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Differences in mRNA Expression of Selected Cytochrome P450, Transporters and Nuclear Receptors Among Various Rat Models of Metabolic Syndrome

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ABSTRACT

Metabolic syndrome (MetS) is a cluster of risk factors that increase the likelihood of developing cardiovascular, metabolic and other diseases. The pharmacological management of MetS often involves polypharmacy, making it essential to understand how drug-metabolising enzymes, transporters, transcription factors and other proteins involved are affected under different metabolic conditions. This study investigated the relative mRNA expression of key hepatic and intestinal genes involved in drug metabolism, including *Cyp1a2*, *Cyp3a23*, *Cyp2d1*, *Cyp2c11*, *Cyp2c6*, *Cyp2e1*, *Cyp7a1*, *Cyp2b1*, *Cyp2a1*, *Abcg5*, *Abcg8*, *Abcb1*, *Nr1i3*, *Nr1i2*, *Ahr*, *Gsta1* and *Comt*, in four nonobese rat models of MetS: hereditary hypertriglyceridaemic (HHTg), spontaneously hypertensive rat (SHR), SHR expressing transgenic human C-reactive protein (SHR-CRP), and bilaterally ovariectomised Wistar (W-OVX), compared to Wistar controls. Gene expression was quantified by RT–PCR with data normalised using the $^{\Delta\Delta}$Ct method. Between the models studied, measurements showed significant differences in the liver. The upregulation of *Cyp2c6* and *Cyp3a23* was observed only in SHR; upregulated *Cyp2d1* was found in SHR as well as in HHTg rats. The downregulated *Cyp1a2* was measured in a condition of hypertriglyceridemia, postmenopause or hypertension. These findings highlight model-specific alterations in gene expression that may affect drug metabolism and interactions. The HHTg may be, in particular, a suitable model for preclinical studies focusing on intestinal drug–drug interactions in MetS-related conditions.

1 | Background

Metabolic syndrome (MetS) represents a cluster of interrelated metabolic disorders, which together increase the risk of developing type 2 diabetes mellitus and cardiovascular diseases [1]. Due to its complex and multifactorial nature, MetS can alter drug metabolism and elimination, primarily via changes in the expression and function of hepatic and intestinal proteins involved in the mentioned mechanisms [2]. The global prevalence of MetS is estimated to be up to one quarter of the world population [3]. Therefore, the therapy of MetS is the subject of intensive study.

Summary

Patients suffering from metabolic syndrome often take multiple medications. Understanding how their bodies process drugs is vital for effective treatment. Our study utilised various rat models of metabolic syndrome to investigate changes in gene expression of key drug-metabolising enzymes, transporters and transcription factors under different metabolic conditions, such as high blood pressure or inflammation. Our findings show significant variations in mRNA expression in these drug-handling components across the models. This research offers crucial insights into why drug responses can differ among metabolic syndrome patients, potentially helping to *develop* safer, more personalised medication strategies.

A key role in the pathogenesis of MetS is attributed to obesity, hypertriglyceridaemia, hypertension, oxidative stress and the activation of inflammatory processes. However, obesity is not always associated with metabolic syndrome. Epidemiological surveys have shown a high prevalence of metabolic syndrome even among normal-weight individuals [4, 5]. Since no single therapeutic agent addresses all features of MetS, patients are often treated with multiple drugs targeting individual components, such as antihypertensives, insulin sensitizers, lipid-lowering agents, and antioxidants. This polypharmacy raises the risk of drug–drug interactions, many of which involve cytochrome P450 (CYP) enzymes. These enzymes metabolise approximately 80% of clinically used drugs [6]; they are also involved in the transformation of endogenous substrates such as fatty acids, sterols and vitamins [7]. Their expression is modulated by various physiological and pathological conditions, including obesity, diabetes and aging [8]. To date, the role of CYPs in regulating the metabolism of drugs and endogenous substrates in metabolic syndrome has been studied mainly in obese individuals [9, 10], whereas in individuals with MetS, which is not associated with obesity, or in individuals with metabolic disorders accompanying MetS, the involvement of CYPs has not been elucidated.

Therefore, in this study, the relative mRNA expression of selected CYPs was investigated in three animal models of metabolic syndrome derived from Wistar rats, which are not associated with obesity induced by excessive fat intake. A number of rat models are used to study pharmacogenetic changes in MetS, each capturing various aspects of the condition. For example, the Zucker Diabetic Fatty (ZDF) rat has developed obesity, insulin resistance and hyperlipidaemia, and it is widely used for type 2 diabetes research [11]. The Zucker rat traits are caused by mutations in the leptin receptor gene, which are rare in humans [12]. The nonobese Zucker Lean (ZL) rat strain exhibits hepatic steatosis and metabolic alterations, providing a model for less overt forms of metabolic syndrome. This model has a normal fat composition and serves primarily as a control for comparison with the Zucker Obese (ZO) rat, another obese rat strain used to study obesity and insulin resistance [12]. The polygenic Zucker Diabetic Sprague–Dawley (ZDSD) rat does not carry the leptin receptor mutation. Thus, the mechanisms leading to the obesity and type 2 diabetes in ZDSD rats might be more similar to those in humans [13]. The Wistar Bonn Kobori Diabetic Fatty (WBKDF) rat similarly shows hyperglycaemia and dyslipidaemia when fed a high-fat diet [14]. Nonobese Goto-Kakizaki (GK) rats, which spontaneously develop type 2 diabetes at an early age without the need for a special diet, are also used for type 2 diabetes research. However, intestinal inflammation has been reported in this animal model as well [15].

The nonobese hereditary hypertriglyceridemic (HHTg) rat exhibits almost all the disorders typical of metabolic syndrome and its organ complications, such as increased serum concentrations of triglycerides and nonesterified fatty acids, ectopic triglyceride accumulation in the liver and muscles, impaired glucose tolerance, mild hyperinsulinaemia, resistance of muscle and adipose tissue to insulin action, oxidative stress and activation of low-grade chronic inflammation. The detailed characteristics of this well-established experimental model have been described in previous studies [16–20].

Hypertension is also a significant risk factor for metabolic syndrome and often occurs together with it. The spontaneously hypertensive rat (SHR) is a nonobese model widely utilised as it mimics many aspects of human hypertension that develops around 5–6 weeks of age [21, 22]. This model is well-characterised by a sustained elevation in blood pressure, typically reaching severe hypertension, often plateauing at 195 mmHg [23], in adult animals. They can also exhibit a range of associated metabolic disturbances, which may include mildly altered glucose metabolism, such as myocardial glucose uptake and oxidation [24]. Furthermore, there are studies indicating that SHR rats can develop changes in their lipid metabolism, elevated oxidative stress and inflammatory markers [24, 25], making the SHR a suitable model for MetS studies.

Metabolic syndrome is also characterised by low-grade inflammation and elevated levels of its biomarker, C-reactive protein (CRP). These are considered risk factors for the development of metabolic syndrome and its complications, such as hepatic steatosis, type 2 diabetes and cardiovascular disease. It is crucial to understand the mechanism of transgenic human CRP, which is expressed in the liver. Human CRP is functionally and structurally different from rat CRP, and its induced expression produces much more pronounced adverse effects on acute phase response and complement activation [26]. The SHR with transgenic human CRP exhibits insulin resistance in skeletal muscles, as well as oxidative stress, inflammatory tissue damage and other disorders that have been previously described in detail [27, 28].

Clinical studies have shown a high prevalence of MetS in postmenopausal women. While this disorder affects 20–30% of women before menopause, its incidence doubles during menopause [29, 30]. Deficiency in ovarian hormones leads to changes in the expression of enzymes involved in lipid metabolism [31]. Bilateral ovariectomy in Wistar female rats (W-OVX) is the most used model for the study of pathogenesis and therapeutic approaches for cardiometabolic complications associated with MetS in the menopausal period [32].

While the role of cytochrome P450 enzymes in obesity-related metabolic syndrome has been explored, less is known about how specific nonobese MetS phenotypes, such as isolated dyslipidaemia, hypertension, oestrogen deficiency or low-grade inflammation, affect the expression of drug-metabolising enzymes and related regulatory pathways. Therefore, in the present study, we conducted a comparative analysis of hepatic and intestinal mRNA expression of selected CYP enzymes, ATP-binding cassette (ABC) transporters and nuclear receptors in four distinct rat models representing these conditions. Our aim was to identify model-specific alterations that may influence pharmacokinetics and support the use of appropriate preclinical models in metabolic research.

In this study, rat nonobese models were selected for their representation of the spectrum of metabolic syndrome-related disorders, such as dyslipidaemia and impaired glucose tolerance, and also mild hyperinsulinaemia (HHTg rat), hypertension (rat model SHR), mild inflammation (rat model SHR-CRP) and hormonal imbalance (ovariectomised Wistar rat model W-OVX), without obesity, providing a complementary perspective for investigating their influence on drug metabolism.

In selected rat models of MetS, a comparative analysis of the gene expression of selected CYPs, ABC transporters and nuclear receptors was performed in the liver, where the majority of the metabolic transformation of endogenous and exogenous substrates takes place. Owing to the growing evidence of a link between the gut microbiome and metabolic events that contribute to MetS, investigations of gene expression in the tissue of the small intestine have been performed as well [33].

