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IVIVE of Transporter-Mediated Renal Clearance: Relative Expression Factor (REF) vs Relative Activity Factor (RAF) Approach

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BCA: bicinchoninic acid; BCRP: breast cancer resistance protein; BW: body weight; BSA: bovine serum albumin; B/P: blood to plasma ratio; CL: clearance; $CL_{int,sec,in\ vivo}$: *in vivo* intrinsic secretory clearance; CL_r: renal clearance; $CL_{r,plasma}$: renal plasma clearance; $CL_{sec,plasma}$: renal secretory clearance; DTT: dithiothreitol; ESI: electron spray ionization; F_{reabs}: fraction reabsorbed in kidney tubules; f_t: fraction transported; f_u: unbound fraction; $f_{u,blood}$: unbound fraction in blood; $f_{u,plasma}$: unbound fraction in plasma; GFR: glomerular filtration rate; HEK: human embryonic kidney; IAA: iodoacetamide; IVIVE: *in vitro* to *in vivo* extrapolation; LC-MS/MS: liquid chromatography tandem mass spectrometry; MATE: multi-antimicrobial extrusion protein; ME: mean error; MPPGC: mg

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protein per gram cortex; MRP: multi-drug resistance protein; MSE: mean squared error; OAT: organic anion transporter; OATP: organic anion transporting polypeptide; OCT: organic cation transporter; Q_r: renal blood flow; P-gp: p-glycoprotein; PSF: physiological scaling factors; RAF: relative activity factor; REF: relative expression factor; RMSE: root mean squared error; TEC: transporter expressing cells; UPLC: ultra-performance liquid chromatography

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Abstract

About 30% of approved drugs are cleared predominantly by renal clearance (CL_r). Of these, many are secreted by transporters. For these drugs, *in vitro*-to-*in vivo* extrapolation of transporter-mediated renal secretory clearance ($CL_{sec,plasma}$) is important to prospectively predict their renal clearance and to assess the impact of drug-drug interactions and pharmacogenetics on their pharmacokinetics. Here we compared the ability of the relative expression factor (REF) and the relative activity factor (RAF) approaches to quantitatively predict the *in vivo* $CL_{sec,plasma}$ of 26 Organic Anion Transporter (OAT) substrates assuming that OAT-mediated uptake is the rate-determining step in the $CL_{sec,plasma}$ of the drugs. The REF approach requires protein quantification of each transporter in the tissue (e.g. kidney) and transporter-expressing cells (TEC) while the RAF approach requires the use of a transporter-selective probe substrate (both *in vitro* and *in vivo*) for each transporter of interest. For the REF approach, 50% and 69% of the $CL_{sec,plasma}$ predictions were within 2- and 3-fold of the observed values, respectively; the corresponding values for the RAF approach were 65% and 81%. We found no significant difference between the two approaches in their predictive capability (as measured by accuracy and bias) of the $CL_{sec,plasma}$ or CL_r of OAT drugs. We recommend that the REF and RAF approaches can be used interchangeably to predict OAT-mediated $CL_{sec,plasma}$. Further research is warranted to evaluate the ability of the REF or RAF approach to predict $CL_{sec,plasma}$ of drugs when uptake is not the rate-determining step.

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Significance Statement

This is the first direct comparison of the REF and RAF approaches to predict transporter-mediated CL_r. The RAF, but not REF, approach requires transporter-selective probes and that the basolateral uptake is the rate-determining step in the CL_r of drugs. Given that there is no difference in predictive capability of the REF and RAF approach for OAT-mediated CL_r, the REF approach should be explored further to assess its ability to predict CL_r when basolateral uptake is not the sole rate-determining step.

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Introduction

Accurate prediction of *in vivo* clearance (CL) is important to support drug candidate selection during early-stage development and to evaluate the impact of drug interactions and pharmacogenetics in clinical development. A comprehensive analysis of 391 drugs found that 31% of compounds were predominantly renally cleared (CL_r) (i.e. CL_r > 50% of total clearance) (Varma et al., 2009). Renal clearance is mediated by active secretion, filtration, and tubular reabsorption. Active secretion of drugs includes passive and transporter-mediated uptake and efflux CL respecitvley across the basal and apical membrane of the proximal renal tubule cells. Organic anion transporters (OAT1-3), located on the basal membrane, are important contributors to the renal secretion of many renally cleared drugs including drugs such as antibiotics and antivirals (Feng et al., 2010). Filtration clearance is a passive process that depends on glomerular filtration rate (GFR) and fraction of the drug unbound in the plasma (f_u). While tubular reabsorption (active or passive or both) can occur, it cannot be determined *in vivo* and is therefore assumed to be passive and minimal.

Common predictive preclinical methodology used to estimate metabolic CL in humans are *in vitro-in vivo* extrapolation (IVIVE) using primary cells (e.g. hepatocytes) and physiological or relative activity factor (RAF) scaling. While IVIVE using RAF or physiological scaling factors (PSF) has been shown to be relatively successful in predicting metabolic clearance, such predictions for transporter-mediated clearance, including active secretion clearance, needs to be verified (Rostami-Hodjegan and Tucker, 2007; Soars et al., 2007; Rowland et al., 2011; Ke et al., 2014). Moreover, unlike human hepatocytes, validated primary human kidney epithelial cells for transport studies are not routinely available. Although, human CL_r predictions can be conducted by allometric scaling of *in vivo* renal CL data in animals, due to inter-species differences in transporter abundance and activity, allometry can lead to inaccurate prediction of human CL_r (Paine et al., 2011; Chu et al., 2013). Other methods for IVIVE of human CL_r that

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have been used are human kidney slices (Watanabe et al., 2011; Scotcher et al., 2016a). However, kidney slices underestimated OAT3-mediated intrinsic renal secretory clearance of 7 OAT3 transported drugs and IVIVE of their renal secretory CL required a scaling factor of 10.

Recently, the RAF approach was successfully used by Mathialagan et al. to predict the *in vivo* human OAT-mediated renal secretory CL and total CL_r of 31 drugs (Mathialagan et al., 2017). Using cells expressing the transporter(s) of interest (e.g. OAT1-expressing cells), the RAF approach scales the *in vitro* transporter uptake CL of the drug of interest to its *in vivo* clearance. To do so, the RAF approach requires that the *in vitro* uptake CL of a probe drug be available in the transporter-selective (e.g. OAT1) cells as well as *in vivo* (Fig. 1). However, a shortcoming of the RAF approach is that such transporter-selective drugs are often not available for many transporters (e.g. breast cancer resistance protein (BCRP), organic anion transporting polypeptides (OATPs)). An alternative approach, the relative expression factor (REF) approach, has recently begun to be explored for IVIVE of drug CL (Ishida et al., 2018; Kumar et al., 2018; Sachar et al., 2020). Unlike the RAF approach, the REF approach does not require a transporter-selective probe substrate. Instead, it requires information on the *in vivo* and *in vitro* abundance of the transporter in the tissue of interest (e.g. kidneys) and in the cells used to determine the drug's *in vitro* transport CL (Fig. 1) (Kumar et al., 2018). Quantitative targeted proteomics, due to its selectivity, sensitivity, and lack of need for protein standards, has become the preferred method for quantification of abundance of transporters, both in *in vitro* systems and tissue samples (Prasad et al., 2016). Then, this abundance is used to scale the *in vitro* transport CL in cells expressing the transporter of interest to that *in vivo* assuming that Jmax of the transporter is directly proportional to the abundance of the transporter and the Km of the drug for the transporter *in vivo* is identical to that *in vitro*. Another advantage of the REF over the RAF approach is that it can handle the involvement of multiple rate-determining transport steps in the CL of the drug, irrespective of whether the transporters are located at the basal,

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apical or both membranes of the kidney epithelial cells (Patilea-Vrana and Unadkat, 2016). In this event, the RAF method would require multiple probe substrates, each reporting the individual rate-determining step, a scenario that is nearly impossible to achieve.

