DMD Fast Forward. Published on November 13, 2012 as doi:10.1124/dmd.112.048504

In vitro cytochrome P450 activity decreases in children with high Paediatric End-Stage Liver Disease scores

Lies De Bock, Koen Boussery, Myriam Van Winckel, Peter De Paepe, Xavier Rogiers, Xavier Stephenne, Etienne Sokal, Jan Van Bocxlaer

Laboratory of Medical Biochemistry and Clinical Analysis, Faculty of Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, 9000 Gent, Belgium – **LDB, KB, JVB**

Ghent University Hospital, Paediatric Gastroenterology Dpt., De Pintelaan 185, 9000 Gent, Belgium – **MVW**

Heymans Institute of Pharmacology, Ghent University, De Pintelaan 185, 9000 Gent, Belgium – **PDP**

Ghent University Hospital, Surgery Dpt., De Pintelaan 185, 9000 Gent, Belgium – **XR**

Catholic University of Louvain and St Luc Clinics, Paediatric Department (HPED), PEDI unit, Laboratory of Paediatric Hepatology and Cell Therapy, Hippocrate Avenue 10, 1200 Brussels, Belgium – **XS, ES**

RUNNING TITLE:

Decreasing CYP activity with increasing PELD score

CORRESPONDING AUTHOR

Lies De Bock

Harelbekestraat 72

9000 Gent

Belgium

Lies.DeBock@UGent.be

Tel: +32 (0) 9 264 81 31

Fax: +32 (0) 9 264 81 97

COUNTS

Number of text pages: 28

Number of table: 4

Number of figures: 7

Number of references: 41

Number of words in abstract: 208

Number of words in introduction: 759

Number of words in abstract: 1470

NONSTANDARD ABBREVIATIONS

A1AD: alfa-1 antitrypsin deficiency

CYP: Cytochrome P450

PELD: Paediatric End-Stage Liver Disease score

OLT: Orthotopic Liver Transplantation

PFIC: Progressive Familial Intrahepatic Cholestasis

Abstract

To improve modelling and simulation of the pharmacokinetics (PK) in paediatric patients, there is a need for research on developmental and disease-specific determinants. This article describes the evaluation of the in vitro cytochrome P450 activity, an important enzyme family in drug metabolism, in children with hepatic dysfunction. The activity of 6 CYP isoforms, CYP1A2, 2C9, 2C19, 2D6, 2E1, and 3A4 was evaluated in thirty-one patients with different pathologies, mainly biliary atresia (n=23). A hypervariable activity was observed for all the isoforms. Compared to an average adult activity, low activities were seen for CYP1A2, 2C19, 2E1, and 3A4. For CYP2E1 and 3A4, a positive correlation between activity and abundance was observed. In this population, age, co-medication, and genotype could not be used as predictors for the CYP activity. In contrast, the Paediatric End-stage Liver Disease score was negatively correlated with the ln(activity). This suggests a decrease in CYP activity with deteriorating hepatic function. Moreover, the activity of all isoforms was correlated, demonstrating a concomitant decrease of all isoforms in young patients with liver disease. To our knowledge, this is the first study to evaluate CYP activity in children with hepatic impairment. The presented data may provide support in the further optimization of a disease-specific model in this patient population.

Introduction

There is a paucity of approved drugs to treat children and young infants. It is estimated that 50 to 75% of drugs in children are used off-label (Rocchi and Tomasi, 2011). Many of the dosing regimens of drugs currently used in children are derived using coarse methodologies, such as linear extrapolations from adult doses on the basis of mere body weight. Information on the behaviour of pharmacology of drugs in children is difficult to acquire, due to the ethical and practical restraints of performing clinical studies in the paediatric population (Kauffman and Kearns, 1992). Modelling and simulation of the pharmacokinetics (PK) and pharmacodynamics (PD) of drugs in children has therefore gained a lot of interest. Many attempts have been made to create models to predict the PK and/or PD of specific drugs in children. These models, integrating information on the paediatric biological system, are predicted to replace the currently used empirical and allometric models (Knibbe et al., 2011), albeit that more in-depth model optimization is still an obvious necessity. Determinants of the PK in paediatric patients should be investigated, such as drug absorption in neonates and infants, the ontogeny of transporters, or age-related changes in PD (Barrett et al., 2012; Johnson and Rostami-Hodjegan, 2011). Besides the important developmental changes, variability due to environmental, genetic and disease related factors also need to be incorporated (Knibbe and Danhof, 2011). The latter have been shown to influence both PK and PD of drugs, underlining he need for disease-specific models.