2 | Materials and Methods

2.1 | Experimental Animals

For this study, liver and small intestine tissues from Wistar rat males ($n = 5$) obtained from AnLab (Prague, Czech Republic), HHTg rat males ($n = 6$), sham-operated Wistar rat females (W-SHAM) ($n = 5$), ovariectomised Wistar rat females (W-OVX) ($n = 5$) provided by the Institute for Clinical and Experimental Medicine (Prague, Czech Republic), SHR rat males ($n = 5$) and transgenic human C-reactive protein SHR rat males (SHR-CRP) ($n = 7$) obtained from the Institute of Physiology of the Czech Academy of Science (Prague, Czech Republic) were used. The male Wistar and female W-SHAM groups were used as controls. At the age of 8 weeks, female Wistar rats were anesthetised with ketamine (70 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally and then bilaterally ovariectomised via a midline incision (W-OVX). Sham-operated animals underwent the entire surgery, except for the removal of ovaries. The animals were saturated with oxygen throughout the procedure, followed by subcutaneous analgesia (meloxicam 1-mg/kg BW). The health status of the animals was monitored after the surgery. The SHR-CRP strain was derived by microinjecting SHR rats with a construct containing cDNA for human CRP under the control of the ApoE promoter [34]. The animals were maintained under a 12 h/12-h light–12-h dark cycle at a temperature of 22°C–25°C and fed a standard laboratory diet for 4–6 months ad libitum. At the end of the study, all the animal subjects were decapitated after light anesthetization (Zoletil 5 mg/kg b.wt.) in a postprandial state, and the collected organs were washed in saline. Parts of the tissues were transferred to RNAlater Stabilisation Solution (Invitrogen, Vilnius, Lithuania) and RIPA Lysis and Extraction Buffer (Santa Cruz, CA, USA) and then stored at −80°C for further analyses. The study was conducted in accordance with the Basic and Clinical Pharmacology and Toxicology policy for experimental and clinical studies [35]. All animal experiments were performed in accordance with the Animal Protection Law of the Czech Republic 501/2020, which follows the European Community Council recommendations 86/609/ECC for the use of laboratory animals. All procedures involving laboratory animals were approved by the Ethics Committee of the Ministry of Health, Czech Republic.

2.2 | Biochemical Analysis

Serum levels of triglycerides, glucose, ALT, AST and total and HDL cholesterol were measured via commercially available kits (Erba Lachema, Brno, Czech Republic and Roche Diagnostics, Mannheim, Germany). Rat and human hsCRP in serum were analysed via ELISA (Alpha Diagnostics International, San Antonio, TX, USA).

For the determination of triglycerides and cholesterol in the liver, samples were extracted in chloroform/methanol. The resulting pellet was dissolved in isopropyl alcohol, after which the triglyceride content was determined via an enzymatic assay (Erba Lachema, Brno, Czech Republic).

2.3 | Tissue Preparation and Real-Time Quantitative RT–PCR

Total RNA was extracted from 30–50 mg of liver and small intestine tissue homogenates from each subject via the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). One microgram of mRNA was then used for cDNA synthesis via reverse transcription with an EvoScript Universal cDNA Master Kit (Roche Diagnostics, Mannheim, Germany). The main RT–qPCR assay was performed with a LightCycler 1536 instrument (Roche, Basel, Switzerland), and the data obtained were normalised with the $^{\Delta\Delta}$Ct method. Gene expression was determined with TaqMan probes combining primers and probes with FAM or VIC dye and a quencher, which were purchased from Life Technologies (Carlsbad, CA, USA). The batch numbers of primers are listed in (Table S1). Relative gene expression changes were normalised to the internal control gene hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) [36]. The housekeeping gene *Hprt1* was selected for normalisation based on its demonstrated stability across various tissues. Dragon et al. evaluated multiple candidate reference genes and identified *Hprt1* as one of the most stable across different rat tissues [37]. Similarly, Kim et al. reported that HPRT1 protein expression exhibited low variability across multiple organs, with a coefficient of variation below 10%. These findings support the suitability of

Hprt1 as a reliable reference gene for gene expression studies in our experimental models.

2.4 | Statistical Analysis

Statistical analysis was performed with TIBCO Statistica software (DataBon, Prague, Czech Republic, ver. 14.0.0.15). One-way ANOVA with a subsequent post hoc Bonferroni correction was used for the comparison of the Wistar, HHTg and SHR groups. For the comparison of the two groups (SHR and SHR-CRP, W-SHAM and W-OVX) and the table of metabolic characteristics, an unpaired Student's *t*-test was used. Statistical significance was defined as $p < 0.05$.

3 | Results

3.1 | Metabolic Characteristics of the Rat Models

As shown in Table 1 and Figure 1, severe dyslipidaemia in the HHTg strain of rats was associated with markedly elevated hepatic triglyceride accumulation. This strain of rat also presented increased nonfasting glucose and mildly increased proinflammatory CRP. Although the rats were nonobese, abdominal adiposity was significantly elevated.

Transgenic expression of CRP in SHR increased the serum levels of triglycerides and cholesterol; however, hepatic triglyceride accumulation was not significantly different (Table 1).

Ovariectomy in female Wistar rats (W-OVX) resulted in increased body weight, abdominal adiposity and serum cholesterol levels (Table 1). Although the serum triglyceride levels were not significantly altered in W-OVX compared to control W-SHAM rats, liver triglyceride levels were significantly increased (Figure 1).

3.2 | Comparison of Hepatic CYP mRNA Expression in Wistar, HHTg and SHR Rats

A comparison of the mRNA expression of selected hepatic CYP isoforms is displayed in Figure 2. Compared to the Wistar group, *Cyp1a2* was significantly downregulated in the HHTg (by 81%; $p < 0.01$) and SHR (by 70%; $p < 0.01$) groups, and *Cyp2a1* was also downregulated in the SHR group (by 68%; $p < 0.01$). Additionally, significant upregulation of *Cyp3a23* was observed in SHR rats (by 170%; $p < 0.01$), significantly increased *Cyp2d1* was measured in HHTg (by 135%; $p < 0.01$) and SHR (by 170%; $p < 0.01$) rats, and *Cyp2c6* was also significantly increased in SHR (by 151%; $p < 0.01$) compared to the control group as is shown in Figure 2.

3.3 | Effect of Human C-Reactive Protein on Hepatic CYP mRNA Expression in SHR Rats

The results of a comparison of CYP mRNA expression in SHR and SHR-CRP rats (Figure 3) were not statistically significant. Transgenic human CRP does not seem to affect mRNA expression of these enzymes. A trend toward an increase in *Cyp7a1* was observed in SHR-CRP rats compared to control SHR rats.

3.4 | Effects of Ovariectomy on Hepatic CYP mRNA Expression in Female Wistar Rats

As shown in Figure 4, there was no statistically significant difference in the mRNA expression of the CYP isoforms in the ovariectomised Wistar rats. A slight trend towards increasing *Cyp2b1* was observed in W-OVX rats in comparison with sham-operated controls. On the contrary, the mRNA expression of other CYPs had a trend towards downregulation, namely, *Cyp1a2*, *Cyp3a23* and *Cyp7a1*.

TABLE 1 | Metabolic characteristics of rat models of metabolic syndrome.

	W-SHAM	W-OVX	Wistar	HHTg	SHR	SHR-CRP
Age (months)	**7**	**7**	**7**	**7**	**9**	**9**
Body weight (BW) (g)	341 ± 19	422 ± 13**	488 ± 23	448 ± 3	410 ± 7	415 ± 7
Adiposity index (g/100 g BW)	1.78 ± 0.20	3.12 ± 0.38*	1.34 ± 0.01	2.09 ± 0.11***	0.86 ± 0.06	0.90 ± 0.05
Liver weight (g/100 g BW)	3.25 ± 0.09	2.37 ± 0.13***	2.74 ± 0.10	3.09 ± 0.03	3.18 ± 0.04	3.30 ± 0.07
Serum triglycerides (mmol/L)	1.82 ± 0.57	1.70 ± 0.37	1.54 ± 0.22	5.01 ± 0.24***	0.51 ± 0.02	0.77 ± 0.12*
Serum cholesterol (mmol/L)	2.35 ± 0.19	3.20 ± 0.26*	2.32 ± 0.10	2.04 ± 0.06	1.43 ± 0.04	1.67 ± 0.13***
HDL-C (mmol/L)	1.71 ± 0.16	2.26 ± 0.14*	1.27 ± 0.08	0.81 ± 0.06**	1.15 ± 0.02	1.21 ± 0.06
Nonfasting glucose (mmol/L)	7.64 ± 0.38	7.66 ± 0.20	6.56 ± 0.19	9.30 ± 0.38***	6.54 ± 0.06	6.30 ± 0.06
hsCRP human (μg/mL)	n.d.	n.d.	n.d.	n.d.	1.25 ± 0.12	240.00 ± 4.78***
hsCRP rat (μg/mL)	1.30 ± 0.13	1.02 ± 0.06	1.25 ± 0.07	1.53 ± 0.06*	1.08 ± 0.10	0.55 ± 0.03**
ALT (μkat/L)	2.23 ± 1.21	2.89 ± 0.78	1.36 ± 0.38	1.24 ± 0.06	1.88 ± 0.03	2.43 ± 0.19*
AST (μkat/L)	3.85 ± 1.20	4.12 ± 0.64	3.21 ± 0.40	3.13 ± 0.28	3.74 ± 0.16	4.16 ± 0.20

Note: The data are presented as the mean ± SEM. Significance was determined via an unpaired Student's *t*-test (* denotes $p < 0.5$; ** denotes $p < 0.01$; *** denotes $p < 0.001$ compared to the control group according to colour; blue represents female W-SHAM rats; black represents male Wistar rats; red represents male SHR rats, $n = 5–7$).

