadone displays considerable unexplained interindividual pharmacokinetic variability. Methadone metabolism clinically occurs primarily by N-demethylation to 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), catalyzed predominantly by CYP2B6. Retrospective studies suggest that the common allele variant CYP2B6*6 may influence methadone plasma concentrations. The catalytic activity of CYP2B6.6, encoded by CYP2B6*6, is highly substrate-dependent. This investigation compared methadone N-demethylation by CYP2B6.6 with that by wild-type CYP2B6.1. Methadone enantiomer and racemate N-demethylation by recombinant-expressed CYP2B6.6 and CYP2B6.1 was determined. At substrate concentrations (0.25–2 μM) approximating plasma concentrations occurring clinically, rates of methadone enantiomer N-demethylation by CYP2B6.6, incubated individually or as the racemate, were one-third to one-fourth those by CYP2B6.1. For methadone individual enantiomers and metabolism by CYP2B6.6 compared with CYP2B6.1, Vmax was diminished, Ks was greater and the in vitro intrinsic clearance was diminished 5- to 6-fold. The intrinsic clearance for R- and S-EDDP formation from racemic methadone was diminished approximately 6-fold and 3-fold for R- and S-methadone, respectively. Both CYP2B6.6 and CYP2B6.1 showed similar stereoselectivity (S->R-methadone). Human liver microsomes with diminished CYP2B6 content due to a CYP2B6*6 allele had lower rates of methadone N-demethylation. Results show that methadone N-demethylation catalyzed by CYP2B6.6, the CYP2B6 variant encoded by the CYP2B6*6 polymorphism, is catalytically deficient compared with wild-type CYP2B6.1. Diminished methadone N-demethylation by CYP2B6.6 may provide a mechanistic explanation for clinical observations of altered methadone disposition in individuals carrying the CYP2B6*6 polymorphism.

Introduction

The long-acting opioid methadone is used to treat opiate addiction, as well as acute and chronic pain related to cancer and other conditions. Clinical use of methadone is challenging, however, because of considerable and unpredictable inter- and intraindividual variability in pharmacokinetics, including metabolism, clearance, and susceptibility to drug interactions (Ferrari et al., 2004; McCance-Katz et al., 2010). This can result in opiate withdrawal, inadequate analgesia, or drug accumulation and toxicity. Indeed, with increasing methadone use over the past decade, there has been an epidemic of toxicity, including a nearly 1800% increase in adverse events and a 390% increase in fatalities, which persist today (CDC, 2012).

Methadone in humans is cleared primarily by hepatic cytochrome P450 (P450)-catalyzed metabolism to the pharmacologically inactive metabolite 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), with some urinary excretion of unchanged drug. Methadone clearance and N-demethylation are stereoselective. After considerable research, a consensus has emerged that methadone N-demethylation in vitro, both by human liver microsomes and expressed CYP isoforms, is catalyzed most efficiently by CYP2B6 and CYP3A4 (whereas CYP3A5 is comparatively inactive), and CYP2B6 but not CYP3A4 N-demethylates methadone stereoselectively (Gerber et al., 2004; Kharasch et al., 2004; Kharasch et al., 2004; Totah et al., 2007, 2008; Chang et al., 2011). Clinical drug interaction studies indicate that CYP2B6, rather than CYP3A4, is a major or predominant CYP isoform responsible for clinical methadone disposition. CYP3A induction (Vourvahis et al., 2012) and very strong CYP3A inhibition (Kharasch et al., 2004; Kharasch et al., 2008; van Heeswijk et al., 2011; Kharasch et al., 2012) had no influence on (or in some studies even increased) methadone N-demethylation or clearance. However, CYP2B6 induction or inhibition did correspondingly modulate methadone plasma concentrations, metabolism, and clearance (Kharasch et al., 2004; Kharasch et al., 2008; Kharasch and Stubbert, 2013).