In the development of disease-specific models, there is a particular interest in hepatic failure. Hepatic impairment has been identified as a condition leading to changes in the PK of drugs through various mechanisms, such as changes in the hepatic blood flow (portal-systemic shunts in cirrhosis), or an impaired metabolic clearance (as in biliary obstruction, where hepatocellular damage can be seen) (Verbeeck, 2008). The specific mechanisms of the alterations in PK can be elucidated through in vitro studies each focusing on one particular aspect of the PK, such as e.g. the activity of drug metabolizing enzymes. Johnson et al. recently published a semi-mechanistic model for the prediction of drug clearance in adult patients with liver cirrhosis (Johnson et al., 2010).The model was based on specific mechanistic data that were previously published, such as the study conducted by George et al. (George et al., 1995). In this study, the activity and abundance of several cytochrome P450 (CYP) isoforms, the main enzymes involved in drug metabolism, were evaluated in patients with severe

chronic liver disease (George et al., 1995). Based on these in vitro data, they concluded that the CYP isoforms were selectively altered in liver disease, with some isoforms showing a profound decrease, whereas others were only slightly or even not affected. Frye et al. demonstrated a similar selective regulation of the various enzymes in liver disease in vivo, with variable and non-uniform alterations in CYP activity (Frye et al., 2006). The results from comparable studies were reviewed by Villeneuve and Pichette (Villeneuve and Pichette, 2004), and Elbekai et al. (Elbekai et al., 2004)

In these previously published studies investigating the effect of hepatic impairment on drug PK, no children were included. Moreover, several studies concluded that the changes in PK depend on the aetiology of the disease, as well as the degree of hepatic impairment (as reviewed by Villeneuve and Pichette (Villeneuve and Pichette, 2004)). The aetiology of severe hepatic dysfunction differs considerably between adults and children. In adults, the main indication for liver transplantation are noncholestatic liver cirrhosis (± 60%) due to alcoholism or hepatitis C, liver cancers (± 10%), cholestatic diseases (± 10%), acute hepatic failure and metabolic disorders (Adam and Hoti, 2009). In children however, biliary atresia is the main indication for liver transplantation, followed by fulminant liver failure, other cholestatic diseases, such as progressive familial intrahepatic cholestatis (PFIC) and Alagille syndrome, and other metabolic diseases (Adam and Hoti, 2009; Muiesan et al., 2007; Sokal et al., 2008).

The available data on the influence of liver disease on CYP activity in adults cannot be extrapolated to the paediatric population, due to the aforementioned ontogeny of several systems, as well as to the differences in aetiology of the liver disease. Information on the influence of liver disease on the CYP activity is pivotal for the development of disease-specific models for this particular patient population. This study therefore aimed to evaluate the in vitro CYP activity in samples of liver explants of children with severe hepatic dysfunction, in view of it being a determinant parameter in such disease-specific physiology based pharmacokinetic (PBPK) models.

Materials and Methods

This study was approved by the Ethics Committee of Ghent University Hospital (B67020084281) and in accordance with the Ethical Committee approval of Saint-Luc Clinics Brussels.

Sample collection

Liver samples were obtained from the explanted liver of 31 paediatric patients who underwent liver transplantation at Ghent University Hospital or Saint-Luc Clinics Brussels (see Table 2). The clinical record was consulted for clinical and laboratory data, such as age, gender, weight, pre-operative medication, and relevant liver function tests. Part of this information was used to calculate the Paediatric End-stage Liver Disease (PELD) score (McDiarmid et al., 2002).

From each liver, small blocks of tissue (1-4 cm^3) were taken on four different sample sites (superficial and central of both right and left lobe). The tissue samples were snap frozen within 15 minutes after explantation of the liver, and were stored at -80°C until processing.

Preparation of liver microsomes

Part of the liver tissue sample was processed into microsomes using a modification of the method of Wilson et al. (Wilson et al., 2003). The samples were thawed on iced, rinsed with homogenization buffer (0.25 M phosphate buffer pH 7.25, 1.15% KCl) and blot dried. After weighing (0.6 – 4 g), the tissue was minced with scissors and homogenized in homogenization buffer (4 ml g^{-1} tissue) using an automated Potter-Elvehjem system. Tissue homogenates were centrifuged at 10,000 *x g* for 15 minutes at 4°C (Beckman L8-70M Ultracentrifuge, Beckman Coulter Limited, High Wycombe, Buckinghamshire,
UK). In order to form a microsomal pellet, the resulting supernatant (S9 fraction) was centrifuged at 100,000 *x g* for 75 minutes at 4°C. The supernatant (cytosolic fraction) was kept aside, and the microsomal pellet was re-suspended and washed using 4 ml homogenization buffer per gram tissue. Centrifugation at 100,000 *x g* was repeated, and the final microsomal pellet was re-suspended in 1.5 volumes of resuspension buffer (homogenization buffer, containing 30% *v/v* glycerol). All fractions were snap frozen in liquid nitrogen and stored at -80°C until analysis.

The total protein content of the microsomes was estimated using the method of Bradford (Bradford, 1976).

Cytochrome P450 activity determinations

In order to exclude possible zonal differences in activity, a microsomal pool, consisting of the four different zones according to their relative weight, was prepared for each patient sample. Microsomal activities of CYP1A2, 2C9, 2C19, 2D6, 2E1, and 3A4 were determined by incubating the microsomes with specific probe substrates that are mainly metabolized to marker metabolites by one of the studied isoforms (see Table 1). Incubations were performed in triplicate (with CV% always < 15%), and in parallel with each of the specific substrates individually. The final reaction medium (total volume 250 µl) consisted of 1 mM NADPH, 0.2 M phosphate buffer (pH 7.4), 0.25 mg protein/ml and one of the substrates at its apparent Km (Yuan et al., 2002). The samples were incubated in a shaking heating block at 37°C for 15 minutes, except for the CYP2C19 assay, which was incubated for 40 minutes. The enzymatic reactions were stopped with 25 µl of ice cold stopreagent containing the internal standard (chlorpropamide), 3% *v/v* formic acid, and 55% *v/v* acetonitrile. The terminated reaction mixtures were vortex mixed and placed on ice. In order to pellet the proteins, samples were centrifuged at 20,000 *x g* for 10 minutes at 4°C. The formed metabolites were quantified with a previously described UPLC-MS/MS method (De Bock et al., 2012a), and activities were expressed as pmol metabolite formed/(minute x mg protein).