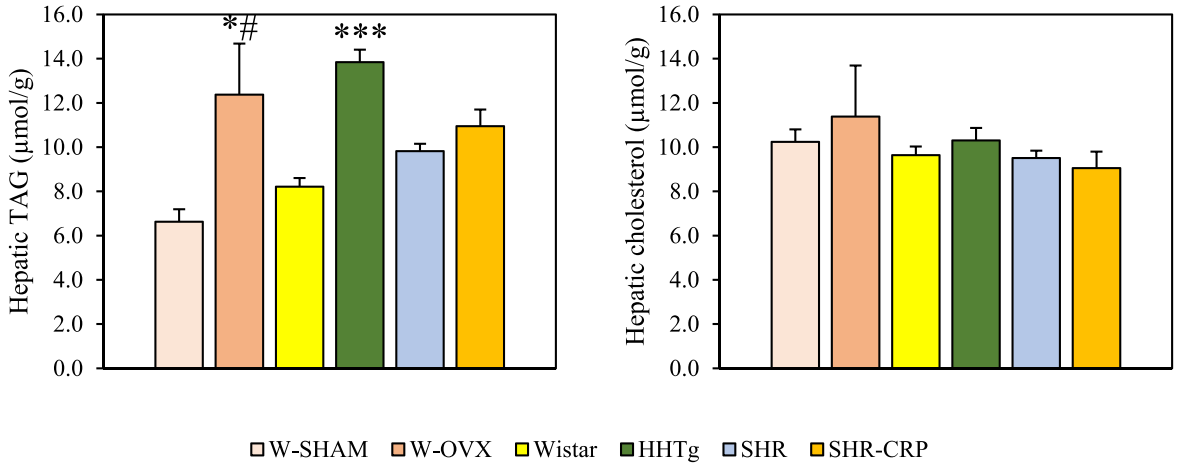

FIGURE 1 | Hepatic triglyceride (TAG) and cholesterol accumulation in rat models of metabolic syndrome. Data are expressed as the mean ± SEM, $n = 5–7$. * denotes $p < 0.05$ compared to the Wistar group; *** denotes $p < 0.001$ compared to the control Wistar males; # denotes $p < 0.05$ compared to W-SHAM.

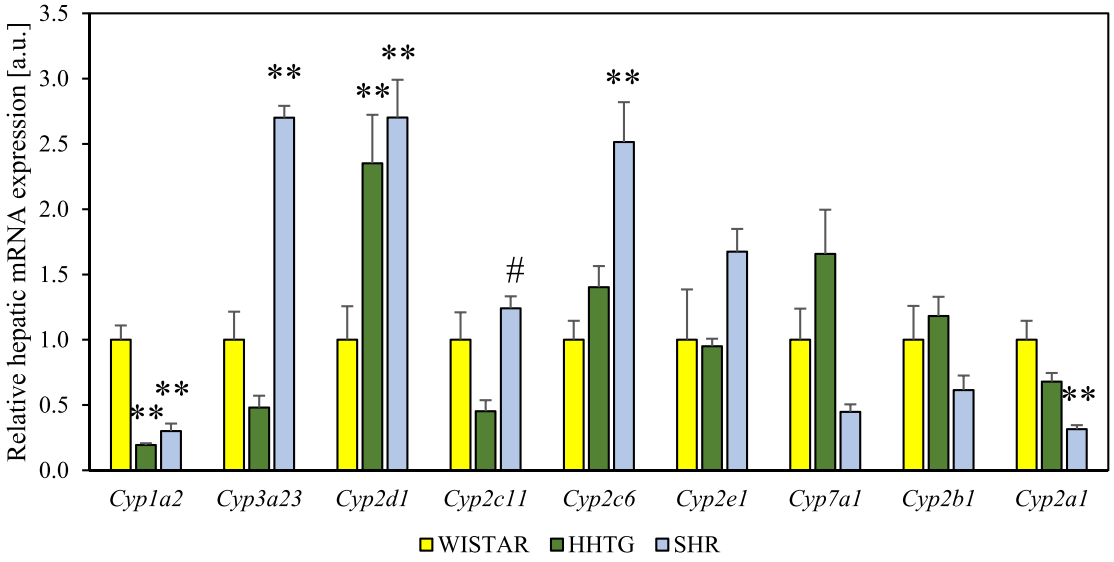

FIGURE 2 | Differences in the relative mRNA expression of cytochromes P450 in the liver tissues of male HHTg and SHR rats compared with control male Wistar rats, with the values normalised to 1.0. Data are expressed as the mean ± SEM $n = 5–7$. ** denotes $p < 0.01$ compared with the Wistar group; # denotes $p < 0.05$ compared with the HHTg group.

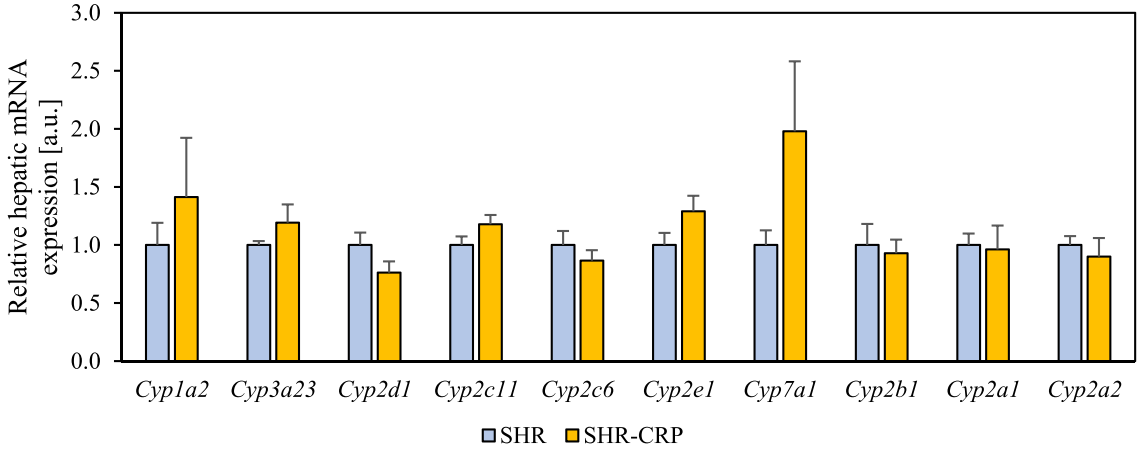

FIGURE 3 | Differences in the relative mRNA expression of CYP in the liver tissues of male SHR-CRP rats compared with male SHR rats. Data are expressed as the mean ± SEM, $n = 5–7$.

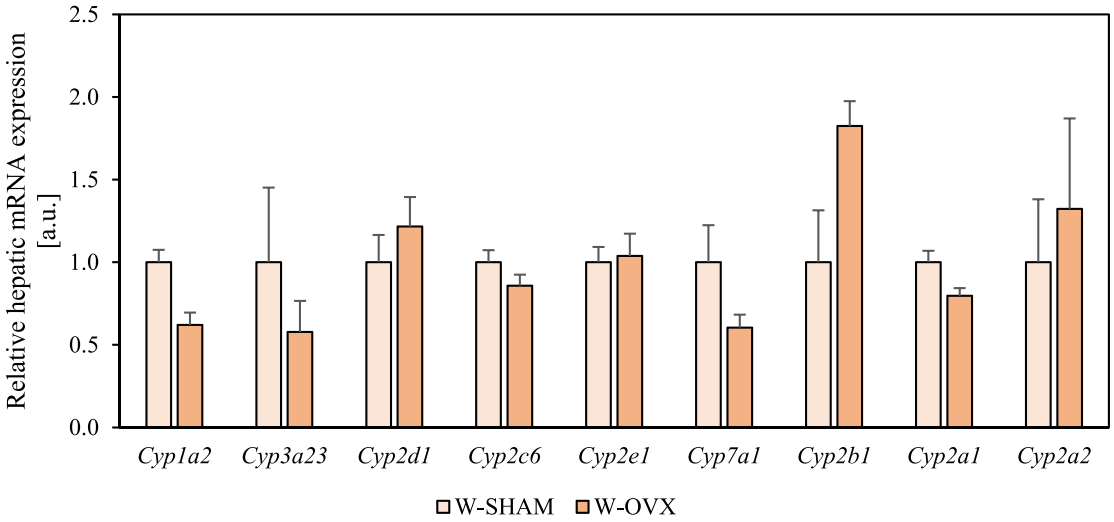

FIGURE 4 | Differences in relative mRNA expression of cytochromes P450 in the liver tissues of ovariectomised female Wistar rats (W-OVX) compared to SHAM-operated controls (W-SHAM), with the values normalised to 1.0. Data are expressed as the mean ± SEM, $n = 5$.

3.5 | Comparison of Hepatic ABC Transporters, Nuclear Receptors and Enzymes Involved in Hormone Regulation mRNA Expression in Wistar, HHTg and SHR Rats

The relative quantification at the mRNA level was also tested for ABC transporters, transcription factors and other enzymes. Most visible changes were observed in *Abcg5* and *Abcg8*. A decreased mRNA expression of both transporters was measured in the HHTg as well as in SHR groups compared to control Wistar rats; however, these differences were not statistically significant, probably due to high variability within the tested animal groups. Likewise, there is a trend toward downregulation of the *Nr1i2* gene (that encodes a ligand activated transcription factor, i.e., pregnane x receptor [PXR]) in both experimental strains compared to control Wistar rats.

