Sexual Dimorphism in the Expression of Cytochrome P450 Enzymes in Rat
Heart, Liver, Kidney, Lung, Brain, and Small Intestine

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Sex Differences in Levels of CYP Enzymes in Rat Organs

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Abstract:

Cytochrome P450 (CYP) enzymes are monooxygenases that are expressed hepatically and extrahepatically and play an essential role in xenobiotic metabolism. Substantial scientific evidence indicates sex-specific differences between males and females in disease patterns and drug responses, which could be attributed, even partly, to differences in the expression and/or activity levels of CYP enzymes in different organs. In this study, we compared the mRNA and protein expression of CYP enzymes in different organs of male and female Sprague Dawley rats by real-time polymerase chain reaction and Western blot techniques. We found significant sex and organ-specific differences in several enzymes. Hepatic Cyp2c11, Cyp2c13, and Cyp4a2 showed male-specific expression, while Cyp2c12 showed female-specific expression. Cyp2e1 and Cyp4f enzymes demonstrated higher expression in the female heart and kidneys compared to males; however, they showed no significant sexual dimorphism in the liver. Male rats showed higher hepatic and renal Cyp1b1 levels. All assessed enzymes were found in the liver, but some were not expressed in other organs. At the protein expression level, CYP1A2, CYP3A, and CYP4A1 demonstrated higher expression levels in the females in several organs including the liver. Elucidating sex-specific differences in CYP enzyme levels could help better understand differences in disease pathogeneses and drug responses between males and females, and thus improve treatment strategies.

Significance statement: In this study, we characterized the differences in the mRNA and protein expression levels of different CYP enzymes between male and female rats in the heart, liver, lung, kidney, brain, and small intestine. We demonstrated unique sex-specific differences in the different organs. This study is considered a big step towards elucidating sex-specific differences in CYP enzyme levels, which is largely
important for achieving a better understanding of differences between males and females in diseases processes and treatment outcomes.
Introduction:

Cytochrome P450 (CYP) is a superfamily of membrane-bound hydrophobic heme enzymes that play a pivotal role in health, homeostasis, and metabolism. CYP enzymes are expressed in almost all biological systems (El-Sherbeni and El-Kadi, 2017). They are so-called because their heme pigment absorbs light at a wavelength of 450 nm following reduction and exposure to carbon monoxide (Lynch and Price, 2007). The discovery of CYP enzymes started in the early 1950s and continued until the 1960s (El-Sherbeni and El-Kadi, 2017). CYP enzymes are classified into families, subfamilies, and individual enzymes based on the structural homology of their amino acid sequences (Nebert et al., 1987; Elbekai and El-Kadi, 2006). Microsomal CYPs, which are attached to the endoplasmic reticulum membrane, comprise the majority of human CYP enzymes and catalyze a wide array of biological reactions (El-Sherbeni and El-Kadi, 2017).

CYP enzymes are mainly expressed in the liver, but they are also found in several extrahepatic tissues including the heart (Elbekai and El-Kadi, 2006), kidney (Fan et al., 2015), lungs (Hukkanen et al., 2002), brain (Dutheil et al., 2008), and other tissues (Zhu and Zhang, 2012; Alonso et al., 2015; Ibrahim et al., 2020). The induction or inhibition of CYP enzymes by xenobiotics or disease states is a major mechanism underlying drug-drug and drug-disease interactions. Moreover, genetic polymorphisms in CYP genes could lead to differences in CYP enzymes that might explain individual and ethnic variations in disease pathogeneses and drug responses (Manikandan and Nagini, 2018). Given their significant biological effects, CYP enzymes have been the focus of many clinical, experimental, and drug development studies.
CYP enzymes play an essential role in the detoxification and activation of both xenobiotics and endogenous molecules. In humans, there are 18 CYP families with more than 50 individual CYP isoenzymes, 9 of which are involved in the metabolism of several drugs (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5) (Wilkinson, 2005). In addition to their essential role in xenobiotic metabolism, CYP enzymes are also largely involved in the synthesis and metabolism of endogenous molecules such as steroids and prostaglandins. For example, CYPs metabolize polyunsaturated fatty acids like arachidonic acid (AA) by the insertion of either an epoxide or hydroxyl group, based on which they can be classified into CYP epoxygenases or hydroxylases, respectively. AA metabolism by CYP enzymes to give multiple epoxy and hydroxy metabolites has been extensively studied in health and diseases, particularly in the cardiovascular system (Elbekai and El-Kadi, 2006; Shoieb et al., 2019; Gerges and El-Kadi, 2021).