(Table 1)

Besides the incubation of the patient microsomes, two commercially available adult microsomal pools were incubated under the same conditions (Xtreme 200 HLM, Xeno Tech, Lenexa KS, USA, and BD UltraPool HLM 150, BD Biosciences, Bedford MA, USA).

DNA extraction and genotyping

DNA was extracted from 25 mg of the remaining liver tissue sample using the QIAamp DNA Mini kit from Qiagen (Santa Clarita, CA, USA). The samples were genotyped using TaqMan® Drug Metabolism Genotyping Assays from Applied Biosystems for the following NCBI dbSNP identification numbers: rs3892097 (2D6*4, 1846G>A), rs1065852 (2D6*10, 100C>T), rs28371725 (2D6*41,

2988G>A), rs1799853 (2C9*2, 3608C>T), and rs1057910 (2C9*3, 42614A>C), rs4244285 (2C19*2, 19154G>A), and rs12248560 (2C19*17, -806C>T).

CYP3A4 and 2E1 abundance determination

The abundance of CYP3A4 was determined in the microsomal suspension using an indirect ELISA (De Bock et al., 2012b). CYP2E1 abundance was determined using a similar method with optimized calibration range (4 – 256 pmol CYP2E1/mg microsomal protein), primary and secondary antibody concentration (1:1,600 and 1:10,000, respectively), and incubation times (secondary antibody incubation at 37°C, and incubation with substrate for 60 minutes).

Data analysis

The individual activities of the 6 isoforms were compared graphically. Subsequently, the isoform activities were grouped based on their percentage of the mean isoform activity in the adult microsomal pools. Five groups were selected: <25%; 25-55%, 55-85%, 85-115% (considered equal to adult activity, based on the allowed analytical variability of ± 15%), and >115%. The possible influence of age, weight, co-medication, and genotype was also evaluated graphically. The relationship between ln(activity) and ln(abundance), and ln(activity) and PELD score was evaluated graphically using locally weighted scatterplot smoothing (LOWESS). Moreover, the Pearson's correlation coefficient was calculated for these pairs of variables. Furthermore, the correlation between the activities of the isoforms was evaluated (graphically and with Pearson's correlation). All data analysis was performed using R® v.2.13 (R foundation for statistical computing, Vienna, Austria), except for the influence of co-medication, which was evaluated using ANOVA analysis with post-hoc Bonferoni correction using SPSS Statistics 20 (SPSS Inc, Chicago, IL, USA). For all statistical analyses, $p < 0.05$ was accepted as indicating a significant difference.

Results

Patient characteristics

Thirty-one patients were included in the study. The patients suffered from biliary atresia (BA; n = 23), α−1 antitrypsin deficiency (A1AD; n = 1), a combination of BA and A1AD (n=1), progressive familial intrahepatic cholestasis (PFIC; n = 2), cystic fibrosis (CF; n = 1), Caroli's disease (CAR; n = 1), acute liver failure (ALF; n = 1), or neonatal hemochromatosis (NH; n = 1). A summary of the patient characteristics is given in Table 2.

(Table 2)

Cytochrome P450 enzyme activities

The results of the individual enzyme activity measurements are depicted in Figure 1. Due to the non-normal distribution, activities were ln transformed. The mean adult ln(activity) of the isoforms, determined in the two commercially available adult pools, was added to the graph as a point of reference (dashed line). For all isoforms, a high inter-individual variability was observed. A low ln(activity) of CYP3A4, 1A2, 2E1 and 2D6 was seen in many patients, compared to the adult reference. The CYP2C9 ln(activities) were spread around the adult reference, with both very low and very high activities in some patients.

Figure 2 shows the same activity data after grouping the results into five groups, based on the percentage of the mean reference adult activity. A different pattern can be seen between the different isoforms. Fifty percent or more of the patients show a CYP3A4, 2E1, 1A2 and 2C19 activity below 25% of the adult activity. In contrast, about 50% of the patients had a CYP2C9 activity higher than in adults. As for the CYP2D6 activity, the patients were more or less equally distributed over all five groups.

The evaluation of the relation between the activity and the age and weight of the patients showed no correlation (data not shown).

Possible influence of co-medication

A list of the known inducing or inhibiting drugs taken in the 2 weeks prior to the transplantation is presented in Table 3. Graphical comparison of the ln(activity) of the 6 isoforms between patients

receiving inhibitors, inducers, both, or no co—medication is depicted in Figure 3. Statistical comparison showed significant differences ($p<0.05$) in ln(activity) of CYP2C9 between the patients receiving no co-medication and those receiving both an inhibitor and an inducer, as well as between the patients receiving an inhibitor and those receiving both. The same results were obtained for CYP2C19. For CYP3A4, significant differences were seen between: (1) no co-medication and inhibitor + inducer; (2) no co-medication and inducer; (3) inhibitor and inhibitor + inducer; (4) inhibitor and inducer.