As with CYP enzymes, the differences in mRNA expression of selected proteins, examined in this chapter, were also observed between experimental groups. Enzyme catechol-*O*-methyltransferase (*Comt*) was decreased at the mRNA level in HHTg rats (by 68%; $p < 0.05$) compared to control Wistar rats. This effect was not found in the second experimental group with SHR rats (Figure 5). The mRNA expression of constitutive androstane receptor (NR1I3), aryl hydrocarbon receptor (AhR) and glutathione S-transferase alpha 1 (GSTA1) was slightly (nonsignificant due to the interindividual variability present) decreased in HHTg rats compared to the control group. In contrast to the experimental group with SHR rats, there were no changes in the mRNA expression of these proteins as well.

3.6 | Effect of Human C-Reactive Protein on Hepatic ABC Transporters, Nuclear Receptors and Enzymes Involved in Hormone Regulation of mRNA Expression in SHRs

A comparison of the SHR and SHR-CRP groups is presented in Figures 6. The presence of human CRP caused a significant increase in mRNA expression of *Nr1i3* in the SHR-CRP group (by 82%; $p < 0.05$). Among the other genes, only slight changes were observed, such as an increasing trend of *Ahr* or a slight decrease of *Gsta1*, both of which were not statistically significant.

3.7 | Effects of Ovariectomy on Hepatic ABC Transporters, Nuclear Receptors and Enzymes Involved in Hormone Regulation of mRNA Expression in Wistar Female Rats

As shown in Figure 7, only a significant change was observed in *Gsta1*, where the relative mRNA expression was decreased by 26.5% ($p < 0.05$) in W-OVX compared with control W-SHAM. There was no significant difference for the other studied genes, as the variability within groups was too high. Visible trends indicated a downregulation of *Abcg5*, *Abcg8*, *Ahr* and upregulation of *Nr1i3*.

3.8 | Comparison of Intestinal CYP mRNA Expression in Wistar, HHTg and SHR Rats

Differences in intestinal mRNA expression between the models are displayed in Figure 8. The expression of CYP isoforms in the small intestine is considerably lower than in the liver; hence, greater deviations were observed in our measurements, and no significant change was calculated. Except for *Cyp2b1*, all the studied isoforms tended to have upregulated mRNA expression in the HHTg group compared with the Wistar group. In the SHR model, a trend toward upregulation of *Cyp7a1* was detected.

3.9 | Effect of Transgenic CRP on Intestinal CYP mRNA Expression in SHRs

As in the previous paragraph, the variability within the SHR-CRP was too high. The lack of significant changes indicated the

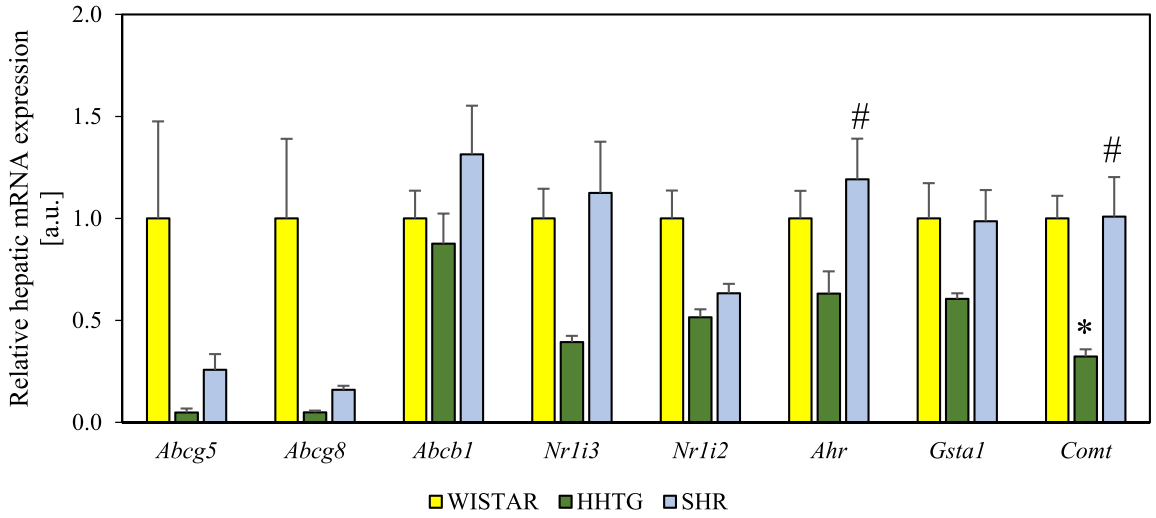

FIGURE 5 | Differences in the relative mRNA expression of ABC transporters, nuclear receptors and enzymes involved in drug metabolism regulation in the liver tissues of HHTg and SHR male rats compared with male Wistar rats, with their values normalised to 1.0. Data are expressed as the mean ± SEM, $n = 5–7$. * denotes $p < 0.05$ compared with the Wistar group; # denotes $p < 0.05$ compared with the HHTg group.

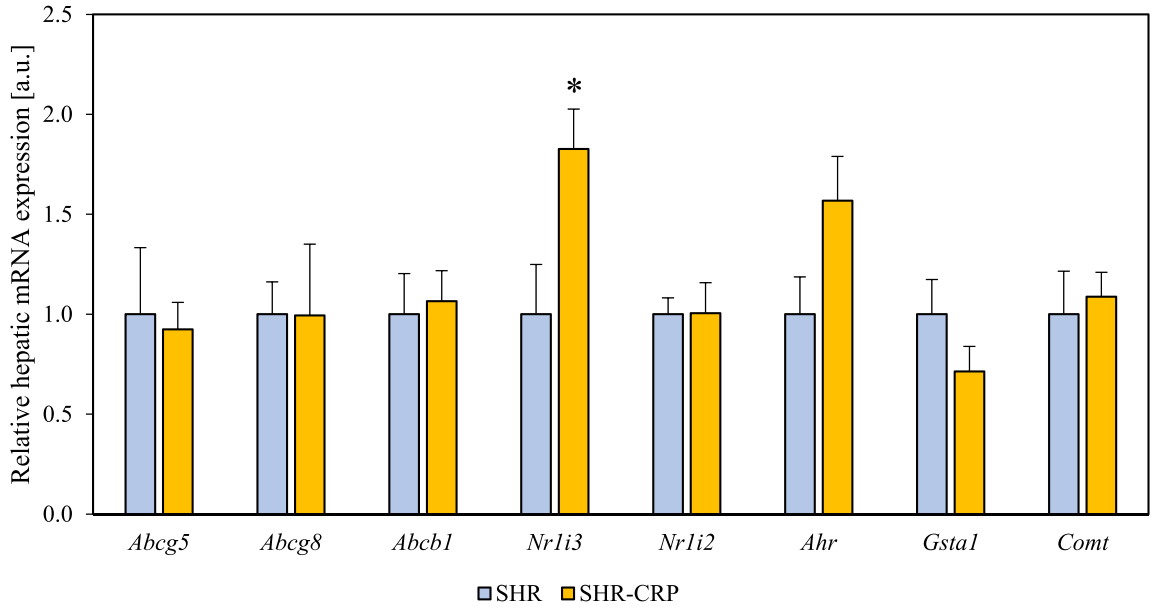

FIGURE 6 | Differences in the relative mRNA expression of ABC transporters, nuclear receptors and other enzymes involved in drug metabolism regulation in the liver tissues of male SHR-CRP rats compared to male SHR rats. Data are expressed as the mean ± SEM, $n = 5–7$. * denotes $p < 0.05$ compared to the Wistar group.

effect of human CRP on CYP expression in the small intestine. Figure 9 shows that *Cyp2d1* was upregulated and *Cyp2e1* was downregulated in the SHR-CRP group compared with those in the SHR group.

3.10 | Effects of Ovariectomy on Intestinal CYP mRNA Expression in Wistar Female Rats

As already mentioned in the previous paragraphs, the amount of CYP mRNA expression in the small intestine was not sufficient to provide statistically significant data; therefore, only trends indicating a change could be assumed. As shown in Figure 10, the most significant difference between W-SHAM and W-OVX was in *Cyp1a2*, which also had the highest SEM. The slight or no differences in the expression of other genes between the groups were observed.