CYP2B6 comprises a small fraction of hepatic CYP content but is responsible for metabolizing a much larger percentage of drugs and xenobiotics (Wang and Tompkins, 2008; Mo et al., 2009). CYP2B6 is a highly polymorphic enzyme (Zanger et al., 2007), with numerous single nucleotide polymorphisms encoding 30 CYP2B6 protein variants identified to date. The CYP2B6*6 allele (516G>T, Q172H; 785A>G, K262R) is of considerable interest, owing to its common occurrence (particularly in populations or descendants of Africans, Asians, and Hispanics) and therapeutic significance (Zanger et al., 2007). For example, CYP2B6*6 influences the metabolism, pharmacokinetics, and clinical effects of efavirenz, nevirapine, cyclophosphamide, and bupropion (Mo et al., 2009). CYP2B6*6 has been associated with diminished CYP2B protein expression in human liver microsomes

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ABBREVIATIONS: Clint, intrinsic clearance; EDPD, 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine; HLM, human liver microsome; Ks, equilibrium dissociation constant for the enzyme-substrate complex; P450, cytochrome P450.
(Xie et al., 2003; Desta et al., 2007; Hofmann et al., 2008). Expressed CYP2B6.6, compared with CYP2B6.1, has diminished catalytic activity toward bupropion and efavirenz (Zhang et al., 2011; Xu et al., 2012), modest-to-moderately increased activity toward cyclophosphamide (Xie et al., 2003; Ariyoshi et al., 2011; Raccor et al., 2012) and artemether (Honda et al., 2011), and indifferent metabolism of selegiline (Watanabe et al., 2010). Human liver microsomes from CYP2B6*6 carriers had diminished metabolism of mephentoin (Lang et al., 2001), bupropion (Hesse et al., 2004; Xu et al., 2012), and efavirenz (Desta et al., 2007; Xu et al., 2012), but not cyclophosphamide (Xie et al., 2003; Raccor et al., 2012). Thus, the influence of CYP2B6*6 genotype on the direction (increased or decreased metabolism), magnitude, pharmacokinetic consequence, clinical implications, and mechanism of CYP2B6-dependent biotransformation are complex and substrate-dependent.

Available clinical evidence suggests that CYP2B6 polymorphisms can influence methadone disposition. Specifically, associations between CYP2B6*6 genotype and higher dose-adjusted steady-state plasma methadone concentrations (Crettol et al., 2005; Crettol et al., 2006; Eap et al., 2007; Wang et al., 2011), and a need for lower methadone doses (Hung et al., 2011; Levran et al., 2012) have also been reported.

Despite these pharmacogenetic association studies evaluating single steady-state plasma methadone concentrations, the influence of CYP2B6*6 alleles (or CYP2B6 polymorphism in general) on clinical methadone metabolism and clearance is unknown, as is their influence on human liver microsomal methadone metabolism. In addition, the activity of CYP2B6.6 toward methadone metabolism is also unknown. Therefore, the purpose of this investigation was to evaluate methadone N-demethylation by CYP2B6.6 and to compare it with that by wild-type CYP2B6.1.

Materials and Methods

Chemicals and Reagents. EDDP and EDDP-d3 were purchased from Cerilliant (Round Rock, TX). Insect cell microsomes (Supersomes) containing CYP2B6.1 coexpressed with human P450 reductase and human cytochrome b5 were purchased from BD Gentest Corporation (Woburn, MA). Microsomes containing CYP2B6.6 coexpressed with P450 reductase and cytochrome b5 were a generous gift from BD Gentest Corporation. All other reagents were purchased from BD Gentest Corporation (Woburn, MA). Microsomes containing CYP2B6.6 coexpressed with human P450 reductase and human cytochrome b5 were purchased from BD Gentest Corporation. Insect cell microsomes (Supersomes) containing CYP2B6.1 coexpressed with human P450 reductase and human cytochrome b5 were purchased from BD Gentest Corporation (Woburn, MA). Microsomes containing CYP2B6.6 coexpressed with P450 reductase and cytochrome b5 were a generous gift from BD Gentest Corporation. All other reagents were from Sigma-Aldrich (St. Louis, MO).