(Table 3)

Correlation genotype and activity

A comparable graphical analysis was performed for the evaluated polymorphisms. For all SNPs, a reduced activity was expected compared to the wild type, except for CYP2C19*17, where an increased activity was anticipated. As seen in Figure 4, none of these expected trends could be observed.

Correlation abundance and activity

In Figure 5, the correlation between the ln(activity) and the ln(abundance) of CYP3A4 and CYP2E1 is shown. For CYP3A4, a clear positive correlation could be observed graphically. Even though a positive correlation between CYP2E1 activity and abundance could be seen, it was less convincing than for CYP3A4. This is also reflected in the Pearson's correlation coefficient, being 0.869 and 0.371 for CYP3A4 and 2E1, respectively.

Correlation of the CYP enzyme activity with PELD score

The ln(activity) was correlated with the PELD score at time of transplantation. This can be seen graphically (LOWESS line) as depicted in Figure 6. This negative correlation is confirmed by the Pearson's correlation coefficients of -0.769 for CYP1A2, -0.461 for CYP2C9, -0.721 for Cyp2C19, -0.492 for CYP2D6, -0.545 for CYP2E1, and -0.643 for CYP3A4..

Correlation of the 6 isoforms

A possible correlation in ln(activity) between the different isoforms was evaluated. As shown in Figure 7, all isoform activities are positively correlated.

Discussion

The cytochrome P450 enzyme system has shown to be altered in adults with liver dysfunction (Verbeeck, 2008). As in vivo studies in the paediatric population are fraught with ethical and practical considerations, in vitro data can be used to optimize models that may be helpful in determining optimal dosing strategies, in both pre-clinical and clinical settings. This study is the first to describe the CYP activity in children with severe hepatic dysfunction, demonstrating a high inter-individual variability for all studied isoforms. Comparison with the average adult activity showed a mainly lower activity for CYP1A2, 2C19, 2E1, and 3A4, and an activity more spread around adult activity for CYP2C9 and 2C19. Nevertheless, merely relating activities to a “reference activity” obscures the true nature of these physiological findings. Besides the possible influence of liver disease, its aetiology and the degree of impairment, many other factors such as age, inhibiting or inducing co-medication, and genetic polymorphisms, are known to affect enzyme activity, precluding the interpretation of activities as such.

About 75% of the children included in the study suffered from biliary atresia (BA). A large group of patients with this condition was expected, as this is the main cause for OLT in children (Adam and Hoti, 2009; Muiesan et al., 2007; Sokal et al., 2008). Contradictory to the expected large group of fulminant hepatic failure, only one patient was included during the time period of collection. This is probably due to the type of recruitment in one of the hospitals, where only planned transplantations by living donor were included. Due to the small number of patients in some groups, the results of patients with different diseases were analysed together. The aetiology of liver disease has been shown to be an important factor in the changes in CYP activity in adults (Villeneuve and Pichette, 2004). However, as shown in Figure 1, the variability in ln(activity) in the BA group is larger than the variability between the different groups. Therefore, the patients were considered as one large group for data analysis. Consequently, these results should be interpreted with some caution in respect to these disease types.

The patients included in the study covered a wide age range (from 0.7 months to almost 12 years), with the majority of the patients being age 2 or younger. Age is an important characteristic to take into account in the interpretation of the results, as some of the important CYP isoforms show a certain maturation during development (Alcorn and McNamara, 2002; de Wildt, 2011; Hines, 2008). The in

vitro activity of the 6 enzymes studied, show a gradual increase in activity during the first 2 years of life in healthy children. However, considerable inter-individual differences were observed in the maturation (Hines, 2007). Nevertheless, a certain correlation between the activity and the age of the patients in our study was expected. It was, however, not observed (data not shown). The lack of correlation between age and activity suggests a disturbance of the normal maturation pattern in children with hepatic dysfunction, or a stronger influence of another parameter than age. This failure to show a correlation is an important point in the mechanistic elucidation of the influence of hepatic dysfunction on CYP activity in children. Evaluation of the relation between activity and other patient related characteristics, such as weight, also showed no correlation.

Most of the children included in the study are chronically ill and thus often receive chronic medication. Most of the CYPs have been shown to be sensitive to induction or inhibition by several drugs (Boobis et al., 2009). The influence of inducing or inhibiting pre-operative medication should therefore also be taken into account. Some of the effects of known inducers were observed either graphically or statistically. In contrast, inhibiting effects were not observed. However, we cannot exclude that during the preparation of the microsomes the competitive inhibitors are (partially) removed from the microsomal pellet. This may cancel out the influence of (competitive) inhibitors in our experimental set up.

Another issue in the evaluation of CYP activity is the highly polymorphic nature of the CYP enzymes. , Three of our studied isoforms, CYP2C9, 2C19 and 2D6 are Class II CYPs, or highly polymorphic CYPs. The other 3 isoforms, CYP3A4, 1A2 and 2E1 are well conserved and do not have important functional polymorphisms, and are therefore Class I CYPs(Rodriguez-Antona and Ingelman-Sundberg, 2006).. The presence of one or more polymorphic sites in the genome of the patients, may cause significant changes in the observed enzyme activity. The previously mentioned classification could not be perceived in the obtained activities in this study, as the variability in the class I CYPs was similar to that of the Class II CYPs. Moreover, no reduced or increased activity was seen in patients carrying a SNP known to affect enzyme activity. In conclusion, genetic polymorphism is also a poor predictor for the CYP activity in these patients.