To date, a large body of evidence points out to significant differences between males and females in the pathogenesis and outcomes of different diseases, as well as in drug metabolism and responses (Dahan et al., 2008; Waxman and Holloway, 2009; Ngo et al., 2014; Regitz-Zagrosek and Kararigas, 2017; Holingue et al., 2020; Tramunt et al., 2020; Gerges and El-Kadi, 2021; Madla et al., 2021). Moreover, it was established previously that there are significant sex differences in the expression or activity of several drug metabolizing enzymes in animals and humans, and that this could be responsible for differences in clinical drug effects between men and women (Waxman and Holloway, 2009). For example, multiple studies have shown higher mRNA and protein expression levels of hepatic CYP3A4 in women than in men, which could explain the higher clearance rates of CYP3A4 substrates in women (Tanaka, 1999; Greenblatt and Von Moltke, 2008; Waxman and Holloway, 2009). On
the other hand, some CYP1A2, CYP2E1, and CYP2D6 substrates were found to have higher clearance rates in men than in women (Franconi et al., 2007; Schwartz, 2007). Cardiovascular diseases are among the diseases that demonstrate significant sex-specific discrepancies, which could be mediated, even in part, by different expression or activity levels of cardiac CYP enzymes and their metabolites (Gerges and El-Kadi, 2021).

Elucidating sex differences in the expression levels of CYP enzymes in different organs could help explain observed sex differences in diseases and drug effects, decrease the incidence of adverse effects and improve the efficacy of different medications, and approach precision medicine. Thus, the current study is one of a series of studies aiming at investigating sex-specific differences in the expression and activity levels of different CYPs, as well as the levels of their metabolites. The aim of the current study was to compare the mRNA and protein expression levels of different CYP enzymes in the heart, liver, lung, kidney, brain, and small intestine between male and female rats.

**Material and Methods:**

**Nomenclature:**

The nomenclature used throughout the manuscript is following the Guidelines for Formatting Gene and Protein Names released in 2014. Briefly, enzyme symbols were written in sentence case and italicized when referring to genes or mRNA of mice or rats, and were capitalized and non-italicized when referring to the proteins (A, 2014).

**Animals:**
Adult (8 weeks old) male (260-280 g, n = 6) and female (200-220g, n = 6) Sprague Dawley (SD) rats were purchased from Charles River Canada (Montreal, QC, Canada). All animals were allowed access to food and water ad libitum throughout the experiment period and were maintained on a 12-hour light/dark cycle. Rats were kept in the animal facility for an acclimatization period of 1 week, after which they were euthanized under isoflurane anesthesia. The liver, heart, lung, kidney, brain, and small intestine (20 cm extending from the stomach distally) were isolated and immediately frozen in liquid nitrogen then stored at -80 °C. All procedures involving experimental animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and were approved by Alberta Health Sciences Animal Policy and Welfare Committee.

Chemicals:

TRIzol reagent used for mRNA extraction was Invitrogen brand (Thermo Fisher Scientific, Carlsbad, CA). High Capacity cDNA Reverse Transcription Kit and SYBR Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA). Real-time polymerase chain reaction (PCR) primers were formulated by and purchased from Integrated DNA Technologies (Coralville, IA). Trans-Blot Turbo RTA Transfer Kit and 2X Laemmli Sample Buffer were purchased from Bio-Rad Laboratories (Hercules, CA). CYP1A2, CYP3A, and CYP4A1 mouse monoclonal primary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX); CYP2C23, CYP2E1, and CY4F2 rabbit polyclonal primary antibodies were purchased from Abcam (Cambridge, UK); and CYP2J rabbit polyclonal primary antibody was purchased from MilliporeSigma (St. Louis, MO). Chemiluminescence
Western blotting detection reagents (ECL) were obtained from Cytiva (MA, USA). All other chemicals used were obtained from Sigma Aldrich (St. Louis, MO).

RNA extraction and cDNA synthesis:

RNA extraction and cDNA synthesis were performed according to the method described by Elshenawy and El-Kadi (Elshenawy and El-Kadi, 2015).

Quantification of mRNA expression by real-time PCR:

The resulting cDNA was subject to PCR amplification using 384-well optical reaction plates in the QuantStudio 5 (Applied Biosystems). The 20-µL reaction mix contained 0.04 µL of 10 µM forward primers and 0.04 µL of 10 µM reverse primers (20 nM final concentration of each primer), 10 µL of SYBR Green Universal Master Mix, 8.92 µL of nuclease-free water, and 1 µL of cDNA sample. Thermocycling conditions were as described in previous reports (Shoieb et al., 2022). The rat CYP enzymes and their human orthologs are listed in Table 1. Rat primer sequences used in this study are listed in Table 2. Analysis of the real-time PCR data was performed using the relative gene expression (ΔΔCT) method (Livak and Schmittgen, 2001). In short, the fold change in the level of target genes between female and male rats, corrected for the level of the housekeeping gene, was determined using the following equation: Fold change = 2−ΔΔCt, where ΔCt = Ct(target gene) − Ct(housekeeping gene) and Δ(ΔCt) = ΔCt(females) − mean ΔCt(males). For comparison of all genes’ expression within the same organ, fold change was calculated relative to the least expressed gene.