Although both IVIVE scaling approaches (REF and RAF) have been successfully used to predict hepatic uptake clearance of drugs mediated by OATPs (Kunze et al., 2014a; Ishida et al., 2018), a direct comparison of the REF and RAF approach for IVIVE of renal secretory CL has never been reported. Therefore, the primary aim of this study was to compare the ability of the REF and RAF approaches to successfully predict the *in vitro* intrinsic renal secretory CL of several OAT-transported drugs. Our secondary aims were to test the ability of both these approaches to predict the total renal secretory CL (active and passive) and the total renal CL of drugs. For these comparisons, the data for the RAF IVIVE of renal CL (including intrinsic, total secretory CL and total renal CL) of these OAT drugs were obtained from a previous publication (Mathialagan et al., 2017).

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Materials and Methods

The rationale for choosing the 26 OAT-transported drugs has been provided by Mathialagan et al (Mathialagan et al., 2017). Briefly, they were chosen because they are selectively transported by OATs. Detailed materials and methods used to conduct IVIVE of renal secretory CL of drugs using the RAF approach have been previously published (Mathialagan et al., 2017). Of note, tenofovir, acyclovir/ganciclovir and oseltamivir/benzylpenicillin were used as probe substrates for OAT1, OAT2 and OAT3, respectively. Therefore, here we describe the materials and methods used for IVIVE of renal CL of drugs using only the REF approach.

Materials

Bovine serum albumin (BSA), ammonium bicarbonate (98% purity), iodoacetamide (IAA), dithiothreitol (DTT), and trypsin protease (MS grade) were obtained from Thermo Fisher Scientific (Rockford, IL). Stable isotope-labeled (heavy) peptides and synthetic unlabeled peptides were purchased from Thermo Fisher Scientific (Rockford, IL) and New England Peptides (Boston, MA), respectively. ProteoExtract™ native membrane protein extraction kit was purchased from (Calbiochem, Temecula, CA). Optima MS-grade acetonitrile, methanol, chloroform, formic acid, and bicinchoninic acid (BCA) protein assay kit were purchased from Fisher Scientific (Fair Lawn, NJ). HEK293 Cells were obtained from Pfizer Inc.

Determination of OAT-mediated Uptake CL of drugs using OAT-Overexpressing HEK Cells

Since the OAT-mediated active uptake and passive diffusion CL of the 26 OAT-transported drugs was used for both the REF and RAF approach, the reader is referred to the previous publication as to how these values were experimentally obtained (Mathialagan et al., 2017).

Transporter Quantification

The values of protein abundance of renal transporters in the human renal cortex were previously generated by our laboratory (Prasad et al., 2016). The same quantification protocol

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was used to quantify the transporters in the OAT-overexpressing HEK cells and is detailed here. Briefly, membrane protein extraction of 3 to 5 million HEK293 cells overexpressing OAT1, OAT2 or OAT3 and three adult kidney cortex samples (~50-100 mg) was performed as follows. Membrane proteins (2 mg/ml) were denatured (heating), reduced (DTT), alkylated (IAA) and digested using trypsin as per optimized conditions described previously (Prasad et al., 2016). The unlabeled synthetic surrogate peptides for each transporter (light peptides) were used as the calibrators. The corresponding peptides, labeled with [$^{13}C_6{}^{15}N_2$]-lysine and [$^{13}C_6{}^{15}N_4$]-arginine residues, were used as the internal standards (heavy peptides). Each trypsin digested sample (5 µL) was injected onto the column (ACQUITY UPLC HSS T3 1.8 µm, C18 100A; 100 × 2.1 mm, Waters, Milford, MA). Peptide quantification was performed using a triple-quadrupole MS instrument (Sciex Triple Quad™ 6500, Concord, ON) in ESI positive ionization mode coupled to an Acquity UPLC, I-class (Waters, Milford, MA) (Supplementary Table 1). The parent to product ion transitions for the light and heavy peptides were monitored using optimized LC-MS/MS parameters in ESI positive ionization mode as described previously. The LC-MS/MS data were processed using Analyst 1.6.2 version software (Sciex, Concord, ON) as described previously (Li et al., 2019).

Prediction of Renal Clearance

The REF value for each transporter was calculated using Eq. 1.

$$REF_{OATx} = \frac{Renal\ Cortex\ OATx\ Abundance\left(\frac{pmol}{mg\ protein}\right)}{In\ Vitro\ Cell\ OATx\ Abundance\left(\frac{pmol}{mg\ protein}\right)}$$

Eq. 1

Where x is 1, 2 or 3. The renal cortex OATx abundance was that in the pooled sample of three kidneys that was assayed simultaneously with the OATx abundance in the HEK cells (see

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Supplementary Table 2). When the *in vivo* intrinsic secretory clearance ($CL_{int,sec,in\ vivo}$) of the drug was calculated by scaling only active uptake, Eq. 2 was used.

$$CL_{int,sec,in\ vivo}\left({mL}/{min * kg}\right) =$$

$$\Big(\big(hOAT1\ Active\ Uptake\ CL\big) * (REF_{OAT1}) + \big(hOAT2\ Active\ Uptake\ CL\big) * (REF_{OAT2}) +$$

$$\big(hOAT3\ Active\ Uptake\ CL\big) * (REF_{OAT3})\Big) * MPPGC * Cortex\ Weight/kg\ BW \quad \text{Eq. 2}$$

Where the active uptake CL represents the transporter-mediated uptake CL of the drug, the mg of protein per gram cortex (MPPGC) was 300 mg/g, and the grams of cortex per kilogram body weight (BW) was 3 g/kg (Bouchet et al., 2003; Kumar et al., 2018). However, when passive diffusion secretory was take into consideration in addition to the active uptake for $CL_{int,sec,in\ vivo}$ calculations, Eq. 3 was used.

$$CL_{int,sec,in\ vivo}\left({mL}/{min * kg}\right) =$$

$$\Big(\big(hOAT1\ Active\ Uptake\ CL\big) * (REF_{OAT1}) + \big(hOAT2\ Active\ Uptake\ CL\big) * (REF_{OAT2}) +$$

$$\big(hOAT3\ Active\ Uptake\ CL\big) * (REF_{OAT3}) + Passive\ Diffusion\ CL_{in\ vitro}\Big) * MPPGC *$$

$$Cortex\ Weight/kg\ BW \quad \text{Eq. 3}$$

Where passive diffusion $CL_{in\ vitro}$ was obtained from Mathialagan et al.(Mathialagan et al., 2017). Once the $CL_{int,sec,in\ vivo}$ predicted by the REF approach was calculated by either Eq. 2 or 3, it was used to predict the total renal secretory clearance ($CL_{sec,plasma}$) of the drug using Eq. 4.