The analysis of the abundance of the isoforms in the microsomes may give information on the nature of the possible changes in enzyme activity. The positive correlation between activity and abundance of

CYP3A4 and 2E1 could infer that the observed changes are due to pre-translational or translational alterations in patients with hepatic dysfunction. The poorer correlation between CYP2E1 activity and abundance could be due to the small range of the observed abundances.

The Paediatric End-Stage Liver Disease (PELD) score was calculated for each patient at the time of transplantation. The PELD score is used in the liver allocation system for paediatric transplant patients. This severity-of-illness score expresses the urgency for transplantation, based on the objective and measurable elements age (< 1 year), serum albumin and total bilirubin, INR, and growth failure (McDiarmid et al., 2002). The diagnosis is not incorporated in the calculation of the PELD score, as it may lead to discrimination of certain subgroups of patients of the transplant waiting list. In this study, the PELD score was used to estimate the degree of hepatic impairment. Evaluation of the relationship between the PELD score (ranging from 0 to 38) and the ln(activity) showed a negative correlation for all six isoforms. The PELD score appears to be the only factor that could give a certain idea on the CYP status of the patient. The observed negative correlation suggests a progressive decrease in enzymatic activity with deteriorating hepatic disease. This negative correlation is in accordance with the in vitro findings in adults (Elbekai et al., 2004; Villeneuve and Pichette, 2004), where the CYP activity was shown to decrease with the Child-Pugh score, an indicator for hepatic dysfunction in adults. Moreover, this negative effect of disease on the CYP activity has recently been described in two in vivo studies evaluating the midazolam clearance in children. The clearance of midazolam is determined by CYP3A4/5-mediated clearance, uridine diphosphate glucuronosyltransferase (UGT)-mediated clearance, and renal clearance. Ince et al. (Ince et al., 2012) studied the age-related changes in midazolam clearance in relation to other covariates, such as specific subpopulations or severity of disease. They showed that critical illness is a major determinant for midazolam clearance in children. Simulations using a PBPK model suggested a major impact of reduced CYP3A4 and 3A5 enzyme abundance on the midazolam clearance. These results were confirmed by another study by Vet et al. (Vet et al., 2012), where a reduced midazolam clearance was observed with increasing critical illness in children on the paediatric intensive care unit. The authors suggested that a reduced CYP3A activity could be the cause for the reduced midazolam clearance. Our in vitro data on CYP3A4 activity support this hypothesis.

The activities of all isoforms in our dataset were correlated positively, meaning that a decrease in activity in one isoform often implies a concomitant decrease in the activities of the others. This

correlation, however, gives no information on the degree of the change in activity. In previously published studies evaluating the effects of hepatic impairment on CYP activity in adults, some of the CYPs have been shown more sensitive to the effects of hepatic dysfunction than others. Frye et al (Frye et al., 2006) suggested a sequential progressive model of hepatic dysfunction in adults, as CYP2C19 was shown to be affected very early in the development of liver failure, whereas CYP2E1 activity decreased later in disease progression. Further research should be conducted in order to develop a similar model for the course of CYP decrease in children with hepatic dysfunction.

In order to evaluate the effects on CYP activity of pathology alone, without confounding contribution of the other abovementioned factors except inducers or inhibitors, the presented activity data could be compared with activity data obtained in healthy children. This would allow a paired comparison between a patient from the study and a healthy child with the same characteristics (such as age, gender, genotype,..). However, the availability of healthy paediatric liver tissue is, for obvious reasons, limited.

Conclusion

This article is to our knowledge the first to describe the in vitro CYP activity in children with hepatic dysfunction. The severity of hepatic dysfunction, expressed as the PELD score, seems to be a major determinant of the in vitro CYP activity in children. Although our results show a wide variability in activity of all CYP studied, we believe this study delivers an important contribution to the increase in knowledge on the pharmacokinetics in a particular paediatric population, i.e. children with severe hepatic dysfunction. These results may provide some support in the improvement of paediatric pharmacokinetic models, but also highlight the difficulties in developing such models.

Acknowledgments

We wish to thank Dr. Ruth De Bruyne, and the transplant teams of Ghent University Hospital (led by Prof. Dr. X Rogiers) and Cliniques Universitaires Saint-Luc (led by Prof. Dr. R. Reding) for their cooperation. We also thank the staff of the Laboratory of Paediatric Hepatology and Cell Therapy (UCL) and Prof. Dr. M. Struys (RUGroningen) and his colleagues for their valuable feedback.

Authorship Contributions

Participated in research design: De Bock, Boussery, Van Winckel, De Paepe, Van Bocxlaer

Conducted experiments: De Bock

Contributed new reagents or analytical tools: De Bock, Rogiers, Stephenne, Sokal

Performed data analysis: De Bock, Boussery, Van Bocxlaer

Wrote or contributed to the writing of the manuscript: De Bock, Boussery, Van Winckel, De Paepe, Rogiers, Stephenne, Sokal, Van Bocxlaer

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Footnotes

This project was financially supported by the Ghent University Special Research Fund, grant [BOF B/09042/02].