Preparation of microsomal protein:
Microsomal fractions were prepared by differential centrifugation of the homogenized organs. Briefly, a weighed mass of each organ was homogenized in cold sucrose solution (0.25 M in distilled water, 0.5 g tissue in 2 mL sucrose solution) containing protease inhibitor cocktail (5 µL/1 mL sucrose solution). The homogenate was centrifuged at 10,000g for 20 min. The resulting supernatant was centrifuged again at 100,000g for 60 min to obtain the microsomal pellet. The pellets were dissolved in the homogenization sucrose solution containing protease inhibitor cocktail and stored at -80°C. Lowry method was used to determine microsomal protein concentrations using bovine serum albumin as a standard (LOWRY et al., 1951).

**Western blot analysis:**

We determined the protein expression of important CYP metabolizing enzymes (CYP1A2, CYP3A) as well as some main arachidonic acid epoxygenases (CYP2C23, CYP2J) and hydroxylases (CYP2E1, CYP4A1, and CYP4F) using denaturing gel electrophoresis. Briefly, isolated proteins from the different organs of male and female rats (15 µg from the liver, 50 µg from the kidney, lung, and brain, 60 µg from the heart, and 75 µg from the small intestine) were diluted with an equal amount of 2X Laemmli Sample Buffer, boiled for 5 min, and separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described by Shoieb et al. (Shoieb et al., 2022). Then, the blots were incubated with the primary antibody: mouse anti-rat CYP1A2 (sc-53241), rabbit anti-rat CYP2C23 (ab53944), rabbit anti-rat CYP2E1 (ab28146), rabbit anti-rat CYP2J (ABS1605), mouse anti-rat CYP3A (sc-271033), mouse anti-rat CYP4A1 (sc-53248), and rabbit anti-human CYP4F2 (ab230709) for 2 h or overnight at 4°C. Then, blots were incubated with a horseradish peroxidase-conjugated horse anti-mouse or goat anti-rabbit IgG secondary antibody.
for 45 min at room temperature. Bands were visualized using the ChemiDocTM Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) using the enhanced chemiluminescence method.

**Statistical analysis:**

All results are presented as mean ± SEM. Comparisons between male and female groups were carried out using unpaired student t test. Differences were considered significant at p < 0.05. All statistical analyses and graphs plotting were performed using GraphPad Prism software, version 8.4.3. (GraphPad Software, Inc. La Jolla, CA, USA).

**Results:**

**Sex-specific differences in the mRNA and protein expression levels of CYP enzymes in the heart:**

The mRNA expression levels of different CYP enzymes were determined by real-time PCR. Some CYP enzymes were found to not be expressed in the heart (Cyp2a1, Cyp2c12, Cyp2d2, Cyp2d3, Cyp3a1, Cyp3a9, Cyp3a18, and Cyp4a8). Cyp4f5 is the most highly expressed CYP in the hearts of both male and female rats, while Cyp4a2 is the least expressed (Figure 1). Generally, female hearts showed higher CYP expression levels than male hearts. Cyp2e1 showed the most marked difference, being nearly 4-fold higher in female hearts than male hearts. Cyp1a1, Cyp1a2, Cyp4a1, Cyp4f1, and Cyp4f4 are all approximately 3-fold higher in female than male hearts. Cyp4a2, Cyp4a3, and Cyp4f5 showed no significant difference, while all other Cyp4 family members showed significantly higher expression in female hearts. Only Cyp2c23 was significantly higher in male hearts than female
hearts (5.3-fold) (Supp. Table S.1.1-S.1.23). Figure 2 shows the mRNA expression levels of different CYP enzymes in male hearts compared to female hearts.

Since mRNA expression does not always correlate with protein levels of enzymes, we measured the protein expression of certain CYP enzymes (CYP1A2, CYP2C23, CYP2E1, CYP2J, CYP3A, CYP4A1 and CYP4F) in all organs in order to investigate sex-specific differences at the protein level. As shown in Figure 3, CYP2J, CYP4A, and CYP4F enzymes were detected in the heart. In agreement with the mRNA results, CYP4A and CYP4F enzymes showed higher protein levels in female rat hearts (2.8 and 2.4-fold higher than the male level, respectively). However, CYP2J showed no significant difference (Figure 3).

Sex-specific differences in the mRNA and protein expression levels of CYP enzymes in the liver:

All the investigated 31 CYP enzymes were found to be expressed in the liver. The expression of Cyp2c12 and Cyp3a18 was found to be limited to the liver, with no extrahepatic expression. Cyp2c23 was the highest expressed CYP in the male liver, while Cyp2e1 was the highest in the female liver. Cyp4a8 was the lowest in both sexes (Figure 4). In contrast to the heart, Cyp2c11 and Cyp2c13 levels in the liver are male-specific, with dramatically higher expression in males, around 1700 and 1300-fold the female hepatic expression levels, respectively. In contrast, Cyp2c12 in female livers is nearly 200-fold compared to male livers. In CYP3 family, Cyp3a9 is nearly 10-fold higher in the female liver, while Cyp3a18 is nearly 25-fold higher in the male liver. Most CYP4 family enzymes are significantly higher in male livers, especially Cyp4a2, which is also male-specific; 3000-fold in the male liver compared to the
female liver (Supp. Table S.2.1-S.2.31). Figure 5 shows the mRNA expression levels of different CYP enzymes in male versus female livers.