3.11 | Comparison of the mRNA Expression of Intestinal ABC Transporters, Nuclear Receptors and Enzymes Involved in Hormone Regulation in Wistar, HHTg and SHR Rats

The pathological conditions of high triglyceridaemia and hypertension had a significant effect on transcription

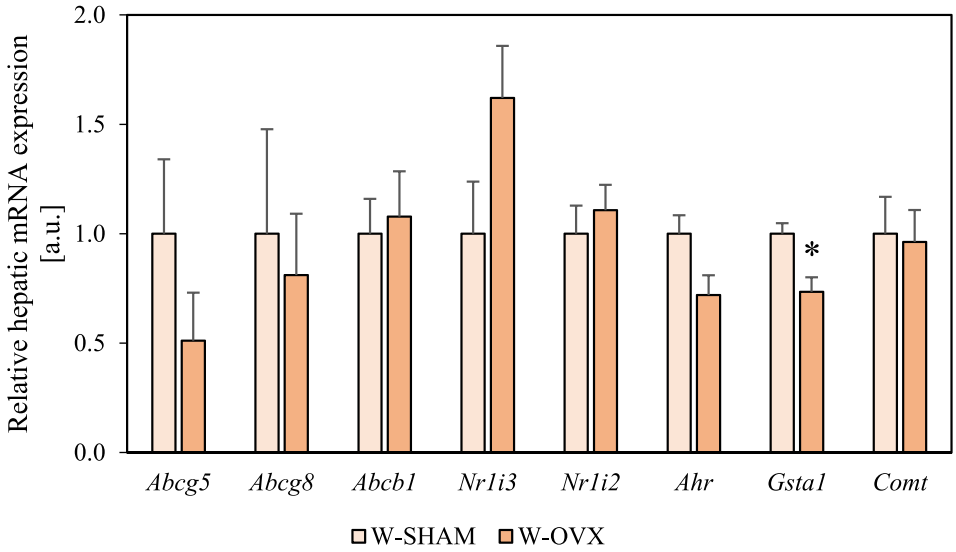

FIGURE 7 | Differences in the relative mRNA expression of ABC transporters, nuclear receptors and enzymes involved in drug metabolism regulation in the liver tissues of ovariectomised female Wistar rats (W-OVX) compared to SHAM-operated controls (W-SHAM). Data are expressed as the mean ± SEM, $n = 5$. * denotes $p < 0.05$ compared with the W-SHAM group.Differences in the relative mRNA expression of ABC transporters, nuclear receptors and enzymes involved in drug metabolism regulation in the liver tissues of ovariectomised female Wistar rats (W-OVX) compared to SHAM-operated controls (W-SHAM). Data are expressed as the mean ± SEM, $n = 5$. * denotes $p < 0.05$ compared with the W-SHAM group.

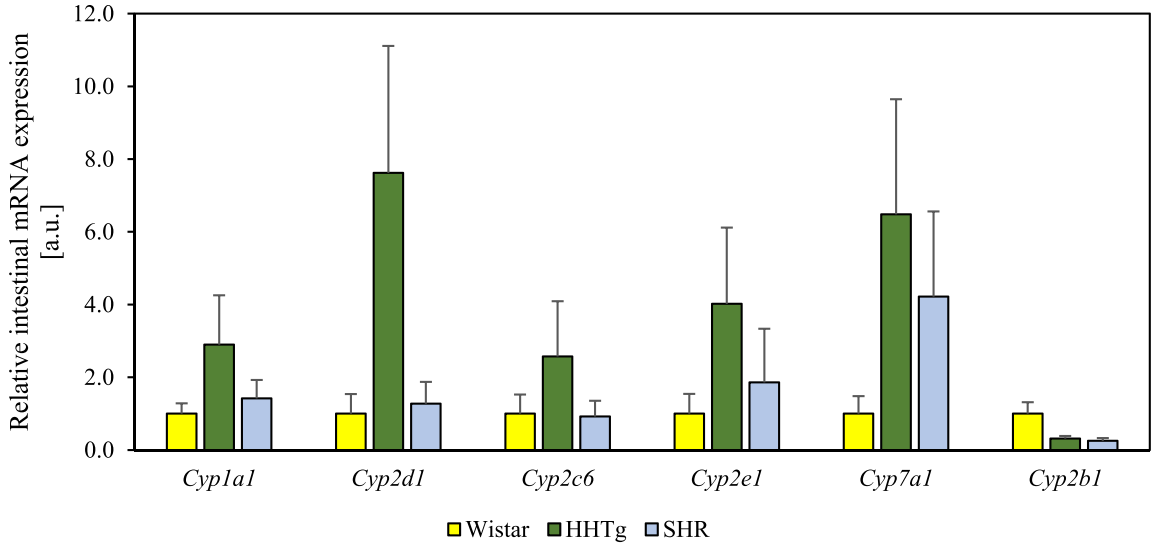

FIGURE 8 | Differences in the relative mRNA expression of cytochromes P450 in the small intestine tissues of male HHTg and SHR rats compared with male Wistar rats. Data are expressed as the mean ± SEM, $n = 5–7$.

factors in the small intestine, as shown in Figure 11. There was significant upregulation of *Nr1i3* (by 113%; $p < 0.05$), *Nr1i2* (by 50%; $p < 0.05$) and *Ahr* (by 32%; $p < 0.05$) in the HHTg group compared with the Wistar control group. The mRNA expression of AhR was also upregulated in the SHR group (by 34%; $p < 0.05$) compared to the Wistar group. For other genes, there were trends indicating changes in mRNA expression; however, these changes were not statistically significant.

3.12 | Effect of CRP on Intestinal ABC Transporters, Nuclear Receptors and Enzymes Involved in Hormone Regulation of mRNA Expression in SHRs

As shown in Figure 12, a significant upregulation of *Nr1i3* by 325% ($p < 0.01$) and *Nr1i2* by 130.7% ($p < 0.01$) in the SHR-CRP group was found compared to the SHR group. For the other observed genes, no significant difference was detected.

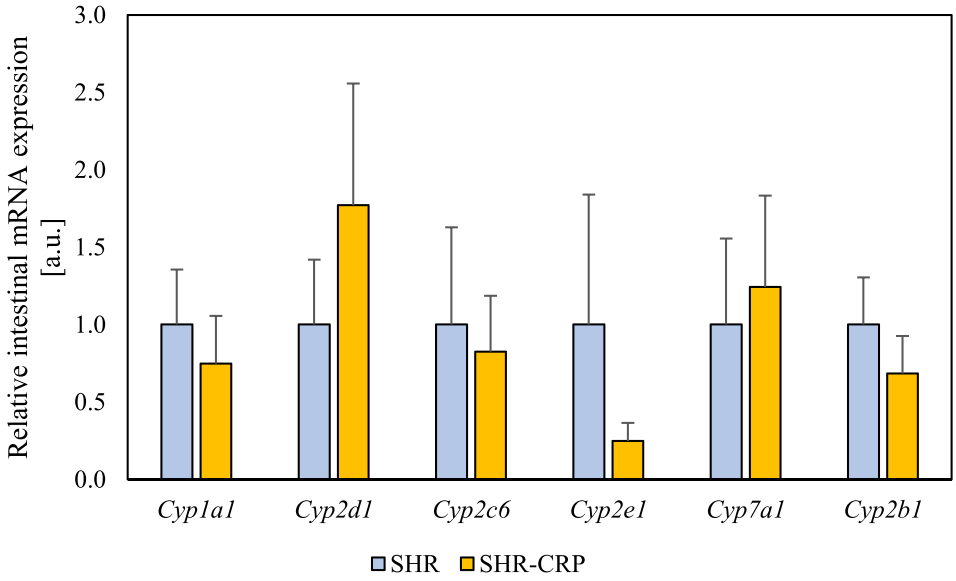

FIGURE 9 | Differences in the relative mRNA expression of CYP in the small intestine tissues of the male SHR-CRP rats compared with the male SHR rats. Data are expressed as the mean ± SEM, $n = 5–7$.Differences in the relative mRNA expression of CYP in the small intestine tissues of the male SHR-CRP rats compared with the male SHR rats. Data are expressed as the mean ± SEM, $n = 5–7$.

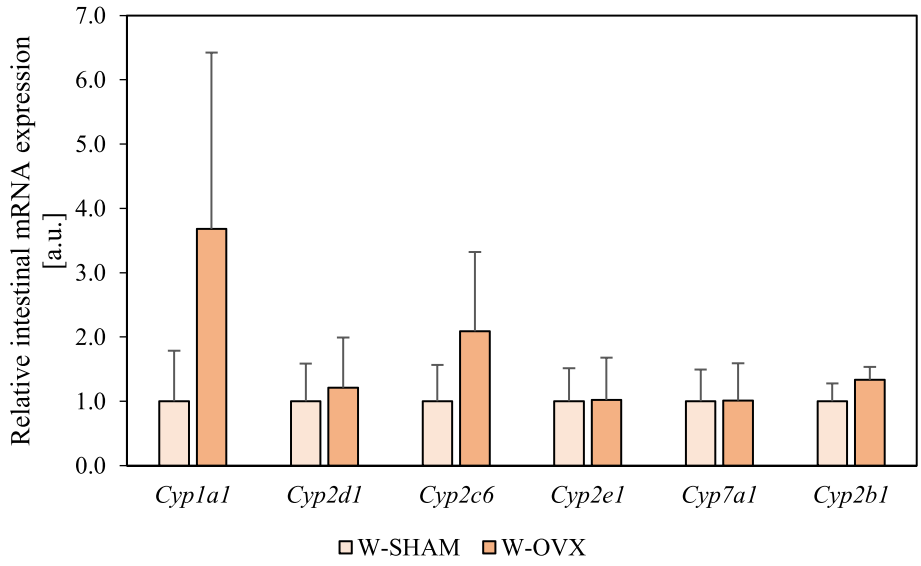

FIGURE 10 | Differences in the relative mRNA expression of cytochromes P450 in the small intestine tissues of female W-OVX Wistar rats compared to female SHAM-operated Wistar rats. Data are expressed as the mean ± SEM, $n = 5$.Differences in the relative mRNA expression of cytochromes P450 in the small intestine tissues of female W-OVX Wistar rats compared to female SHAM-operated Wistar rats. Data are expressed as the mean ± SEM, $n = 5$.