Methadone Metabolism. Incubations (200 μl, 10 pmol/ml CYP2B6) with RS-, R-, or S-methadone were performed as previously described (Totah et al., 2007, 2008), with minor modifications. Reactions (10-minute) were quenched with 20 μl 20% trichloroacetic acid containing internal standard (d3-EDDP, final concentration 4.55 ng/ml) and centrifuged; the supernatant (200 μl) was processed immediately by solid-phase extraction as described previously (Karshas et al., 2004), except that Strata-X-C 33 μm plates (Phenomenex, Torrance, CA) were used. EDDP analysis was performed on an API 3200 triple-quadrupole mass spectrometer with Turto Ion Spray source (Applied Biosystems/MD Sciex, Foster City, CA), Shimadzu high-pressure liquid chromatograph (Columbia, MD), Gerstel autosampler (Baltimore, MD), and Chrom Tech chiral AGP column (3 × 50 mm, 5 μm) with chiral AGP guard cartridge (3 × 10 mm) (Apple Valley, MN), as described (Sharma et al., 2011), except that the mobile phase gradient was 20 mM ammonium formate (pH 5.0) and methanol. Retention times were 10.4 and 11.2 minutes, respectively, for R- and S-EDDP. EDDP was quantified using peak area ratios and standard curves prepared using calibration standards in buffer. Control incubations lacking NADPH and protein were included for all reactions to determine the background EDDP, which was subtracted from all results.

Data and Statistical Analysis. Results are the mean ± S.D. (3-6 replicates) unless otherwise indicated. Differences between groups were determined by analysis of variance followed by the Student-Newman-Keuls test (SigmaPlot 12.3; Systat, San Jose, CA). EDDP formation versus substrate concentration data were analyzed by nonlinear regression analysis (SigmaPlot 12.3) as described previously (Totah et al., 2007, 2008) using the Adair-Pauling model, based on the recognition that CYP2B6 contains at least two binding sites. Results are the parameter estimate ± standard error of the estimate.

Results

N-demethylation of racemic methadone and individual enantiomers was evaluated at concentrations (0.25–2 μM) approximating those in patients receiving low and high doses of methadone, respectively, for treatment of pain (typically 10–20 mg) or substance abuse (60–100 mg). Rates of methadone enantiomer N-demethylation by CYP2B6.6 were typically one-third those by CYP2B6.1 when enantiomers were incubated individually (Fig. 1A). When racemic methadone N-demethylation was evaluated (Fig. 1B), rates of R- and S-EDDP formation by CYP2B6.6 were even lower, approximately one-fourth those by CYP2B6.1. N-demethylation by CYP2B6.1 was stereoselective, with S-methadone metabolism exceeding that of R-methadone, both with individual enantiomers and the racemate. Although EDDP formation by CYP2B6.6 was much lower than that by CYP2B6.1, stereoselectivity (S>R-methadone) was preserved, with individual enantiomers and the racemate.

Concentration-dependence of methadone N-demethylation was determined for racemic methadone and the individual enantiomers (Fig. 2). As observed previously (Totah et al., 2008), Eadie-Hofstee plots for racemic methadone N-demethylation by CYP2B6.1 were not strictly linear, nor were those for R- and S-methadone, indicating that the nonlinearity of the racemate did not represent the two enantiomers interacting differently with a single enzyme site, but rather, each enantiomer interacting with two apparent enzyme sites. Nonlinear Eadie-Hofstee plots were also observed for CYP2B6.6-catalyzed racemic methadone and methadone enantiomers N-demethylation. The Adair-Pauling equation, which allows for two binding sites, was used to model EDDP formation from individual methadone enantiomers, and kinetic parameters are provided in Table 1. For CYP2B6.6, Vmax was diminished to approximately one-third to one-fifth that for CYP2B6.1, Ks was greater than for CYP2B6.1, and the in vitro intrinsic clearance (Clint, Vmax/Ks), was diminished 5- to 6-fold. The Adair-Pauling equation was also used to model EDDP enantiomer formation from racemic methadone. For CYP2B6.6, the apparent Ks for both enantiomers was approximately 50% greater than for CYP2B6.1. Vmax was diminished more for R-methadone (to one-fourth) than S-methadone (to approximately half), and the in vitro Clint was diminished approximately 6- and 3-fold for R- and S-methadone, respectively.