As shown in Figure 6, all the assessed enzymes were found to be expressed in the liver. CYP1A2, CYP3A, and CYP4A1 were significantly higher in female rat livers (2.5-fold, 1.9-fold, and 2.9-fold, respectively) compared to the male livers. In contrast, CYP2J was found to be significantly higher in the male rat liver (1.5-fold female expression level), in agreement with the mRNA result (Figure 6).

**Sex-specific differences in the mRNA and protein expression levels of CYP enzymes in the kidney:**

Cyp2c23 was the most highly expressed, while Cyp3a1 was the least expressed CYP in both male and female kidneys (Figure 7). Cyp2c12, Cyp2c13, and Cyp3a18 are not expressed in the kidney. Several enzymes demonstrated similar sexual dimorphism in the kidney to that shown in the heart, such as Cyp1a1, Cyp2e1, Cyp4f1, Cyp4f4, and Cyp4f6, which all showed higher expression the female organs. Cyp1a1 showed the greatest difference, with the female expression level 26-fold the male level. However, similar to the liver and in contrast to the heart, Cyp2c11 expression was significantly higher in male versus female kidneys (13-fold), and in contrast to both heart and liver, Cyp2c23 expression was significantly higher in female kidneys (approximately 2-fold). In addition, Cyp4a8 showed nearly 7.5-fold higher expression in the females’ kidney, whereas it was not expressed in the heart (Supp. Table S.3.1-S.3.28). Figure 8 shows the mRNA expression levels of different CYP enzymes in male versus female kidneys.

At the protein expression level, CYP2C23, CYP2E1, CYP3A, CYP4A1, and CYP4F2 were detected in the kidney. Similar to the mRNA expression, CYP2E1 was
found to be significantly higher in the female rat kidney (1.7-fold the male expression level). CYP2C23 also appears to be slightly higher in females, but the difference did not achieve statistical significance. Moreover, CYP3A protein levels are significantly higher in the female kidney (2.4-fold), in agreement with CYP3A2 mRNA result (Figure 9).

Sex-specific differences in the mRNA and protein expression levels of CYP enzymes in the lung:

The most expressed CYP mRNA in the lungs of both male and female rats was Cyp2b1, with a marked difference between it and the second enzyme (Cyp2e1) (11.5-fold in males and 27-fold in females). On the other hand, Cyp2j10 and Cyp3a2 were the least expressed (Figure 10). In addition to Cyp2c13 and Cyp4a2 and except Cyp3a9, all the enzymes that were not expressed in the heart were also found to not be expressed in the lung. Except Cyp1a2 and Cyp2b1, all other CYP enzymes expressed in the lung did not show statistically significant sex-specific differences between males and females. Cyp1a2 is around 1.8-fold higher in male lung, while Cyp2b1 is 2.3-fold higher in female lung (Supp. Table S.4.1-S.4.22). Figure 11 demonstrates the male versus female mRNA expression levels of different CYP enzymes in the lungs.

CYP2C23, CYP3A, and CYP4A1 were all found to be significantly higher in the female lung at the protein expression level (3.2, 4.2, and 7.3-fold the male expression level, respectively). On the other hand, CYP2E1, CYP2J, and CYP4F demonstrated no significant difference between males and females, and CYP1A2 was not detected (Figure 12).
Sex-specific differences in the mRNA and protein expression levels of CYP enzymes in the brain:

*Cyp2j3* was found to be the most expressed enzyme in the brain of male and female rats, while *Cyp4a8* and *Cyp2c13* were the least expressed (Figure 13). *Cyp2c12, Cyp2d2, Cyp2d3, Cyp3a18,* and *Cyp4a2* were found to not be expressed in the brain. Similar to the liver, the brain mRNA expression levels of *Cyp2c11* and *Cyp2c13* were significantly higher in males than in females, but with a less marked difference (1.8 and 3.7-fold, respectively). Unlike all other organs, *Cyp1a1* and *Cyp4a3* in the brain were significantly higher in the males (approximately 2 and 6-fold, respectively). On the other hand, *Cyp4f4* and *Cyp4f5* mRNA levels were significantly higher in the females (1.7 and 1.4-fold, respectively) (Supp. Table S.5.1-S.5.26). The mRNA expression levels of different CYP enzymes in male versus female brain are shown in Figure 14.

At the protein expression level, CYP2J and CYP4F enzymes were detected in the brain, and both showed no significant sex-specific difference (Figure 15).