Lies De Bock

Harelbekestraat 72

9000 Gent

BELGIUM

Figure legends

Figure 1: Ln(activity) of the 6 CYP isoforms of the 31 patients. Indications for OLT: A1AD: α-1 antitrypsin deficiency; ALF: acute liver failure; BA: biliary atresia; CAR: Caroli's disease; CF: cystic fibrosis; NH: neonatal hemochromatosis; PFIC: progressive familial intrahepatic cholestasis. The dashed line represents the mean ln(activity) as determined in two commercially available adult microsome pools.

Figure 2: The activity of the CYP isoforms in terms of percentage of the adult activity. The results were classified into 5 groups, and the percentage of the study population (n=31) present in a group is depicted.

Figure 3: Comparison of the ln(activity) between patients receiving co-medication known to induce or inhibit CYP activity and patients who did not receive these drugs. (Inh: inhibitor; ind: inducer; comed: co-medication). (*$p<0.05$, –: median ln(activity) within each group)

Figure 4: Analysis of the effects of SNPs on the activity of CYP2D6 (*4, *10, *41), CYP2C9 (*2, *3), and CYP2C19 (*2, *17). Allelic combinations are arranged from expected lowest (left) to expected highest (right) activity.

Figure 5: Ln(activity) and ln(abundance) of CYP2E1 and 3A4 were positively correlated.

Figure 6: Correlation of ln(activity) and PELD score. A negative correlation was observed for all 6 isoforms.

Figure 7: Graphical representation of the positive correlation of the ln(activities) of the 6 isoforms.

Tables

Table 1: **Specific substrates and conditions for the incubation experiments**.

Total microsomal protein content was 0.25 mg/ml.

Enzyme	Substrate	Concentration (µM)	Time (min)	Metabolite
CYP1A2	Phenacetin	50	15	Acetaminophen
CYP2C9	Tolbutamide	100	15	4-Hydroxytolbutamide
CYP2C19	S-Mephenytoin	100	40	4'-Hydroxymephenytoin
CYP2D6	Dextromethorphan	5	15	Dextrorphan
CYP2E1	Chlorzoxazone	50	15	6-Hydroxychlorzoxazone
CYP3A4	Midazolam	5	15	1-Hydroxymidazolam

Table 2: **Patient information.**

Summary of the gender, age at transplantation, diagnosis and PELD of the 31 included patients (M: male; F: female; OLT: orthotopic liver transplantation; PELD: Paediatric End-Stage Liver Disease; A1AD: α-1 antitrypsin deficiency; PFIC: progressive familial intrahepatic cholestasis).

Patient ID (n = 31)	Gender	Age at transplantation (months)	Indication for OLT	PELD at transplantation
ID1	F	6.3	Biliary atresia	23
ID2	M	0.7	Neonatal hemochromatosis	38
ID3	M	7.3	Biliary atresia	13
ID4	M	9.0	Biliary atresia	26
ID5	M	4.7	Biliary atresia	35
ID6	F	6.5	Biliary atresia	15
ID7	F	9.5	Biliary atresia	7
ID9	F	7.4	Biliary atresia	18
ID10	M	132	Cystic fibrosis with cirrhosis	8
ID11	M	9.2	Biliary atresia	38
ID12	M	15.3	Biliary atresia	20
ID13	M	6.4	Biliary atresia	31
ID14	F	108	Acute liver failure	34
ID15	M	13.0	Biliary atresia	26
ID16	M	23.7	Biliary atresia	1
ID17	M	11.4	Biliary atresia	19
ID18	M	25.4	Biliary atresia	17
ID19	M	11.5	Biliary atresia	19
ID20	M	11.5	A1AD	32
ID21	F	60.7	Caroli's disease	12
ID22	F	7.8	Biliary atresia	24
ID23	F	9.4	Biliary atresia	18
ID24	M	24.5	Biliary atresia	0

ID25	F	51.4	PFIC II	11
ID26	M	11.0	Biliary atresia	37
ID27	M	7.1	Biliary atresia	30
ID28	M	8.1	Biliary atresia	19
ID29	M	78.1	Biliary atresia + A1AD	33
ID30	F	11.6	Biliary atresia	10
ID31	F	8.9	Biliary atresia	3
ID32	M	83.8	PFIC III	6

Table 3: **Medication history.**

Summary of the administered pre-operative medication, known to influence (induce or inhibit) CYP activity.

	Number of patients receiving the drug	Affected CYP isoform(s)	Reference
Inhibiting drugs			
retinol	14	CYP2C9, 2C19	(Hamman et al., 1997; Yamazaki and Shimada, 1999)
cholecalciferol	13	CYP2C9, 2C19, 2D6	(Yamazaki and Shimada, 1999)
somatostatin	4	CYP2D6, 3A4	(Rasmussen et al., 1998)
sulfamethoxazol	1	CYP2C9	(Venkatakrishnan et al., 2000)
fluconazol	2	CYP2C9, 2C19	(Venkatakrishnan et al., 2000)
(es)omeprazol	8	CYP2C9, 2C19	(Li et al., 2004)
amlodipine	1	CYP2C9, 2D6, 3A4	(Ma et al., 2000)
ciprofloxacine	1	CYP1A2, 3A4	(Fuhr et al., 1992; McLellan et al., 1996)
propranolol	5	CYP1A2, 2D6	(Kudo et al., 1997; Tassaneeyakul et al., 1993)
Inducing drugs			
(es)omeprazol	8	CYP1A2	(Rost et al., 1992)
rifampicin	7	CYP2C9, 2C19, 3A4	(Sousa et al., 2008)

Table 4: **Pearson's correlation between the CYP isoforms**.