Discussion

The major finding of this investigation is that the N-demethylation of methadone catalyzed by CYP2B6.6, the CYP2B6 variant encoded by the CYP2B6*6 polymorphism, is catalytically deficient compared with wild-type CYP2B6.1. In comparing CYP2B6.6 with CYP2B6.1, EDDP formation from both individual methadone enantiomers was diminished, Ks was increased, Vmax was reduced, and the in vitro Clint was diminished to approximately one-fifth that for the wild-type enzyme. With racemic methadone, EDDP formation from both methadone enantiomers was also lower with CYP2B6.6, Vmax was reduced, and the in vitro Clint was diminished to approximately one-fifth to one-half that for wild-type CYP2B6.1. At substrate concentrations approximating total plasma methadone concentrations (0.25–0.5 μM each enantiomer) occurring clinically, for both individual enantiomers and racemic methadone, rates of N-demethylation by CYP2B6.6 were generally only one-third those for CYP2B6.1.
Although rates of methadone metabolism by CYP2B6.6 were diminished compared with CYP2B6.1, the stereoselectivity of metabolism (S-methadone > R-methadone) seen previously with CYP2B6.1 (Gerber et al., 2004; Totah et al., 2007, 2008; Chang et al., 2011), was preserved with CYP2B6.6.

Modeling of methadone N-demethylation by CYP2B6 is complex (Totah et al., 2007). Methadone enantiomer N-demethylation by CYP2B6.1 showed apparent multiple-site or multiple-affinity binding with complex allosteric kinetics or homotropic cooperativity, which was best described using the Adair-Pauling equation (Totah et al., 2007). This approach was used to model methadone enantiomer N-demethylation by CYP2B6.1 and CYP2B6.6 in the present investigation. With CYP2B6.6, at the highest substrate concentrations, the possibility of substrate or product inhibition cannot be eliminated; however, there are insufficient data which to evaluate such models in an identifiable fashion. Consequently, this investigation used the simplest model for which there is precedent. In a previous investigation of racemic methadone metabolism by CYP2B6.1, a competitive inhibitory interaction was found; each enantiomer in the racemate (R or S) inhibited the metabolism of its antipode (S or R-methadone) (Totah et al., 2007). The Adair-Pauling model was found to be mis-specified for racemic methadone, and a more complex model was required to describe methadone metabolism; however, this model required evaluating metabolism of a complex matrix of individual

Fig. 1. Recombinant CYP2B6-catalyzed methadone N-demethylation at therapeutic concentrations. Results are shown for (A) metabolism of individual methadone enantiomers (0.25–1 μM each) and (B) racemic methadone (0.5–2 μM, corresponding to 0.25–1 μM of each enantiomer) by CYP2B6.1 and CYP2B6.6. Results are the mean ± S.D. of 3–6 determinations.

*Significantly different versus CYP2B6.1 (P < 0.05).

Fig. 2. Concentration-dependence and kinetics of recombinant CYP2B6-catalyzed N-demethylation of methadone to EDDP. Results are shown for metabolism of (A and B) individual methadone enantiomers (0.25–500 μM each) and (C and D) racemic methadone (0.5–1000 μM, corresponding to 0.25–500 μM of each enantiomer) by CYP2B6.1 and CYP2B6.6. Corresponding Eadie-Hofstee plots are shown in (B and D). For ease of comparison with enantiomers metabolism (A and B), racemic methadone is shown as the concentration of the individual enantiomers (C and D). Symbols represent CYP2B6.1-catalyzed EDDP formation from R-methadone (△) and S-methadone (◇) (single enantiomers or the racemate) and CYP2B6.6-catalyzed EDDP formation from R-methadone (▲) and S-methadone (▼) (single enantiomers or the racemate). Each data point is the mean ± S.D. of 3–6 determinations. Lines represent rates predicted from nonlinear regression analysis of data using the Adair-Pauling equation.
antipode concentrations at each enantiomer concentration. In the present investigation, only racemic methadone metabolism by CYP2B6.6 was evaluated, precluding application of the complex CYP2B6 model. The simpler Adair-Pauling equation was used to model the data, accepting some mis-specification in the parameter estimates. Thus, for racemic methadone, the reported $K_e$ and $V_{max}$ are best considered as apparent parameters. Use of a Michaelis-Menten model did not result in improved fits to the data (not shown).