$$CL_{sec,plasma}\left({mL}/{min * kg}\right) = \frac{Q_r * f_{u,blood} * CL_{int,sec,in\ vivo}}{Q_r + f_{u,blood} * CL_{int,sec,in\ vivo}} * (B/P)$$

Eq. 4 Where the Q_r (15.7 mL/min/kg) is the renal blood flow, $f_{u,blood}$ is the unbound fraction in blood and (B/P) is the blood to plasma ratio. Then, the total renal CL ($CL_{r,plasma}$) was calculated using unbound fraction in plasma ($f_{u,plasma}$), glomerular filtration rate (GFR; 1.78 mL/min/kg) (Varma et al., 2009), $CL_{sec,plasma}$, and the fraction reabsorbed (F_{reabs}) (Eq. 5). Because the passive reabsorption fraction (F_{reabs}) of drugs cannot be determined *in vivo*, it was

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assumed to be zero (Mathialagan et al., 2017). Of note, the probe substrates are also multi-drug resistance protein (MRP) and/or multi-antimicrobial extrusion protein (MATE) substrates. However, in using Eq. 5, we assumed as did Mathialagan et al., that the transporter CL across the basal membrane (mediated by OATs) was the rate-determining step in the renal secretory CL of the drug.

$$CL_{r,plasma}\left({}^{mL}/_{min*kg}\right) = (f_{u,plasma} * GFR + CL_{sec,plasma})(1 - F_{reabs}) \quad \text{Eq. 5}$$

Comparison of the Ability of REF and RAF to Predict $CL_{int,sec,in\ vivo}$, $CL_{sec,plasma}$ and $CL_{r,plasma}$

Two approaches were used to compare the ability of REF and RAF to predict $CL_{int,sec,in\ vivo}$, $CL_{sec,plasma}$ and $CL_{r,plasma}$. First, we determined the number of drugs where the predicted values fell within 2-fold or 3-fold of the observed values. Second, to determine if the two approaches were significantly different from each other, we determined the precision (root mean squared error (RMSE); Eq. 6) and bias (mean error (ME); Eq. 7) of each approach where n is the number of drugs tested. If the 95% confidence intervals of precision and bias of each approach overlapped, we concluded that the two approaches were not statistically different. The above statistics were computed with and without including passive diffusion secretory CL of the drugs. Also, of note, the probe drugs were not included when computing these statistics because they were used to derive the RAF values.

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(predicted_i - observed_i)^2}{n}} \quad \text{Eq. 6}$$

$$ME = \frac{\sum_{i=1}^{n}(predicted_i - observed_i)}{n} \quad \text{Eq. 7}$$

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Results

REF and RAF Values

The REF values determined by quantifying the OAT1, 2, and 3 transporters in HEK293 and renal cortex were 0.16, 0.15, and 0.37, respectively (Supplementary Table 2) (Prasad et al., 2016). The RAF values for OAT1 (tenofovir), OAT2 (acyclovir and ganciclovir), and OAT3 (oseltamivir acid and benzylpenicllin) were previously reported as 0.64, 7.3, and 4.1, respectively (Mathialagan et al., 2017). As indicated in a previous publication, the chosen probe substrates (i.e. tenofovir, acyclovir/ganciclovir and oseltamivir/benzylpenicillin) are selective for the specified transporter and have no significant uptake by the other OAT transporters located on the basal membrane of the kidney epithelial cells (Mathialagan et al., 2017).

Comparison of the REF and RAF Approaches to Predict Secretory and total Renal Clearance of Drugs

For the REF approach, 46% and 62% of the $CL_{int,sec,\textit{in vivo}}$ predictions were within 2- and 3-fold of the observed values, respectively; while the corresponding values for the RAF approach were 62% and 73%, respectively (Table 1, Fig. 2a-b). For the REF approach, 50% and 69% of the $CL_{sec,plasma}$ predictions were within 2- and 3-fold of the observed values, respectively; while the corresponding values for the RAF approach were 65% and 81%, respectively (Table 1, Fig. 2c-d). Finally, for the REF approach, 65% and 92% of the $CL_{r,plasma}$ predictions were within 2- and 3-fold of the observed values; while the corresponding values for the RAF approach were 81% and 88%, respectively (Table 1, Fig. 2e-f). The 95% confidence intervals for the precision (RMSE) and bias (ME) of the REF-predicted $CL_{int,sec,\textit{in vivo}}$, $CL_{sec,plasma}$ and $CL_{r,plasma}$ overlapped with those of the RAF approach (Table 1, Fig. 3). Precision and bias calculations were identical

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for the predicted $CL_{sec,plasma}$ and $CL_{r,plasma}$, as addition of filtration clearance (the same constant value in both approaches) to $CL_{sec,plasma}$ did not alter the predictive power of the two approaches. The REF and RAF $CL_{int,sec,\textit{in vivo}}$, $CL_{sec,plasma}$, and $CL_{r,plasma}$ prediction, observed value, and fold error for each drug are listed in Supplementary Table 3.

Theoretically, the REF approach should be highly sensitive to the value of the PSF used, while the RAF approach should not be as it does not necessarily need to use a PSF. However, although Mathialagan et al. did use PSF, the RAF values are independent of the PSF used as this value cancels out when predicting $CL_{int,sec,\textit{in vivo}}$ (see Eq. 9, in (Mathialagan et al., 2017)). Nevertheless, their PSF values were 15 milligrams of protein per gram of kidney (0.25 mg of protein per million HEK cells, 60 million HEK cells per gram of kidney) and 4.3 grams of kidney per kilogram of body weight (hereafter referred to as the kidney PSF) (Mathialagan et al., 2017). Therefore, we examined the sensitivity of the REF and RAF approach to the value of PSF used. The PSF for the REF approach that we used was the value that we have previously determined in kidney tissue where the aforementioned transporters were quantified, i.e. 210 mg of protein per gram of kidney (hereafter referred to as the cortex PSF). Since these approaches used different PSF, we compared the predictive power of the two approaches using the same PSF. As expected (since the RAF approach is independent of the PSF used), when the cortex PSF was used, the predicted $CL_{sec,plasma}$ by the RAF differed from that by kidney PSF by only 1.6% (Supplementary Fig. 1). In contrast, the predicted $CL_{sec,plasma}$ by the REF using the kidney PSF considerably underpredicted the observed values by an average of about 10-fold (Supplementary Fig. 1).