A positive correlation between the ln(activities) of all six CYP isoforms was observed.

	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP2E1
CYP2C9	0.756				
CYP2C19	0.581	0.768			
CYP2D6	0.693	0.571	0.603		
CYP2E1	0.846	0.794	0.755	0.738	
CYP3A4	0.594	0.648	0.740	0.594	0.682

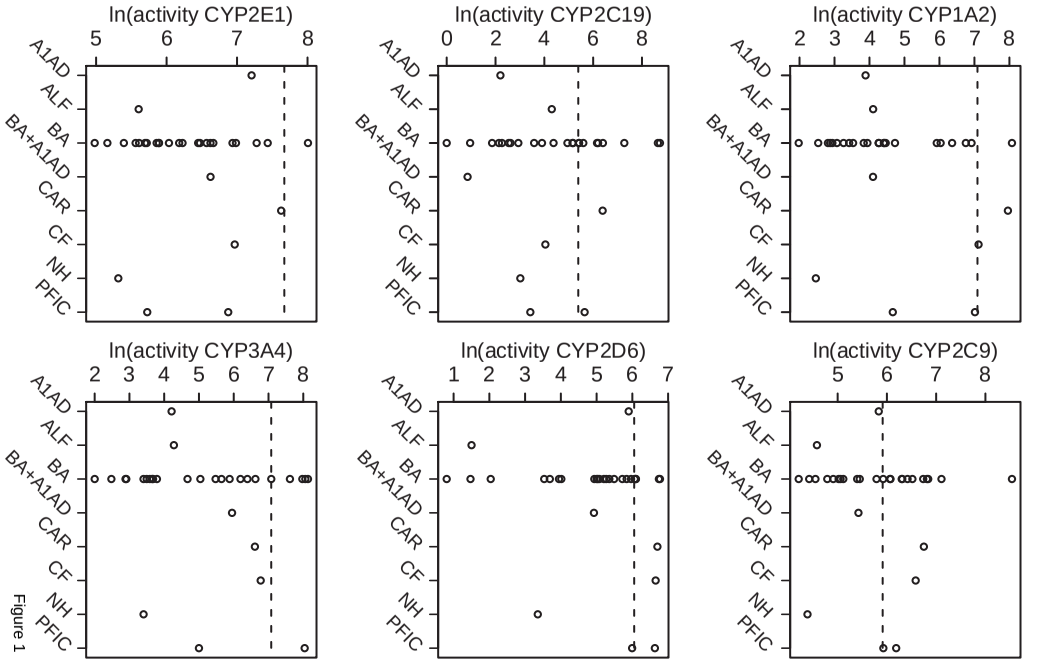

ln(activity CYP2E1)
ln(activity CYP2C19)
ln(activity CYP1A2)
ln(activity CYP3A4)
ln(activity CYP2D6)
ln(activity CYP2C9)
A1AD
ALF
BA
BA+A1AD
CAR
CF
NH
PFIC