The catalytic behavior of CYP2B6.6 is substrate-dependent, and the mechanism of altered CYP2B6.6-catalyzed biotransformation, when it has been observed, is not well understood. Some information is available from studies using expressed CYP2B6.6, from others using liver microsomes of individuals carrying the CYP2B6*6 allele, and from clinical pharmacogenetic studies. In COS-1 cells expressing CYP2B6.6, the $Cl_{int}$ for 7-ethoxy-4-trifluoromethylcoumarin $O$-$de$-ethylation was double that for CYP2B6.1 (Jinno et al., 2003). In COS-7 cells expressing CYP2B6.6, the $Cl_{int}$ for 7-ethoxy-4-trifluoromethylcoumarin $O$-$de$-ethylation, selegiline $N$-demethylation, and selegiline $N$-depropylation was not different from that for CYP2B6.1 (Watanabe et al., 2010); the activity toward bupropion was significantly less (Hofmann et al., 2008), whereas the activity toward artemether was significantly greater (Honda et al., 2011). In an insect cell CYP2B6 expression system co-expressing P450 reductase but not cytochrome $b_{5}$, the $Cl_{int}$ for efavirenz 8-hydroxylation by CYP2B6.6 was half that of CYP2B6.1, whereas the $Cl_{int}$ for cyclophosphamide 4-hydroxylation was 60% greater (Ariyoshi et al., 2011). In a CYP2B6 reconstitution system with P450 reductase but not cytochrome $b_{5}$, the catalytic difference ($k_{cat}/K_m$) of CYP2B6.6 for 7-ethoxy-4-trifluoromethylcoumarin, bupropion 4-hydroxylation, and efavirenz 8-hydroxylation was decreased to, respectively, two-thirds, one-half, and one-fifth of that seen with CYP2B6.1 (Zhang et al., 2011). In a CYP2B6 expression system co-expressing P450 reductase and cytochrome $b_{5}$, CYP2B6.6-catalyzed efavirenz 8-hydroxylation was not significantly different. The $Cl_{int}$ for bupropion 4-hydroxylation was reduced by approximately one-third compared with CYP2B6.1 (Xu et al., 2012). In the absence of $b_{5}$, efavirenz 8-hydroxylation $Cl_{int}$ by CYP2B6.6 was approximately one-half that seen with CYP2B6.1, and bupropion 4-hydroxylation $Cl_{int}$ was approximately 50% greater (Xu et al., 2012). In the present insect cell CYP2B6 expression system, which contained both coexpressed P450 reductase and cytochrome $b_{5}$, the catalytic difference between CYP2B6.6 and CYP2B6.1 for methadone enantiomer metabolism was generally greater than that observed previously for other substrates. The $Cl_{int}$ for $N$-demethylation was approximately 5-fold lower. Thus, methadone appears to be one of the most susceptible substrates to the diminished catalytic efficiency of CYP2B6.6.

In human liver, the CYP2B6*6 allele causes aberrant splicing (Hofmann et al., 2008), resulting in reduced functional mRNA and low hepatic CYP2B expression (Lang et al., 2001; Desta et al., 2007; Hofmann et al., 2008). Thus, both diminished P450 content and deficient catalytic efficiency may combine to cause the phenotype of decreased CYP2B6-catalyzed biotransformation in CYP2B6*6 carriers. Compared with expressed enzyme systems, human liver microsomes may therefore show greater catalytic differences between CYP2B6.1 and CYP2B6.6 and the effect of the CYP2B6*6 polymorphism. Indeed, in human liver microsomes from individuals with *6 genotypes, there was markedly diminished CYP2B6 protein expression, cyclophosphamide 4-hydroxylation (Xie et al., 2003), bupropion hydroxylation (Hesse et al., 2004; Xu et al., 2012), and efavirenz 8-hydroxylation (Desta et al., 2007; Xu et al., 2012).