In the above analyses, for both the REF and RAF approach, passive diffusion secretory CL of the drug was not taken into consideration. Therefore, we compared the predictive capability of the REF and RAF approaches with inclusion of passive diffusion secretory clearance. To be

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consistent across both approaches, the cortex PSF was used to scale the passive diffusion secretory CL. In doing so, for the REF approach, 23% and 50% of the $CL_{int,sec,\textit{in vivo}}$ predictions were within 2- and 3-fold of the observed values, respectively; while the corresponding values for the RAF approach were 35% and 54%, respectively (Table 2, Fig. 4a-b). For the REF approach, 31% and 65% of the $CL_{sec,plasma}$ predictions were within 2 and 3-fold of the observed values, respectively; while the corresponding values for the RAF approach were 46% and 58%, respectively (Table 2, Fig. 4c-d). For the REF approach, 38% and 88% of the $CL_{r,plasma}$ predictions were within 2 and 3-fold of the observed values, respectively; while the corresponding values for the RAF approach were 65% and 85%, respectively (Table 2, Fig. 4e-f). The 95% confidence intervals for the precision (RMSE) and bias (ME) of the REF-predicted $CL_{int,sec,\textit{in vivo}}$, $CL_{sec,plasma}$ and $CL_{r,plasma}$ overlapped with those of the RAF approach. However, the REF approach demonstrated a positive bias for the $CL_{int,sec,\textit{in vivo}}$ predictions (Table 2, Fig. 5) while the RAF approach did not. Nevertheless, even with the addition of passive diffusion secretory CL, the $CL_{int,sec,\textit{in vivo}}$, $CL_{sec,plasma}$, and $CL_{r,plasma}$ predictions for both the REF and RAF approaches were equally as precise and unbiased as the predictions when passive diffusion secretory CL was not taken into consideration (Supplementary Fig. 2). The REF and RAF $CL_{int,sec,\textit{in vivo}}$, $CL_{sec,plasma}$, and $CL_{r,plasma}$ prediction, observed value, and fold error after the inclusion of passive diffusion secretory CL for each drug are listed in Supplementary Table 4.

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Discussion

The goal of this work was to evaluate the capability of the REF approach to predict $CL_{int,sec,\textit{in vivo}}$, $CL_{sec,plasma}$ and $CL_{r,plasma}$ from *in vitro* OAT uptake studies in OAT-overexpressing HEK cells, and compare the predictability to the previously reported RAF approach (Mathialagan et al., 2017). Our primary end-point was comparison of the predicted $CL_{sec,plasma}$ as opposed to $CL_{r,plasma}$. The latter is often used as the prediction endpoint, but this is misleading because $CL_{r,plasma}$ can be well-predicted even when $CL_{sec,plasma}$ is not. This will occur when $CL_{sec,plasma}$ is a relatively small fraction of the total renal CL of the drug (Kunze et al., 2014b).

Three main assumptions were made in our study. First, of the three parts that compose renal clearance, we accounted for filtration and tubular secretion but reabsorption was assumed to be negligible based on a sigmoidal permeability-tubular reabsorption model (Scotcher et al., 2016b; Mathialagan et al., 2017). Second, a well-stirred model was used to predict the $CL_{sec,plasma}$. The well-stirred model postulates instantaneous and complete mixing of the unbound drug between the interstitial space of the renal cortex and blood (Malcolm Rowland, 2011). Third, we assumed that the rate-determining step for $CL_{sec,plasma}$ of the 26 drugs studied was uptake via the designated OAT transporters; other apical or basolateral transporters, if any, were considered insignificant contributors to their $CL_{r,plasma}$ (Watanabe et al., 2011; Mathialagan et al., 2017).

When passive diffusion secretory CL was assumed to be negligible, the predictive power of the two approaches was not statistically different as indicated by the overlapping 95% confidence intervals of the precision and bias of the predictions of $CL_{int,sec,\textit{in vivo}}$, $CL_{sec,plasma}$ and $CL_{r,plasma}$ (Table 1, Fig. 3). As expected, precision in the prediction of $CL_{sec,plasma}$ or $CL_{r,plasma}$ was

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respectively greater than $CL_{int,sec,\textit{in vivo}}$ for the following reasons. First, incorporation of renal blood flow (Eq. 4) in predicting $CL_{sec,plasma}$ dampened the contribution of $CL_{int.sec}$ to the total $CL_{sec,plasma.}$ Second, when predicting $CL_{r,plasma}$, due to contribution of filtration CL, the contribution of $CL_{int.sec,\textit{in vivo}}$ to $CL_{r,plasma}$ further diminishes.

Although the *in vivo* passive diffusion secretory CL may be negligible for some drugs, this may not be the case for other drugs. Thus, the correct approach is to compare the observed $CL_{int,sec,\textit{in vivo}}$ with that predicted using the REF and RAF approaches after including the predicted passive diffusion secretory CL. In doing so, the predictions of all CL parameters by both the REF and RAF approaches worsened as indicated by the greater number of drugs that fell outside the 2 and 3-fold error windows ($CL_{sec,plasma}$: n=9) (Table 2, Fig. 4). For the two approaches, those drugs for which the predicted values of $CL_{sec,plasma}$ were worse and fell out of the 2 or 3-fold window had notable contribution from passive diffusion secretory CL. The drugs that fell out of the 2 or 3-fold window for the REF approach, had a 20 – 50% contribution of passive diffusion secretory CL of the total $CL_{int,sec,\textit{in vivo}}$. The corresponding contribution was 39 – 99% for the RAF approach. Interestingly, where available, this predicted passive diffusion secretory CL was corroborated by the *in vivo* passive diffusion secretory CL as measured by the change in the secretory renal CL of the drug when the drug was co-administered with probenecid to inhibit OATs (Mathialagan et al., 2017). In the presence of probenecid, data were available for renal CL of 3 of the 9 drugs that fell out of either the 2 or 3-fold windows for $CL_{sec,plasma}$ predictions. For those drugs, the *in vivo* passive diffusion secretory CL was estimated to be 32 – 67% of the total renal CL, whereas our predictions for these three drugs ranged 23 – 50%. Nevertheless, even with this deterioration in the precision of predictions, the precision and bias in $CL_{int,sec,\textit{in vivo}}$, $CL_{sec,plasma}$ or $CL_{r,plasma}$ predictions by the REF and RAF approaches did not differ significantly (Supplementary Fig. 2). Irrespective of whether passive diffusion CL was included or not, there were no trends identified regarding the drugs that were

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outside the 2 and 3-fold error windows, when drug dependent characteristics such as molecular weight, ionization state, LogP, f_u, and magnitude of $CL_{sec,plasma}$. However, there was a high degree of overlap between the REF and RAF approaches (9/13 and 14/18 drugs overlapped for the $CL_{sec,plasma}$ predictions with only active uptake CL and with the inclusion of passive diffusion CL, respectively). The overlap is likely due to the similar assumptions made in clearance predictions as discussed earlier. These results do point to an interesting finding that suggests that our approach to predict passive diffusion secretory CL of OAT-transported drugs needs refinement. Until that refinement has been accomplished, because predictions using only the active uptake clearance were more precise, we would suggest IVIVE of OAT-mediated renal CL of drugs based on active uptake clearance alone.