3.13 | Effects of Ovariectomy on Intestinal ABC Transporters, Transcription Factors and Enzymes Involved in Hormone Regulation of mRNA Expression in Wistar Rat Females

As shown in Figure 13, intestinal *Abcg5* (by 124%; $p < 0.01$) and *Abcb1* (by 120%; $p < 0.05$) were significantly upregulated. A trend suggesting the downregulation of *Abcg8* and *Gsta1* was observed, but due to high variability in test subjects, a statistically significant difference was not detected. The other genes did not significantly differ between the studied models.

4 | Discussion

The current study aimed to identify and compare the gene expression of selected CYP isoforms, ABC transporters, nuclear receptors and other enzymes involved in the metabolism of drugs and endogenous substances in three unique rat models, which resemble disorders in humans associated with metabolic syndrome. Knowledge about the function of CYP isoforms is important because the treatment of cluster disorders in MetS increases the risk of drug–drug interactions, and increasing evidence shows the participation of *CYP* gene products in lipid

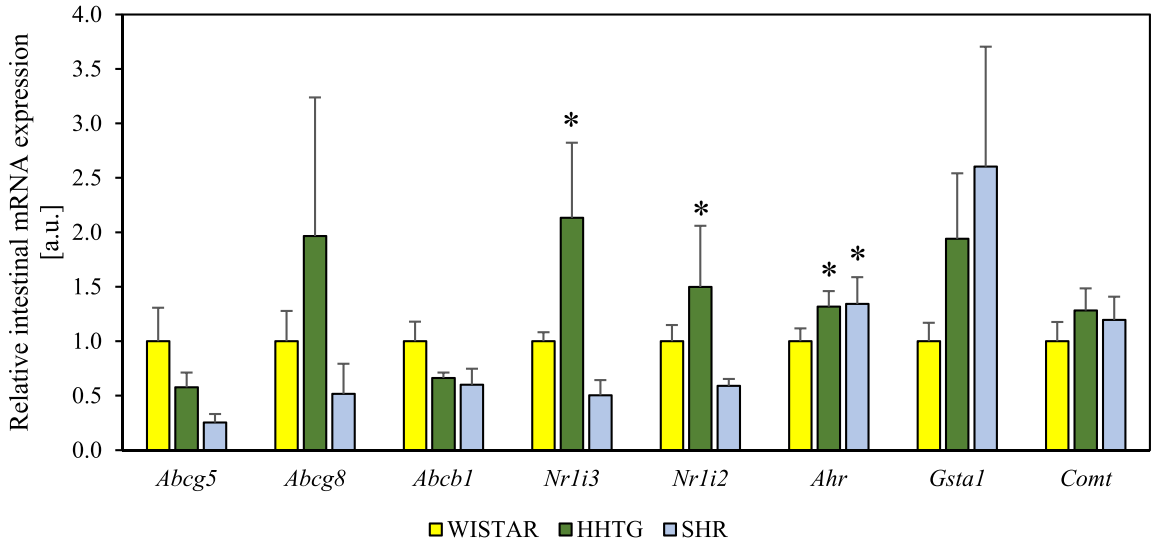

FIGURE 11 | Differences in the relative mRNA expression of ABC transporters, nuclear receptors and other enzymes involved in drug metabolism regulation in the small intestine tissues of male HHTg and SHR rats compared to male Wistar rats. Data are expressed as the mean ± SEM, $n = 5–7$. * denotes $p < 0.05$ compared to the Wistar group.

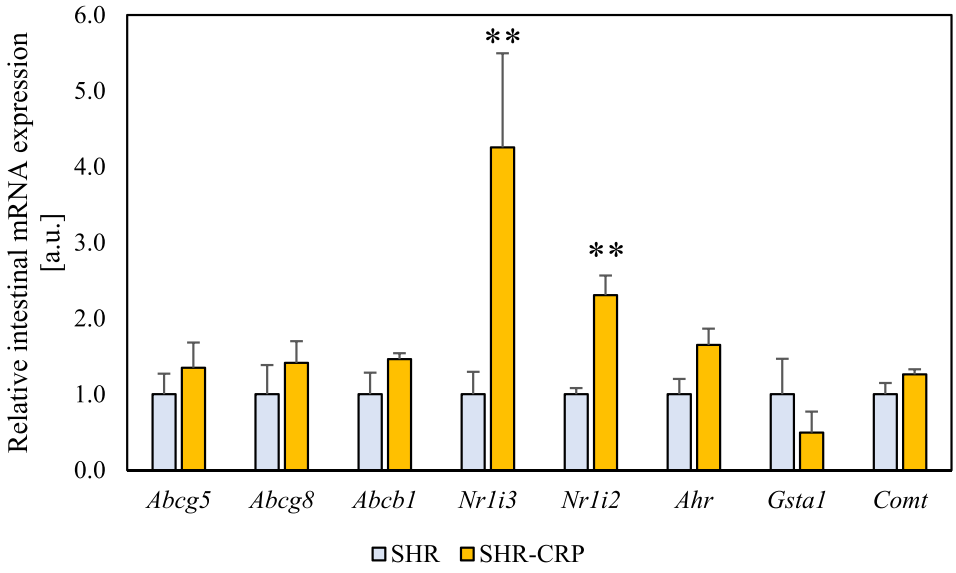

FIGURE 12 | Differences in the relative mRNA expression of ABC transporters, nuclear receptors and other enzymes involved in drug metabolism regulation in the small intestine tissues of male SHR-CRP rats compared to male SHR rats. Data are expressed as the mean ± SEM, $n \geq 5$. ** denotes $p < 0.01$ compared to the SHR group.Differences in the relative mRNA expression of ABC transporters, nuclear receptors and other enzymes involved in drug metabolism regulation in the small intestine tissues of male SHR-CRP rats compared to male SHR rats. Data are expressed as the mean ± SEM, $n \geq 5$. ** denotes $p < 0.01$ compared to the SHR group.

metabolism, including fatty acid oxidation and cholesterol and bile acid metabolism [8].

The selected MetS models were originally derived from Wistar rats, and Wistar rats were used as a control group in this study. The measurements demonstrated interstrain differences in gene expression between the mentioned models, especially in the liver, where most metabolic processes occur. Our measurements point to intermodel differences that could lead to different effects and metabolisms of various drugs used to treat metabolic syndrome and endogenous pathways that are involved in homeostasis.

4.1 | The mRNA Expression of CYPs and Other Selected Proteins in Hypertriglyceridaemic State

Hypertriglyceridaemia is highly prevalent worldwide and can affect up to 25% of adults [38]. This factor is the principal cause of disorders associated with MetS and may lead to ectopic deposition into non-adipose tissue. Lipotoxicity and lipoproteins rich in triglyceride residues may be more atherogenic than LDL on a particle basis [39]. HHTg rats were generated via the selective inbreeding of Wistar rats, and they have elevated hepatic production of triglycerides (increased serum triglycerides

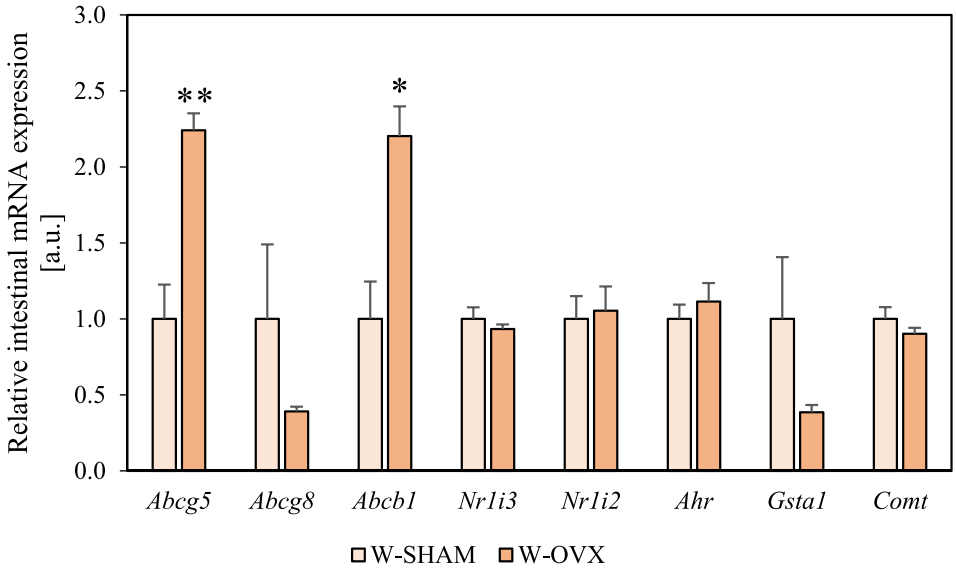

FIGURE 13 | Differences in the relative mRNA expression of ABC transporters, nuclear receptors and other enzymes involved in drug metabolism regulation in the small intestine between ovariectomised female Wistar rats (W-OVX) and female SHAM-operated Wistar rats. Data are expressed as the mean ± SEM, $n = 5$. * denotes $p < 0.05$; ** denotes $p < 0.01$ compared to the W-SHAM group.Differences in the relative mRNA expression of ABC transporters, nuclear receptors and other enzymes involved in drug metabolism regulation in the small intestine between ovariectomised female Wistar rats (W-OVX) and female SHAM-operated Wistar rats. Data are expressed as the mean ± SEM, $n = 5$. * denotes $p < 0.05$; ** denotes $p < 0.01$ compared to the W-SHAM group.

concentration in HHTg rats compared to Wistar rats is shown in Table 1). This model is currently well established for the study of the pathogenesis and therapy of metabolic syndrome [19, 40–42]. In contrast, in our study, rats of the SHR strain (which are also used for studies of MetS) had decreased serum triglycerides, total cholesterol and HDL cholesterol concentration as well as a decreased adiposity index compared to the control group containing male Wistar rats (Table 1) and of course compared to HHTg rats. One of the possible reasons for these effects can be a deficiency of the *Cd36* gene that encodes fatty acid translocase [43]. The glycoprotein CD36 is present in multiple cell types but is highly expressed on the brush border membrane of the enterocytes, primarily in the proximal part of the intestine. One of its functions is a facilitating the absorption of long-chain fatty acids (LCFA) from the diet and directing fatty acids for chylomicron production. A decreased activity of the CD36 transporter could be a significant factor in the absence of hypertriglyceridaemia in our SHR rats, but only theoretically. In fact, animals or humans with a deficiency of *Cd36* have no change or even an increase in serum triglycerides concentration compared to those with normal expression of *Cd36*. It is possible that other intestinal transporters of fatty acids, including fatty acid transport proteins (FATPs) and fatty acid binding proteins (FABPs), could compensate for the reduced LCFA uptake in the absence of *Cd36* [44–46].