Clinically, numerous investigations have shown an association between CYP2B6*6 genotypes and increased efavirenz plasma concentrations, diminished metabolism and clearance, and greater neurotoxicity and hepatotoxicity (Haas et al., 2004; Holzinger et al., 2012; Turpeinen and Zanger, 2012). Bupropion metabolism was similarly diminished in CYP2B6*6 carriers, based on lower plasma hydroxybupropion/ bupropion AUC ratios (Chung et al., 2011).

We previously evaluated the influence of CYP2B6 content and genetic polymorphisms on human liver microsomal methadone metabolism (Totah et al., 2008). There was no apparent consistent relationship between methadone $N$-demethylation and CYP2B6 content (or genotype), and no definitive conclusions could be drawn regarding CYP2B6 genotype and methadone metabolism. Since then, microsomal CYP3A content was requantified (Raccor et al., 2012); when pairs of livers were rematched for CYP3A content but different CYP2B6 content (or genotype) and methadone enantiomer metabolism was again compared, a clear influence of CYP2B6 genotype became apparent (Supplemental Table 1). For example, microsomes from human livers 141 and 144 both had high CYP3A content but high (CYP2B6*1/*4) and moderate CYP2B6 (CYP2B6*1/*6) content, respectively; lower $N$-demethylation of both methadone enantiomers was observed in human liver microsome (HLM) 144. Livers 124 and 148 both had high CYP3A content but high (CYP2B6*1/*6) and low CYP2B6 (CYP2B6*6/*6) content, respectively, and lower methadone metabolism was observed in HLM 148. HLM 142 and 164 both had low CYP3A but moderate (CYP2B6*1/*4) and low CYP2B6 (CYP2B6*1/*6) content, and lower methadone N-demethylation was observed with HLM 164. These data suggest that CYP2B6*6 allele, specifically the *6 allele, can influence human liver microsomal methadone N-demethylation.

Diminished methadone metabolism by expressed CYP2B6.6 and livers from individuals with the CYP2B6*6 allele is consistent with previous reports of a genetic influence of CYP2B6 on methadone plasma concentrations. Dose-adjusted steady-state trough and peak plasma S- methadone concentrations were greater in homozygous carriers of CYP2B6*6 than in heterozygotes and noncarriers (Crettol

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<tr>
<td></td>
<td>CYP2B6.1</td>
<td>CYP2B6.6</td>
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<tr>
<td>$K_e$ (uM)</td>
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<td>$25 \pm 5$</td>
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<td>$Cl_{int}$ (mL/min/mmol)</td>
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$Cl_{int}$, intrinsic clearance; EDDP, 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine; $K_e$, equilibrium dissociation constant for the enzyme-substrate complex; P450, cytochrome P450.
et al., 2005; Crettol et al., 2006; Eap et al., 2007). Dose-adjusted steady-state trough S-methadone concentrations were 2-fold higher in CYP2B6*6 genotypes than in noncarriers (Crettol et al., 2005). Another investigation found that CYP2B6*6 homozygotes similarly needed lower methadone doses (Hung et al., 2011). CYP2B6*6 carriers had higher plasma S-methadone concentrations and a higher concentration-to-dose ratio for both enantiomers (Wang et al., 2011). Mean methadone doses required by methadone maintenance patients were significantly lower in CYP2B6*6/6 genotypes than in heterozygotes or noncarriers (Levran et al., 2012). In a series of methadone-related deaths, whole-blood RS-methadone concentrations were significantly (approximately 2-fold) higher in CYP2B6*6 carriers than in non-carriers (Bunten et al., 2011). Together, these reports suggest that the CYP2B6*6 allele influences methadone disposition, although there have been no published studies investigating the influence of CYP2B6*6 or other polymorphisms on clinical methadone metabolism or clearance. Diminished methadone N-demethylation by CYP2B6.6 further supports these clinical observations and may provide a mechanistic explanation.

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Authorship Contributions
Participated in research design: Gadel, Kharasch. Conducted experiments: Gadel, Crafford, Regina. Performed data analysis: Gadel, Crafford, Regina, Kharasch. Wrote or contributed to the writing of the manuscript: Gadel, Crafford, Regina, Kharasch.

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