Since the prediction capability of the REF and RAF approaches were not significantly different, our results indicate that the two approaches can be used interchangeably to predict the renal secretory CL of OAT substates. When applied to clearance (renal or hepatic) via other transporters, both approaches have their pros and cons. Since the RAF approach requires a probe substrate, the REF approach is more useful when a transporter-selective probe substrate is not available for any one of the transporters of interest. For example, the REF approach can potentially be used to determine the clearance of drugs that are not solely rate-determined by a single apical (MATE1/2K, MRP4, etc.) or basal (OAT1/2/3, organic cation transporter (OCT)2/3, etc.) transporter. Abundance of the basal and apical transporters in HEK293 cells and renal cortex would need to be measured to include them in the REF approach. Verification of the REF approach to test the predictive power of $CL_{sec,plasma}$ and $CL_{r,plasma}$ for the drugs with multiple clearance pathways as the rate-determining steps remains to be tested. In contrast, when a transporter-selective probe substrate is available *in vivo* and *in vitro* (if using primary cells e.g. kidney epithelial cells), the RAF approach will likely perform better. Unfortunately, such probe substrates are rarely available. Even for the OAT substrates studied here, many of them are

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multiple OAT transporter substrates. In that event, data on multiple probe substrates, each selective for a given OAT, are needed. In addition, the RAF approach assumes that the *in vivo* renal secretory CL is the only rate-determining step in the systemic renal CL of the drug. If the apical transporters are involved (e.g. MATEs or P-glycoprotein (P-gp)), this assumption will not hold and therefore the estimation of the renal CL using the RAF approach will be inaccurate. On the other hand, assuming no passive diffusion secretory CL, the REF approach (but not the RAF approach) is highly dependent on the PSF used as demonstrated by the 10-fold difference in the $CL_{sec,plasma}$ predictions when the cortex vs. kidney PSF was used (Supplementary Fig. 1). However, it is important to note that when the passive diffusion secretory clearance is included to predict the $CL_{sec,plasma}$, both approaches, REF and RAF, need to utilize PSF and therefore estimation of this parameter will be highly dependent on the PSF value. Therefore, we gathered literature values on the various PSF determined by us and others (Table 3). The kidney PSF used by Mathialagan et al. is the lowest, while the one used here is the highest reported (15 – 210 mg protein/g kidney; Table 3) (Mitchell et al., 1945; Forbes et al., 1953; Forbes et al., 1956; Snyder, 1979; Pacifici et al., 1988; Knights et al., 2016; Mathialagan et al., 2017; Scotcher et al., 2017; Kumar et al., 2018). Thus, it is imperative that the correct PSF value be used when using both approaches and, going forward, a consensus is needed on the PSF value that should be used.

Human renal clearance predictions are often based on preclinical animal data (i.e. rat and dog CL_r), due to lack of reliable in vitro based approaches (Paine et al., 2011). The REF or RAF approaches, which were demonstrated to provide reasonable IVIVE in our study, can be employed to project renal clearance in drug discovery setting, and thus enable dose predictions. Additionally, this approach allows for quantitating individual transporter contribution (f_t) to the overall renal secretion, which allows for DDI predictions in drug development.

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In conclusion, using the same *in vitro* and *in vivo* dataset, we showed that the REF and RAF approaches were not significantly different in their ability to predict CL_r of OAT substrates. However, for drugs that have renal (or hepatic) CL rate-determined by both basal and apical transporters, the REF approach has an advantage over the RAF approach. This is because the latter is dependent on the availability of *in vivo* renal CL data for a probe drug. It is highly unlikely that such data are possible to obtain with currently approved drugs. Despite the theoretical advantage of the REF approach (i.e. it does not require data on probe drugs), its ability to simultaneously and accurately predict renal clearance when multiple rate-determining steps (and therefore transporters) are involved remains to be tested.

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Authorship Contributions

Participated in research design: Varma, Prasad, Unadkat

Conducted experiments: Prasad, Bhatt, Mathialagan

Performed data analysis: Kumar, Mathialagan, Bhatt

Wrote or contributed to the writing of the manuscript: Kumar, Prasad, Varma, Unadkat, Mathialagan, Bhatt

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Footnotes

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Figure Legends

Fig. 1: For IVIVE of renal $CL_{sec,plasma}$ of drugs, the REF approach scales the drug uptake CL into TEC using the REF (transporter abundance in TEC/transporter abundance in the human kidney). In contrast, the RAF approach scales the drug uptake CL into TEC using the RAF (uptake CL of the probe drug in TEC/*in vivo* probe $CL_{int,sec,in\ vivo}$). PSF – physiological scaling factor; Eq. x indicates the Equation used in the REF approach or the RAF approach (Mathialagan et al., 2017).

Fig. 2: Observed and predicted values of *in vivo* $CL_{int,sec}$ (a-b), $CL_{sec,plasma}$ (c-d) or $CL_{r,plasma}$ (e-f) when using the REF and RAF approaches. The solid line is the line of identity. Note: passive diffusion secretory CL of the drugs was assumed to be negligible.

Fig. 3: Both the REF and RAF approaches were equally precise (RMSE) and unbiased (ME) in predicting the *in vivo* $CL_{int,sec}$ (a, b) as demonstrated by the overlapping 95% confidence intervals (lines). This conclusion remained the same for precision and bias of $CL_{sec,plasma}$ and $CL_{r,plasma}$ predictions (c, d) by the two approaches. Note: passive diffusion secretory CL of the drugs was assumed to be negligible.

Fig. 4: Observed and predicted values of *in vivo* $CL_{int,sec}$ (a-b), $CL_{sec,plasma}$ (c-d) or $CL_{r,plasma}$ (e-f) when passive diffusion secretory CL was included in the REF and RAF approaches. The solid line is the line of identity.

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Fig. 5: After including passive diffusion secretory clearance when predicting $CL_{int,sec,\textit{in vivo}}$, the REF approach demonstrated a positive bias (ME) while the RAF approach did not, but both approaches were equally precise (RMSE) as demonstrated by the overlapping 95% confidence intervals (lines; a, b). In addition, both approaches were equally precise (RMSE) and unbiased (ME) in predicting $CL_{sec,plasma}$ and $CL_{r,plasma}$ of the drugs (c, d).

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Tables

Table 1: The predicted Precision, Bias, and Percent of data within 2- or 3-Fold of the observed value for $CL_{int,sec,in\ vivo}$, $CL_{sec,plasma}$, or $CL_{r,plasma}$ when using the REF or RAF approach[b]

	IVIVE Approach	Percent within 2-fold error	Percent within 3-fold error	Precision[a]	Bias[a]
$CL_{int,sec,in\ vivo}$	REF	46	62	15 [3.1, 26]	3.0 [-2.9, 9.0]
	RAF	62	73	9.8 [0.97, 18]	-3.3 [-7.1, 0.45]
$CL_{sec,plasma}$	REF	50	69	2.0 [0.15, 3.8]	0.40 [-0.41, 1.2]
	RAF	65	81	1.6 [0.058, 3.1]	0.015 [-0.65, 0.67]
$CL_{r,plasma}$	REF	65	92	2.0 [0.15, 3.8]	0.40 [-0.41, 1.2]
	RAF	81	88	1.6 [0.058, 3.1]	0.015 [-0.65, 0.67]

[a] Precision, RMSE (root mean square error) and bias, ME (mean error) are reported as mean and [95% confidence interval]
[b] Passive diffusion secretory CL of the drug was assumed to be negligible

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Table 2: The predicted Precision, Bias, and Percent of data within 2- or 3-Fold of the observed value for $CL_{int,sec,in\ vivo}$, $CL_{sec,plasma}$, or $CL_{r,plasma}$ when passive diffusion secretory CL was included in the RAF or REF approaches