Sex-specific differences in the mRNA and protein expression levels of CYP enzymes in the small intestine:

At the mRNA expression level, *Cyp2b1* and *Cyp2b2* were found to be the most highly expressed CYP enzymes in the small intestine of female and male rats, respectively, while *Cyp3a1* was the least expressed in both sexes, similar to the kidney (Figure 16). *Cyp3a18, Cyp2c12, Cyp2d2, Cyp4a2,* and *Cyp4a3* were found to not be expressed in the small intestine. Only *Cyp2c13* showed significant sex-specific difference in the small intestine, being 1.4-fold higher in the males compared to the
females (Supp. Table S.6.1-S.6.24). The mRNA expression of male-vs-female expression of CYP enzymes in the small intestine is demonstrated in Figure 17.

At the protein expression level, CYP1A2 and CYP3A were demonstrated to be significantly higher in the females, 2.4-fold and 3.7-fold, respectively. This result is in agreement with other organs such as the liver, kidney, and lung. CYP4A1 was also detected at the protein expression level but showed no significant difference between males and females, similar to CYP4A1 expression in the kidney (Figure 18).

**Discussion:**

Previously, many studies were conducted on male animals only and results were generalized to both sexes (Holdcroft, 2007; Lee, 2018). Ignoring sex differences could have undesirable consequences such as increased side effects or decreased efficacy. Thus, sex-specific differences are gaining increasing attention in research, and more studies are starting to include female groups (Wald and Wu, 2010; Lee, 2018). Previous studies have demonstrated sex-specific differences in the expression or activity levels of different CYP enzymes (Waxman and Holloway, 2009; Zhang et al., 2011). However, most of these studies reported just a few enzymes, and investigated sexual dimorphism in response to inducers or inhibitors. In this study, we investigated and compared the mRNA and protein expression of different CYPs in the heart, liver, lung, kidney, brain, and small intestine of male and female SD rats.

Higher expression levels of CYP1A1 were previously reported in the lungs (Lingappan et al., 2013, 2016) and hearts (Zhang et al., 2015) of female versus male mice. Moreover, CYP1A1 was detectable in the lungs and kidneys of female but not male SD rats, and was undetectable in the liver of both sexes (Iba et al., 1999). Our results showed significantly higher cardiac and renal Cyp1a1 mRNA levels in female
rats. Hepatic CYP1A2 activity was reported to be higher in male than in female SD rats (Fonsart et al., 2008). However, a study found higher CYP1A2 levels in female compared to male human liver samples (Zhang et al., 2011), while other studies gave conflicting results (Nafziger and Bertino, 1989; Ou-Yang et al., 2000; Zanger and Schwab, 2013). A study in 2016 demonstrated higher Cyp1a2 brain mRNA levels in female Wistar rats (Nagai et al., 2016), while our results showed no significant difference in the brain. In our study, Cyp1a2 mRNA expression showed significant sex difference only in the lung, being male-dominant, whereas CYP1A2 protein was female-dominant in the liver and the intestine.

CYP1B1 is constitutively expressed in several tissues, most importantly in the heart (Maayah et al., 2015). A previous study showed that treatment of embryonic rat cardiomyocytes with growth hormone (GH) in a pulsatile pattern, which mimics the male secretory pattern, significantly decreased Cyp1a1 and increased Cyp1b1 expression compared to the constant treatment pattern, which mimics the female pattern. Moreover, they found higher Cyp1b1 mRNA levels in male mice hearts compared to female mice (Zhang et al., 2015). Acute doxorubicin exposure in mice also caused a male-specific increase in cardiac Cyp1b1 (Grant et al., 2017). In our study, cardiac Cyp1b1 showed no significant difference, but hepatic and renal Cyp1b1 levels were significantly higher in male rats. Interestingly, human hepatic CYP1B1 was also previously reported to be significantly higher in men (Yang et al., 2012). Hepatic CYP2A1 was previously found to be female-dominant in rats (Martignoni et al., 2006). We found higher hepatic Cyp2a1 expression in females but higher renal expression in males.

The CYP2C family is known to be highly abundant in the rat liver (Martignoni et al., 2006). CYP2C11 and CYP2C13 were previously reported to be male-specific
enzymes in the liver, spleen, and bone marrow, while CYP2C12 was reported to be female-specific (Thangavel et al., 2007; Huang et al., 2011; Babelova et al., 2015). In addition, previous studies demonstrated significantly lower metabolism of CYP2C substrates in female compared to male Wistar (Ohhira et al., 2006) and SD rats (Fukuno et al., 2018). CYP2C11 sexual dimorphism is attributed to sex differences in the circulating GH profile. Female SD rats could express CYP2C11 after hypophysectomy and infusion with GH in the pulsatile male secretory fashion (Legraverend et al., 1992; Thangavel et al., 2007; Banerjee et al., 2021). We also found significantly higher Cyp2c11 levels in male versus female kidney and brain; however, interestingly, it was found to be female-dominant in the heart. CYP2C23 protein was previously reported to be highly abundant in the liver and kidney of SD rats, while it was undetectable in the heart and lungs (El-Sherbeni et al., 2013). We detected CYP2C23 in the lung with a higher expression in female rats.