As regards cholesterol absorption in the intestine, Nassir et al. demonstrated with the use of primary enterocytes that CD36 deficiency reduces cholesterol uptake by 60% in the proximal intestine but not in the distal intestine. Therefore, it is suggested that CD36 mediates cholesterol absorption in the intestine, but its absence can be compensated for by many factors, including slower gastrointestinal emptying, a longer length of small intestine and the presence of other cholesterol transporters, such as Niemann-pick C1-like 1 (NPC1L1). Nassir et al. also observed significant upregulation of NPC1L1 in the middle and distal intestine in *Cd36*-null mice, where it is normally very low [46, 47]. These data probably explain why animals or humans with a deficiency of CD36 have increased serum triglyceride concentration and not changed serum cholesterol concentration compared to those with normal function of CD36. However, it does not explain why SHR rats in our study had lower serum triglyceride, HDL and total cholesterol concentration compared to control Wistar rats. This effect could be explained by different microbial composition in the intestine. For explanation, authors Sun et al. and Dardi et al. reported that the quantity of bacteria belonging to the genus *Bacteroides* is reduced in the gut of SHR, while bacteria belonging to the genera *Bifidobacterium* and *Prevotella* and phylum *Firmicutes* (*Bacillota*) are abundant in the gut of the same rat model [48, 49]. Siais et al. found out that there exists a moderate positive correlation between the *Bacteroidetes* and triglyceride concentration in blood samples obtained from women. They also observed a moderate negative correlation between the amount of *Firmicutes* and *Firmicutes*/*Bacteroides* ratio (B/F ratio) with triglyceride concentration in the same women [50]. Therefore, if rats of model SHR have a reduced presence of *Bacteroides* and an increased abundance of *Firmicutes* in the intestine, the measured lower serum triglyceride concentration may be due to these changes in their intestinal status. While the genus *Lactobacillus*, which is also abundantly represented in the SHR intestine, is responsible for the production of compounds that can downregulate intestinal NPC1L1 at the mRNA and protein levels, thereby inhibiting cholesterol uptake [49, 51]. Npc1l1 mRNA was not measured in these rats; however, based on data obtained from the literature, it can be assumed that changes in the intestinal microbial composition may also lead to a decrease in cholesterol levels.

Our results also revealed significant interstrain differences of CYPs and nuclear receptors at the mRNA level in the liver, namely, decreased *Cyp1a2*, increased *Cyp2d1* and decreased *Comt* in HHTg rats compared to control male Wistar rats. The enzyme CYP1A2 is expressed mainly in the liver and is involved in the metabolism of approximately 10% of clinically used drugs, including β-blockers, caffeine and phenacetin, also in the endogenous metabolism of steroid hormones [52, 53]. Therefore, the downregulation of *Cyp1a2* (Figure 2) could cause a decrease in protein expression of CYP1A2 that can lead to a slower metabolism of these drugs. The CYP1A2 mRNA expression is regulated by the AhR [54] or PXR [55], both showed a downward trend at the mRNA level as well (Figure 5).

Rat CYP2D1 is considered an orthologue of human CYP2D6, which is linked to the metabolism of drugs, neurosteroids, dopamine and serotonin neurotransmitters. The effect of a maternal high-fat and high-carbohydrate diet on hepatic CYP2D1 expression in rat offspring has recently been reported [56].

Another observed difference between HHTg and Wistar rats was the downregulation of the hepatic gene *Comt* encoding COMT with the ability to degrade catecholamines and oestrogens into less active metabolites. The enzyme COMT may play an important role in MetS because it contributes to the conversion of methionine to homocysteine [57] that participates in folic acid metabolism. The elevated homocysteine is associated with an increased risk of cardiovascular disease [58] and dementia [59]. The downregulation of *Comt* in the liver of HHTg rats could protect these rats against these diseases. On the other hand, the downregulation of *Comt* could also be a one of the causes of impaired glucose tolerance [60] as one of the other properties of HHTg rats (increased serum nonfasting glucose in HHTg compared to control Wistar male rats was also observed in this study, Figure 1).

Our measurements also revealed a trend towards an upregulation of *Cyp7a1* (Figure 2) and a trend towards a downregulation of *Abcg5* and *Abcg8* (Figure 5) in the HHTg group compared to controls. Orolin et al. compared Wistar-Kyoto male rats with HHTg rats, and their data revealed significant changes in the expression of these genes [40]. Our data correlate with their study, although without statistical significance. According to Orolin et al., the increase in CYP7A1 mRNA expression could be due to downregulation of the ABCG5/8 transporter at both the mRNA and protein levels. Downregulation of this transporter causes a decrease in sterol transport, leading to an increase in liver cholesterol concentration. Increased cholesterol concentration leads to an increase in the activity of CYP7A1, which metabolises cholesterol and thus reduces its concentration in the body [40]. Due to these mechanisms, cholesterol balance occurs in the body. As can be seen in Table 1 and Figure 1B, the cholesterol concentrations were unchanged in the serum and liver of HHTg rats compared with control rats.

In the intestine, it is known that the distribution of CYP enzymes is changing along the entire intestine [61]. Therefore, the sampling was performed ever in the middle of a small intestine. Even so, the results were highly variable within the group, probably due to sampling a small piece of small intestine. The mRNA expression of *Cyp1a1*, *Cyp2d1*, *Cyp2c6*, *Cyp2e1* and *Cyp7a1* tended to increase in HHTg rats compared to Wistar rats as well as to SHR. This increase was not statistically significant, as was explained above. Additionally, analysis of gene expression in the small intestine revealed a significant increase in mRNA expression of transcription factors (Figure 11), namely, the AhR (regulates CYP1A1), the PXR (NR1I2; regulates, e.g., CYP1A1 or CYP2C6) and the constitutive androstane receptor (NR1I3, regulates CYP1A1) [62], in HHTg rats compared to the control group. The change in mRNA expression of CYPs and transcription factors could also be caused by a different composition of intestinal microflora. An effect of intestinal microflora on CYP mRNA expression was already studied by Zemanová et al. or Wang et al. They found that intestinal bacterial composition has a significant effect on the CYP enzymes at mRNA and protein level [63, 64]. The increased intestinal CYPs mRNA measured in HHTg rats may be useful in the selection of a rat model for the study of drug–drug interactions in the intestine with or without a connection with intestinal microflora and metabolic syndrome because the CYPs mRNA in HHTg rats were shown to be more detectable and better measurable. But these results of higher mRNA levels still need to be complemented with protein level measurements to confirm the suitability of the HHTg model for the studies mentioned above.

4.2 | The mRNA Expression of CYPs and Other Selected Proteins in Hypertensive State

This study does not focus on just one model, but on three selected models. This is because each model differs in certain parameters, even though all three selected models are models suitable for the study of metabolic syndrome. Rats of the HHTg strain have slightly elevated blood pressure according to our measurements, whereas SHR have significantly higher blood pressure values [43, 65]. Lemmer et al. published that SHR have blood pressure of 176/123 mmHg compared to 116/79 mmHg in control normotensive Wistar-Kyoto strain rats (calculated as rhythm-adjusted mean) [65]. Therefore, SHR rats are more suitable for studies of hypertension.

The enzyme CYP1B1 contributes to a hypertension by the production of reactive oxygen species [66]. This enzyme is present in the liver in very low concentration [67]; thus, the mRNA of CYP1B1 was not measured in our study. However, our study reveals a trend towards an increase in mRNA expression of liver CYP2E1 in the SHR group compared to Wistar rats. The enzyme CYP2E1 contributes to the production of reactive oxygen species as well [68]. It is known that people with obesity and insulin resistance have increased activity of CYP2E1 [69, 70]. The enzyme CYP2E1 metabolises alcohol [68], so these people are probably metabolizing alcohol faster and maybe drinking more of it; thus, more reactive oxygen species are produced in their bodies, leading to the development of many diseases. In HHTg rats, the mRNA of CYP2E1 in the liver was not changed in comparison with control Wistar rats.