	IVIVE Approach	Percent within 2-fold error	Percent within 3-fold error	Precision[a]	Bias[a]
$CL_{int,sec,in\ vivo}$	REF	23	50	29 [-0.41, 59]	13 [1.5, 24]
	RAF	35	54	21 [-2.0, 44]	6.1 [-2.1, 14]
$CL_{sec,plasma}$	REF	31	65	2.4 [0.29, 4.6]	0.92 [-0.0032, 1.9]
	RAF	46	58	2.1 [0.14, 4.0]	0.63 [-0.18, 1.4]
$CL_{r,plasma}$	REF	38	88	2.4 [0.29, 4.6]	0.92 [-0.0032, 1.9]
	RAF	65	85	2.1 [0.14, 4.0]	0.63 [-0.18, 1.4]

[a] Precision (RMSE) and bias (ME) values are reported as mean and [95% confidence interval]

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Table 3: Total protein concentration in whole kidney and kidney cortex tissue

Source	Method	Microsomes (mg total microsomal protein/g kidney)		Homogenate (mg total homogenate protein/g kidney)
Mitchell (1945)	Nitrogen mineralization			146.9
Forbes (1953)	Nitrogen mineralization			192.8
Forbes (1956)	Nitrogen mineralization			181.9
Pacifici (1988)	Lowry method	5.3		48.4[a]
Al-Jahdari (2006)	Lowry method	12.8		32.0[b]
Knights (2016)	Cytochrome C reductase activity	9.3		53.8[b]
Mathialagan (2017)	HEK cell protein concentration			15
		Microsomes (mg total microsomal protein/g cortex)	**Homogenate (mg total homogenate protein/g cortex)**	
Scotcher (2017)	BCA assay kit	26.2	89.3[a]	62.5[c]
Kumar (2018)	BCA assay kit		300	210[c]

[a] Extrapolated with the assumption that the theoretical contribution of S9 protein to homogenate protein is 89%

[b] Back calculated from microsomal protein and Cyt C reductase activity in microsomes and homogenate

[c] Extrapolated with the assumption that the cortex weight is 70% of kidney weight

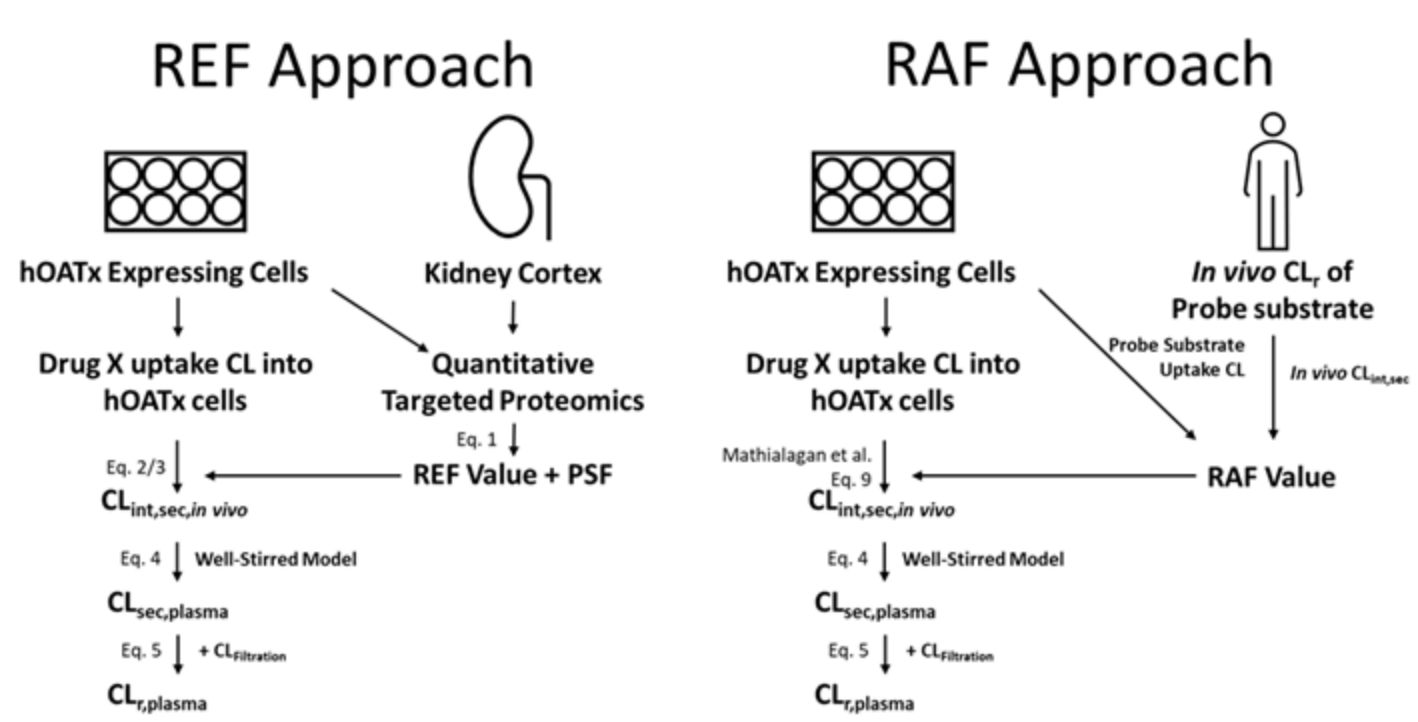
REF Approach
hOATx Expressing Cells
Kidney Cortex
Drug X uptake CL into hOATx cells
Quantitative Targeted Proteomics
Eq. 1
Eq. 2/3
REF Value + PSF
$CL_{int,sec,in\ vivo}$
Eq. 4
Well-Stirred Model
$CL_{sec,plasma}$
Eq. 5
+ $CL_{Filtration}$
$CL_{r,plasma}$
RAF Approach
hOATx Expressing Cells
In vivo CL_r of Probe substrate
Drug X uptake CL into hOATx cells
Probe Substrate Uptake CL
In vivo $CL_{int,sec}$
Mathialagan et al.
Eq. 9
RAF Value
$CL_{int,sec,in\ vivo}$
Eq. 4
Well-Stirred Model
$CL_{sec,plasma}$
Eq. 5
+ $CL_{Filtration}$
$CL_{r,plasma}$