Our results showed female-dominant expression of Cyp2e1 in the heart and kidney. In mice, in contrast, renal CYP2E1 expression was found to be higher in males (Freeman et al., 1992; Speerschneider and Dekant, 1995), while cardiac CYP2E1 showed no sexual dimorphism (Zhang et al., 2015). However, acute doxorubicin exposure was associated with a female-specific increase in heart CYP2E1 in mice (Grant et al., 2017). Hepatic CYP2J2 levels were found to be significantly higher in female subjects compared to male subjects (Yang et al., 2012). In contrast, our results showed male-dominant hepatic expression of CYP2J enzymes. We found Cyp2j4 to be significantly higher in male kidneys. A previous study demonstrated significantly higher CYP2J5 levels in male versus female mice kidneys (Ma et al., 2004). As for cardiac expression, we found significantly higher Cyp2j3 levels in female rat hearts compared to males. In line with that, treatment of rat cardiomyocytes...
with GH in a male secretory pattern significantly decreased $Cyp2j3$ expression compared to the female pattern. Mouse $Cyp2j11$ is also higher in female than in male hearts (Zhang et al., 2015). However, cardiac CYP2J protein levels showed no significant difference.

The CYP3A subfamily of enzymes is considered the most important among human drug metabolizing enzymes. Several studies have shown that women have significantly higher hepatic and intestinal CYP3A enzyme activities compared to men (Tanaka, 1999; Greenblatt and Von Moltke, 2008; Krogstad et al., 2020), and higher hepatic CYP3A4 levels (Lamba et al., 2010; Yang et al., 2010, 2012). CYP3A7, the human ortholog of rat Cyp3a9, was previously found to have significantly higher gene expression in female hepatic samples compared to males (Yang et al., 2012). In agreement with human data, our results showed higher hepatic $Cyp3a9$ mRNA and CYP3A protein levels in female rats; however, we found $Cyp3a2$ mRNA to be higher in male rats. CYP3A2 was previously found to be induced by zolmitriptan in male but not female SD rats (Yu et al., 2008).

A study in 2003 reported $Cyp3a9$ mRNA levels to be significantly higher in the livers and lungs of female SD rats compared to male rats, and that it’s expression is affected by ovariectomy and subsequent estrogen administration (Anakk et al., 2003). In agreement, we found significantly higher $Cyp3a9$ mRNA and CYP3A protein levels in the livers of female rats. In the lung, kidney, and small intestine, $Cyp3a9$ mRNA levels showed no significant difference, while CYP3A protein levels were significantly higher in the females. Similar to our findings, previous reports have identified hepatic CYP3A2 and CYP3A18 to be male-dominant isoenzymes, and CYP3A9 to be a female-dominant isoenzyme in Wistar (Robertson et al., 1998) and SD rats (Kushida et al., 2021).
CYP4A enzymes play an important role in the ω-hydroxylation of AA (El-Sherbeni and El-Kadi, 2017). In line with our results, hepatic Cyp4a2 was previously reported to be significantly higher in male rats of Fischer 344 and obese ZSF1 strains (Sundseth and Waxman, 1992; Babelova et al., 2015). Moreover, the induction of hepatic Cyp4a by endotoxin was found to be male-specific in SD and Fischer 344 rats (Mitchell et al., 2001). Although hepatic Cyp4a1 showed no sex-specific difference at the mRNA level, hepatic CYP4A1 protein levels were found to be significantly higher in female rats.

We found renal Cyp4a2 levels to be significantly higher in male rats. Similarly, previous studies showed significantly higher renal CYP4A2 levels in male versus female Fischer 344 and SD rats (Sundseth and Waxman, 1992; Bleicher et al., 2001). Interestingly, a previous study demonstrated that treatment of SD rats with dihydrotestosterone lowered the renal Cyp4a1 levels, and enhanced Cyp4a2/3 levels (Nakagawa et al., 2003). In addition, clofibrate administration significantly enhanced renal Cyp4a2 expression only in male SD rats (Bleicher et al., 2001). Another study showed an increase of renal CYP4A protein in female SD rats treated with dihydrotestosterone (Zhou et al., 2005).

CYP4F enzymes appear to be female-dominant enzymes. We found significantly higher Cyp4f levels in the heart and kidney of females versus males, and Cyp4f4 and Cyp4f5 were significantly higher in female brains. At the protein expression level, we found significantly higher CYP4F levels in the heart of female versus male rats. Similarly, a study in 2002 found significantly higher expression levels of CYP4F enzymes in female versus male SD rats in the liver, kidney, lung, and brain, and found a significant decrease in hepatic and renal CYP4F expression.
levels in female rats after ovariectomy, which was significantly restored by estrogen treatment (Kalsotra et al., 2002).