Another enzyme of the cytochrome P450 family, human CYP3A4 and CYP2D6, is significantly associated with essential hypertension [71]. In our study, the significant upregulation of *Cyp3a23* was found in SHR liver, and the significant upregulation of *Cyp2d1* was measured in SHR as well as HHTg rat liver compared to control Wistar rats. Rat CYP3A23 is identical to

CYP3A1 [72] and this enzyme is orthologous to human CYP3A4, whereas CYP2D1 is orthologous to human CYP2D6 [73]; therefore, upregulated *Cyp3a23* and *Cyp2d1* (measured in rat liver in our study) could have an association with hypertension. In addition, it is known that there exists a protein–protein interaction between human CYP3A4 and CYP2C9 [74]. If there is a similarity of these human CYPs to rat CYPs, there is a possible explanation for why upregulated *Cyp2c6* was measured in SHR (significantly) compared to control Wistar rats.

In both experimental rats (SHR and HHTg), a trend to decrease of *Pxr* was observed in comparison with control rats. This effect can lead to a decrease of *Cyp3a23* (nonsignificantly observed in HHTg rats) or *Cyp1a2* (significantly observed in both HHTg and SHR). The transcription factor PXR is also connected with CD36 that was mentioned above [75, 76].

Almost 80% of individuals with MetS suffer from hypertension, which is usually treated with a combination of several drugs, and patients are thus at a risk of drug–drug interactions [77]. As results show, the significant upregulation of *Cyp3a23*, *Cyp2d1* and *Cyp2c6* and the significant downregulation of *Cyp1a2* and *Cyp2a1* observed in the liver SHR compared to control Wistar rats could change the activity of CYP enzymes and thus affect the metabolism of drugs used for the treatment of disorders of MetS, especially the treatment of hypertension. Alterations in the levels of these enzymes may affect the course and outcome of pharmacotherapies with various drugs, such as antiarrhythmics or β-blockers in the case of CYP3A23 and CYP2D1 enzymes [78, 79], and clopidogrel in the case of CYP2C6 [80].

4.3 | The mRNA Expression of CYPs and Other Selected Proteins in a Condition of Chronic Inflammation

Metabolic syndrome is characterised by low-grade inflammation. The best characterised biomarker, CRP, contributes to the development of multiple features of metabolic syndrome [27]. Because human CRP, in contrast to rat CRP, can activate rat complement and may be proinflammatory in rats, we investigated the effects of transgenically expressed human CRP in the SHR-CRP model. The upregulation and downregulation of *Cyp* genes in SHR-CRP rats were like those in SHRs. In contrast, a significant upregulation of *Nr1i3* was observed in SHR-CRP rats compared to SHRs (Figure 6). This gene is responsible for the production of the constitutive androstane receptor, and it is involved, together with the PXR (NR1I2), in the regulation of xenobiotic metabolism and also regulates many genes involved in glucose and lipid metabolism [81]. The other measured genes did not significantly differ in terms of mRNA expression.

In the intestine, there are trends in gene expression regulation between SHRs and SHR-CRP, namely, both the *Nr1i3* (+325%, $p < 0.01$) and the *Nr1i2* (+131%, $p < 0.01$). These data could indicate an effect of CRP in the small intestine on the upregulation of CYPs at the mRNA level. Recent publications have shown that CRP negatively modulates *Cyp* gene expression via microRNA upregulation in the liver [82], but little is known about drug metabolism in the intestinal tissue associated with inflammation; thus, additional research is needed.

4.4 | The mRNA Expression of CYPs and Other Selected Proteins in Postmenopausal State

Approximately 20%–30% of premenopausal women have metabolic syndrome, and this number is almost twice as high in postmenopausal women [83]. Ovariectomised rats can be used as a model of menopausal metabolic syndrome to evaluate pathogenesis and discover effective therapeutic approaches [32].

Tissues isolated from the liver and small intestine of sham-operated Wistar (W-SHAM) rats were used as controls for ovariectomised Wistar females (W-OVX), as there was a need to avoid bias caused by the surgery itself.

In W-OVX offspring, a significant downregulation of *Gsta1* in the liver and significant upregulation of *Abcg5* and *Abcb1* in the intestine (compared to control W-SHAM rats) were detected. The expression of other hepatic genes did not significantly change; we only observed an increasing trend in hepatic *Cyp2b1* and *Nr1i3*. Our results are partly in accordance with those of Malinska et al. [31], where significant differences were also observed for several transcription factors, such as *Abcb1b* and *Cyp2e1* in the liver in ovariectomised female rats. On the other hand, in our study, W-OVX female animals presented increased levels of the intestinal *Abcg5*, but Malinska et al. reported increased levels of the hepatic *Abcg8*. Increased mRNA expression of ABCG8 transporter indicated increased cholesterol secretion from hepatocytes into bile. ABCG5/8 transporters not only play important roles in sterol absorption and excretion but also provide an important pathway for cholesterol elimination [84]; however, in both studies, the hepatic cholesterol concentration was not influenced.

Interestingly, the trend towards a decrease in mRNA expression of CYP1A2 (Figure 4) as well as AhR (Figure 7) was observed in the liver of W-OVX compared to female W-SHAM rats. The nuclear receptor AhR regulates CYP1A2, as was already mentioned above. Tsiokou et al. performed an in vivo study with the use of caffeine as a metabolic probe to assess CYP1A2 and CYP2A6 activity. They found a decrease in CYP1A2 activity in postmenopausal compared to premenopausal women. The decrease in CYP1A2 activity (and probably the downregulation of *Cyp1a2* in our study) could be caused by the complex hormonal interplay in the endocrine system of postmenopausal women [85].

On the other hand, the intestinal mRNA expression of *Cyp1a1* and *Cyp2c6* was measured as a trend towards an increase in W-OVX rats in comparison with control W-SHAM rats. As was mentioned above, the high variability in rats within the group was probably caused by the changing composition of cytochrome P450 along the intestine; therefore, it is difficult to take a sample from the exact same organ site in every animal.

The intestinal microflora was also tested in these rats. The culture method was used, and our preliminary results showed a reduction in bacterial incidence to 30% in W-OVX rats compared to control W-SHAM rats. The upregulated *Cyp1a1* and *Cyp2c6* gene expression could be a result of a different microbial composition in the intestine of W-OVX rats.

4.5 | Limitations of the Study

Selected rat models successfully replicate specific features of human MetS, but it is important to recognise that they do not fully capture the complete clinical and pathophysiological complexity observed in humans. Each model provides valuable insight into isolated aspects of the condition: HHTg rats primarily model dyslipidaemia, SHR rats reflect hypertension, W-OVX rats simulate oestrogen deficiency, and the SHR-CRP model introduces a component of low-grade inflammation in addition to hypertension. However, human MetS is multifactorial and polygenic, involving a complex interplay of metabolic disturbances that are not comprehensively represented across these individual animal models.

This inherent difference poses significant challenges for direct application of our preclinical findings to broad human conditions. For instance, while the HHTg model effectively mimics HHTg, its limited capacity to reflect the full spectrum of human dyslipidaemia, including changes in LDL cholesterol and wider systemic metabolic dysfunction, restricts how broadly these specific findings can be generalised. Notably, a complete assessment of dyslipidaemia, including LDL cholesterol levels, was not performed in this study, which further limits the direct translation of our findings to the comprehensive human dyslipidaemic phenotype. Similarly, although the SHR-CRP model is useful for studying certain inflammatory components, its notably restricted metabolic profile, lacking the broader metabolic disturbances common in inflammatory MetS in humans, raises questions about its suitability as a comprehensive model for investigating the systemic impact of inflammation on drug metabolism within the full context of human MetS.

A notable limitation of this study is its sole reliance on mRNA expression analysis. While such changes offer valuable insights, they do not always directly correlate with protein levels or enzyme activity. Confirmation of these findings at the protein and functional levels is therefore crucial for fully verifying the physiological relevance and impact on drug metabolism in vivo.

Furthermore, our study utilised a relatively small sample size for each experimental group ($n = 5–7$), which may limit the statistical power and generalizability of our findings. Therefore, while this research provides new insights into how specific metabolic conditions affect drug-metabolizing enzymes, transporters and transcription factors, these results should be interpreted cautiously when considering their direct relevance to diverse human MetS populations.

5 | Conclusion

Despite these limitations, the study identified several significant differences in the hepatic and intestinal expression of genes involved in drug and hormone metabolism. Hepatic expression of CYP1A2 was consistently decreased under conditions of hypertriglyceridaemia, postmenopause or hypertension. In contrast, CYP2D1 expression was increased in hypertriglyceridaemic and hypertensive states. The upregulation of CYP2C6 and CYP3A23 was observed only in hypertensive rats. These alterations measured in rat livers may influence pharmacokinetic profiles in preclinical drug studies. In the intestine, gene expression patterns were most significantly different in HHTg rats, where elevated mRNA levels for several CYP isoforms were observed. This suggests that the HHTg model may be particularly useful for studies focused on intestinal drug metabolism. However, confirmation at the protein and functional levels will be necessary to validate these findings and their translational relevance.

Author Contributions

Jan Soukop, Iveta Zapletalová, Zuzana Rácová, Martin Poruba, Martina Hüttl, Hana Malínská, Ludmila Kazdová and Rostislav Večeřa conceptualised and designed the experiments. Jan Soukop and Iveta Zapletalová analysed the data. Jan Soukop, with the help and supervision of Rostislav Večeřa, Zuzana Rácová, Martin Poruba and Ludmila Kazdová, wrote the paper.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.