Figure 1

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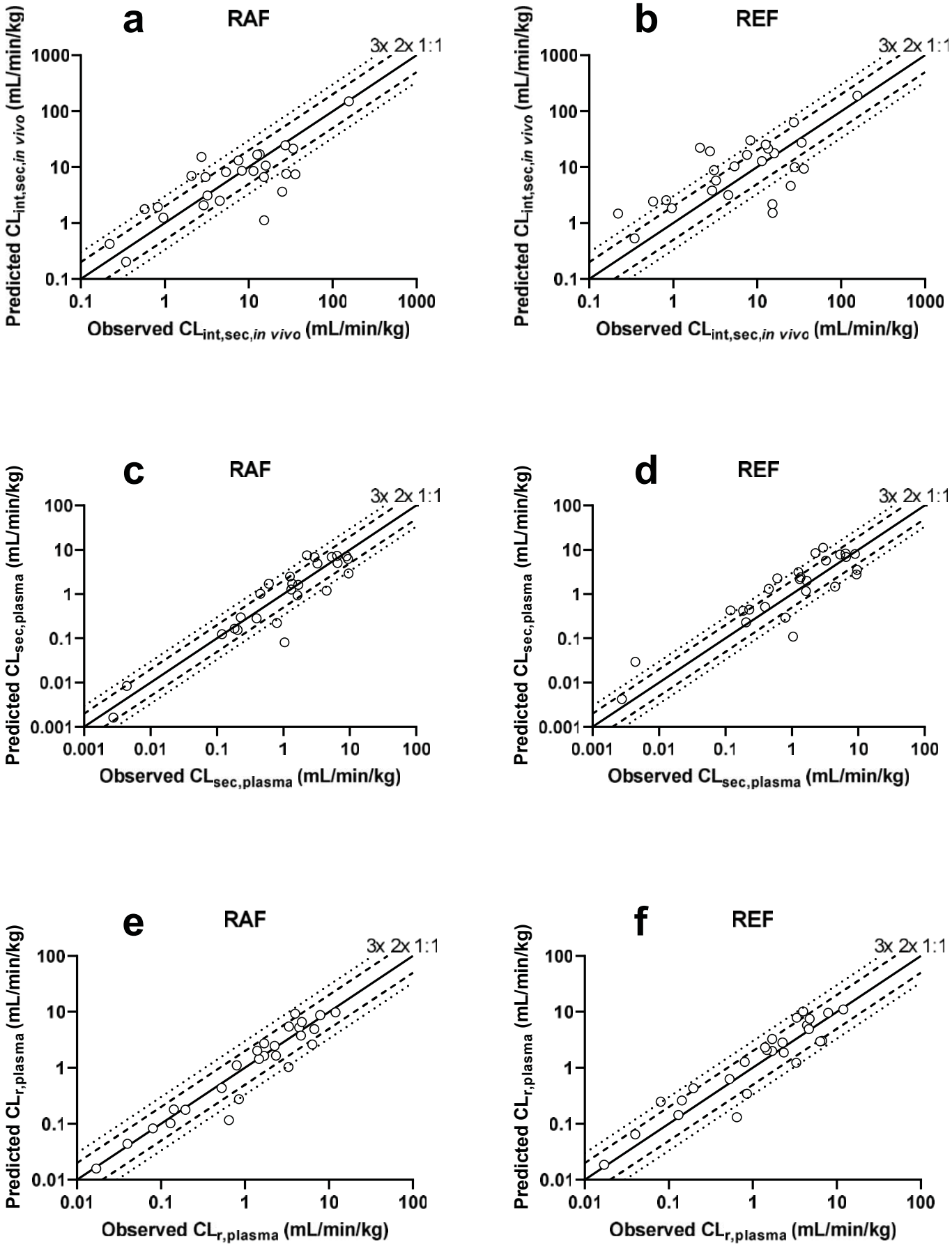

Figure 2

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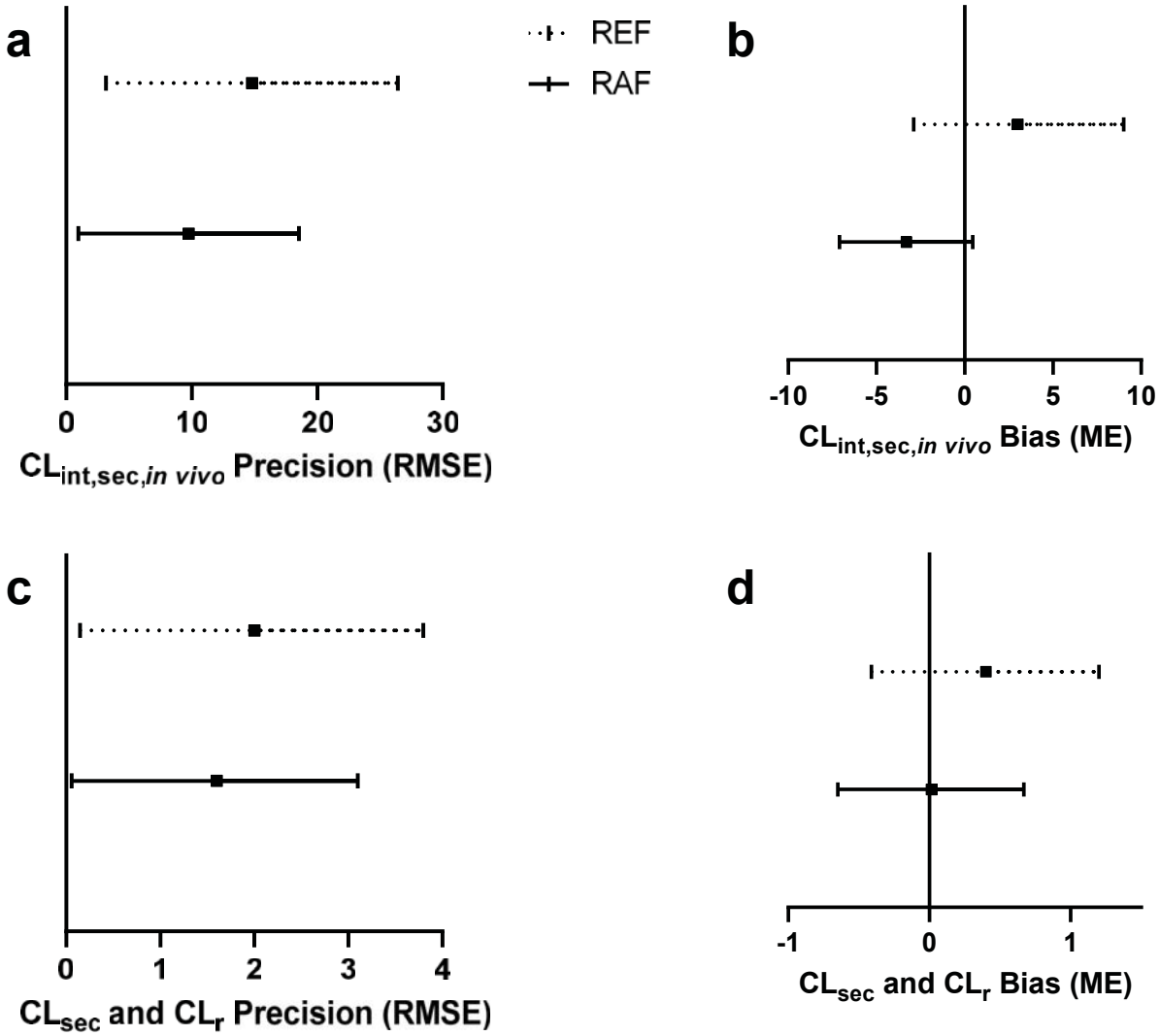