In conclusion, there are significant sex-specific differences in the expression levels of different CYP enzymes. Elucidating sex-specific differences in CYPs is crucial for explaining differences between males and females in diseases processes and treatment outcomes. This study has some limitations. Firstly, species discrepancies in the basal and inducible levels of enzymes could complicate the translation of the results to humans (Hammer et al., 2021). Moreover, CYP expression levels could differ among different rat strains (Nishiyama et al., 2016). Finally, expression levels of enzymes are not necessarily correlated to their activity levels. However, rats are still considered to be valuable models for preclinical development and have previously been used for the study of sex differences in drug metabolizing enzymes and the mechanisms underlying these differences (Waxman and Holloway, 2009; Jung et al., 2015; Blais et al., 2017). Moreover, several rat CYP enzymes (e.g., CYP1A1, CYP1A2, CYP2E1, CYP2J3, CYP4F1) show high degree of structural similarity to their human orthologs (Hammer et al., 2021). Thus, despite the study limitations and some results that are different from human data, we believe that our results could still give valuable insights regarding sex-specific differences in human CYP enzymes. Additional studies investigating the activity of different CYP enzymes and levels of their metabolites in males and females are important for having better insight into sex-specific discrepancies and their potential clinical and therapeutic implications.

Authorship contributions:

Participated in research design: Gerges, El-Kadi.
Conducted experiments: Gerges.

Performed data analysis: Gerges, El-Kadi.

Wrote or contributed to the writing of the manuscript: Gerges, El-Kadi.

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Figure legends:

**Figure 1:** The mRNA expression of different CYP enzymes in male (A) and female (B) rat heart relative to the least expressed. The mRNA expression of CYP enzymes was determined in the heart of adult male and female Sprague Dawley rats by real-time PCR and normalized to β-actin housekeeping gene. Results are presented as mean ± SEM, n = 4-6.
**Figure 2:** Sex-specific differences in the mRNA expression levels of CYP enzymes in the rat heart. The mRNA expression of CYP enzymes was determined in the heart of adult male and female Sprague Dawley rats by real-time PCR and normalized to β-actin housekeeping gene. Results are presented as mean ± SEM., n = 4-6. Data were analyzed using unpaired student t test. *, significant difference from male rats (p < 0.05).

**Figure 3:** Sex-specific differences in the protein expression levels of some CYP enzymes in the rat heart. The protein expression of CYP enzymes was determined in the heart of adult male and female Sprague Dawley rats by Western blot and normalized to GAPDH housekeeping protein. Results are presented as mean ± SEM., n = 4-6. Data were analyzed using unpaired student t test. *, significant difference from male rats (p < 0.05).

**Figure 4:** The mRNA expression of different CYP enzymes in male (A) and female (B) rat liver relative to the least expressed. The mRNA expression of CYP enzymes was determined in the liver of adult male and female Sprague Dawley rats by real-time PCR and normalized to β-actin housekeeping gene. Results are presented as mean ± SEM, n = 4-6.

**Figure 5:** Sex-specific differences in the mRNA expression levels of CYP enzymes in the rat liver. The mRNA expression of CYP enzymes was determined in the liver of adult male and female Sprague Dawley rats by real-time PCR and normalized to β-actin housekeeping gene. Results are presented as mean ± SEM., n = 4-6. Data were analyzed using unpaired student t test. *, significant difference from male rats (p < 0.05).
**Figure 6:** Sex-specific differences in the protein expression levels of some CYP enzymes in the rat liver. The protein expression of CYP enzymes was determined in the liver of adult male and female Sprague Dawley rats by Western blot and normalized to GAPDH housekeeping protein. Results are presented as mean ± SEM., n = 4-6. Data were analyzed using unpaired student t test. *, significant difference from male rats (p < 0.05).

**Figure 7:** The mRNA expression of different CYP enzymes in male (A) and female (B) rat kidney relative to the least expressed. The mRNA expression of CYP enzymes was determined in the kidney of adult male and female Sprague Dawley rats by real-time PCR and normalized to β-actin housekeeping gene. Results are presented as mean ± SEM, n = 4-6.

**Figure 8:** Sex-specific differences in the mRNA expression levels of CYP enzymes in the rat kidney. The mRNA expression of CYP enzymes was determined in the kidney of adult male and female Sprague Dawley rats by real-time PCR and normalized to β-actin housekeeping gene. Results are presented as mean ± SEM., n = 4-6. Data were analyzed using unpaired student t test. *, significant difference from male rats (p < 0.05).

**Figure 9:** Sex-specific differences in the protein expression levels of some CYP enzymes in the rat kidney. The protein expression of CYP enzymes was determined in the kidney of adult male and female Sprague Dawley rats by Western blot and normalized to GAPDH housekeeping protein. Results are presented as mean ± SEM., n = 4-6. Data were analyzed using unpaired student t test. *, significant difference from male rats (p < 0.05).
**Figure 10:** The mRNA expression of different CYP enzymes in male (A) and female (B) rat lung relative to the least expressed. The mRNA expression of CYP enzymes was determined in the lung of adult male and female Sprague Dawley rats by real-time PCR and normalized to β-actin housekeeping gene. Results are presented as mean ± SEM, n = 4-6.