Figure 3

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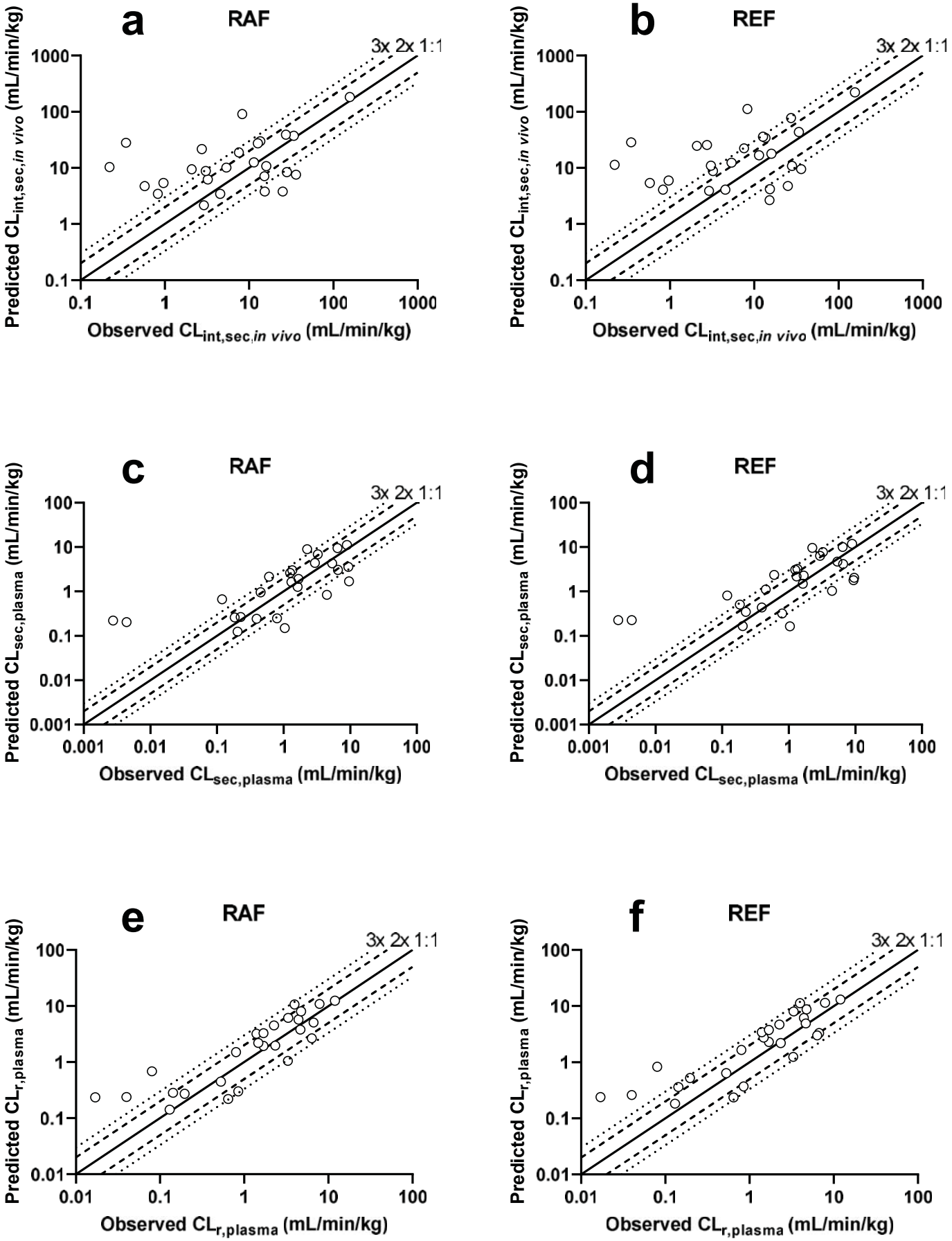

Figure 4

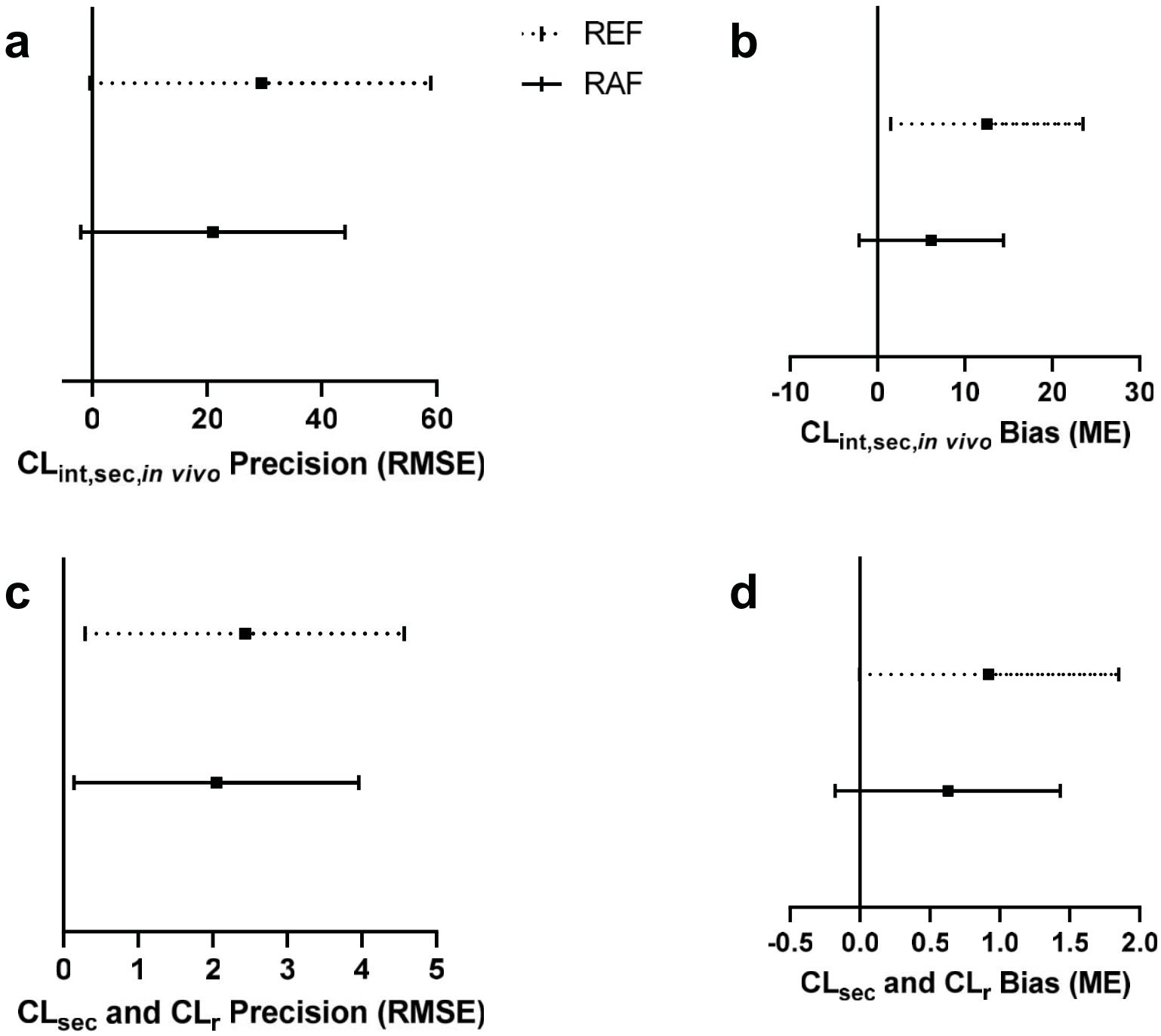

Figure 5