**Figure 11:** Sex-specific differences in the mRNA expression levels of CYP enzymes in the rat lung. The mRNA expression of CYP enzymes was determined in the lung of adult male and female Sprague Dawley rats by real-time PCR and normalized to β-actin housekeeping gene. Results are presented as mean ± SEM., n = 4-6. Data were analyzed using unpaired student t test. *, significant difference from male rats (p < 0.05).

**Figure 12:** Sex-specific differences in the protein expression levels of some CYP enzymes in the rat lung. The protein expression of CYP enzymes was determined in the lung of adult male and female Sprague Dawley rats by Western blot and normalized to GAPDH housekeeping protein. Results are presented as mean ± SEM., n = 4-6. Data were analyzed using unpaired student t test. *, significant difference from male rats (p < 0.05).

**Figure 13:** The mRNA expression of different CYP enzymes in male (A) and female (B) rat brain relative to the least expressed. The mRNA expression of CYP enzymes was determined in the brain of adult male and female Sprague Dawley rats by real-time PCR and normalized to β-actin housekeeping gene. Results are presented as mean ± SEM, n = 4-6.

**Figure 14:** Sex-specific differences in the mRNA expression levels of CYP enzymes in the rat brain. The mRNA expression of CYP enzymes was determined in the brain
of adult male and female Sprague Dawley rats by real-time PCR and normalized to β-actin housekeeping gene. Results are presented as mean ± SEM., n = 4-6. Data were analyzed using unpaired student t test. *, significant difference from male rats (p < 0.05).

**Figure 15:** Sex-specific differences in the protein expression levels of some CYP enzymes in the rat brain. The protein expression of CYP enzymes was determined in the brain of adult male and female Sprague Dawley rats by Western blot and normalized to GAPDH housekeeping protein. Results are presented as mean ± SEM., n = 4-6. Data were analyzed using unpaired student t test. *, significant difference from male rats (p < 0.05).

**Figure 16:** The mRNA expression of different CYP enzymes in male (A) and female (B) rat small intestine relative to the least expressed. The mRNA expression of CYP enzymes was determined in the small intestine of adult male and female Sprague Dawley rats by real-time PCR and normalized to Gapdh housekeeping gene. Results are presented as mean ± SEM, n = 4-6.

**Figure 17:** Sex-specific differences in the mRNA expression levels of CYP enzymes in the rat small intestine. The mRNA expression of CYP enzymes was determined in the small intestine of adult male and female Sprague Dawley rats by real-time PCR and normalized to Gapdh housekeeping gene. Results are presented as mean ± SEM., n = 4-6. Data were analyzed using unpaired student t test. *, significant difference from male rats (p < 0.05).

**Figure 18:** Sex-specific differences in the protein expression levels of some CYP enzymes in the rat small intestine. The protein expression of CYP enzymes was determined in the small intestine of adult male and female Sprague Dawley rats by
Western blot and normalized to GAPDH housekeeping protein. Results are presented as mean ± SEM., n = 4-6. Data were analyzed using unpaired student t test. *, significant difference from male rats (p < 0.05).

**Table 1: Rat cytochrome P450 enzymes and their human orthologs**

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Table 2: Rat primer sequences

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Cyp, cytochrome P450; Gapdh, glyceraldehyde 3-phosphate dehydrogenase.
Fig. 4

(A) Male Liver

(B) Female Liver
Fig. 6
Fig. 7

A

Male Kidney

mRNA level (fold of 3A1)

B

Female Kidney

mRNA level (fold of 3A1)
Fig. 9

**Kidney**

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Protein relative density (fold of male)

- CYP2C23
- GAPDH
- CYP2E1
- GAPDH
- CYP3A
- GAPDH
- CYP4A1
- GAPDH
- CYP4F
- GAPDH

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Fig. 12

Lung

Males

Females

protein relative density
(fold of male)

2C23
2E1
2J
3A
4A1
4F

M
F

CYP2C23
GAPDH

CYP2E1
GAPDH

CYP2J
GAPDH

CYP3A
GAPDH

CYP4A1
GAPDH

CYP4F
GAPDH
Fig. 13

(A) Male Brain

(B) Female Brain

mRNA level (fold of 4A8)
Fig. 15

Brain

- Males
- Females

Protein relative density (fold of male)

2J

4F

CYP2J

GAPDH

CYP4F

GAPDH
Figure 18: Protein relative density (fold of male) for CYP1A2, CYP3A, and CYP4A1 in the small intestine of males (M) and females (F). The image shows bar graphs with error bars indicating the standard deviation. Statistical significance is denoted by asterisks. The GAPDH band is used as a loading control for each sample.