Figure 1

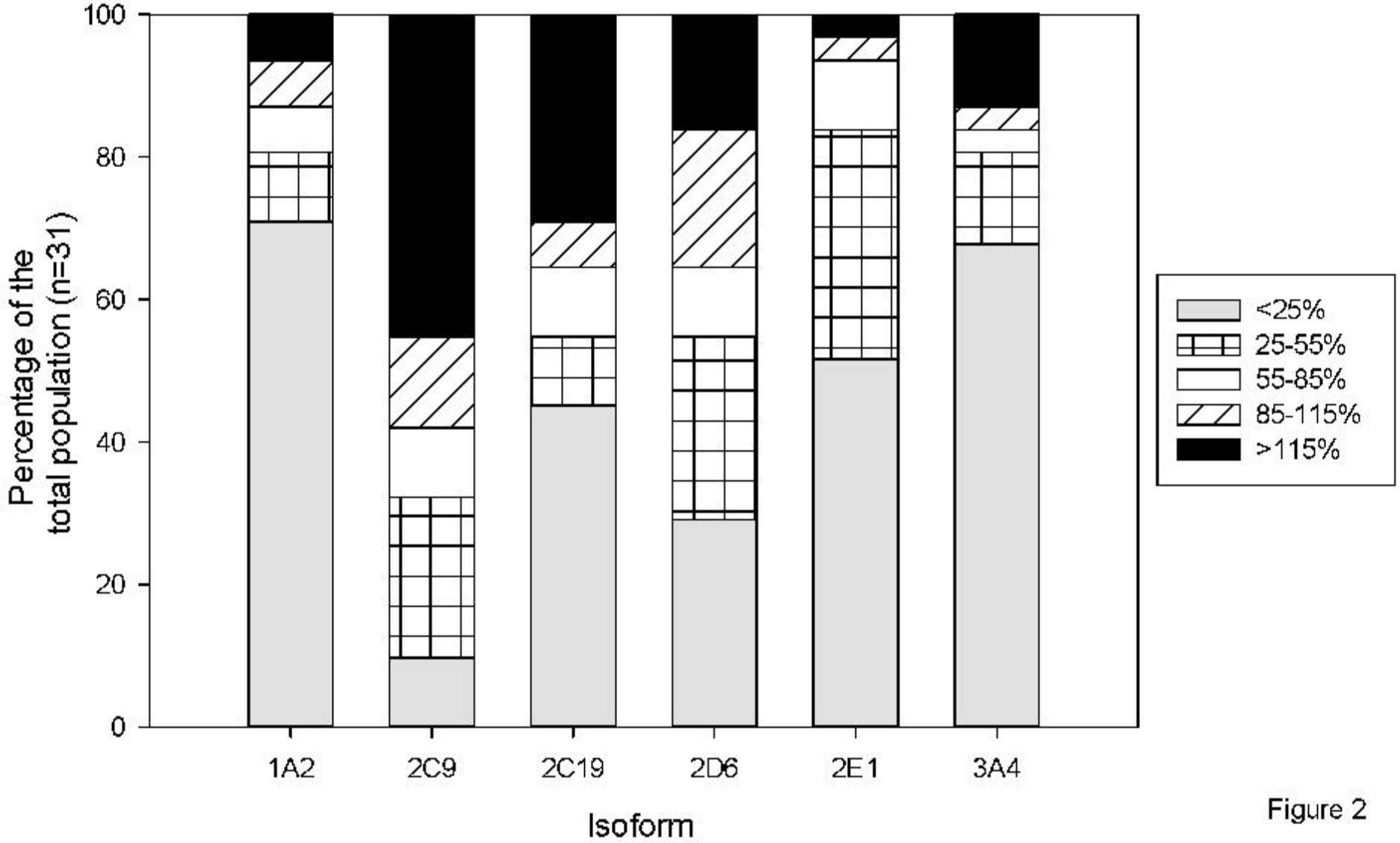

100
80
60
40
20
0
Percentage of the total population (n=31)
1A2
2C9
2C19
2D6
2E1
3A4
Isoform
<25%
25-55%
55-85%
85-115%
>115%

Figure 2

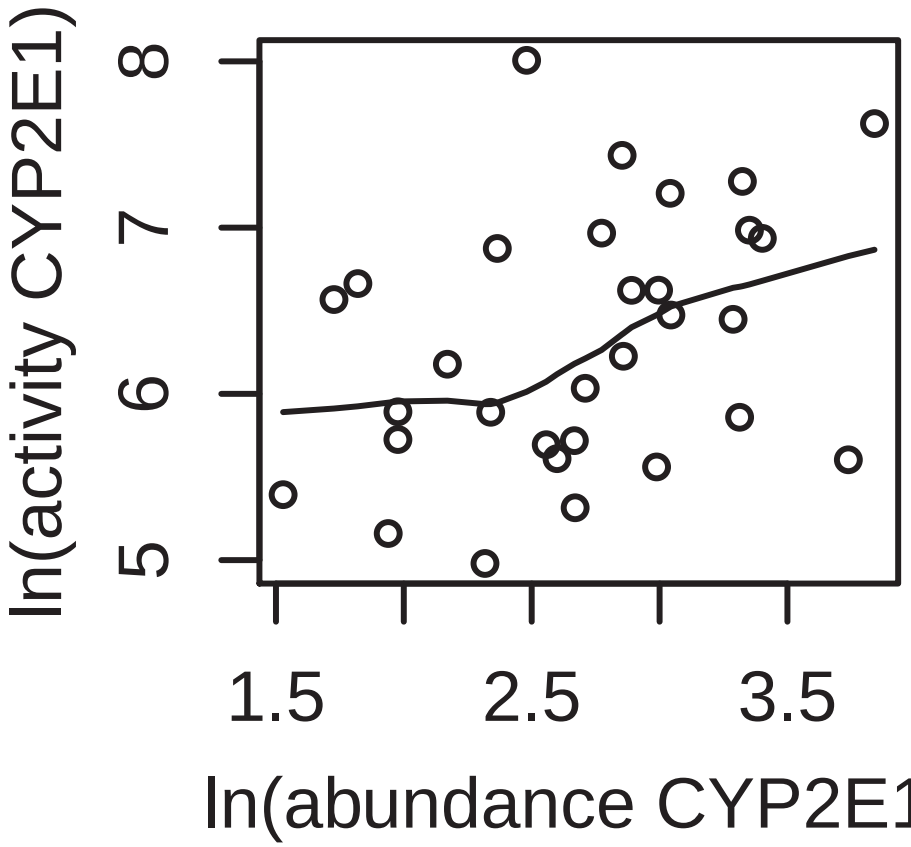
ln(activity CYP1A2)
ln(activity CYP2C9)
ln(activity CYP2C19)
ln(activity CYP3A4)
ln(activity CYP2D6)
inh
no comed
inh + ind
ind
*

Figure 3

Figure 4

ln(activity CYP2D6)
1 2 3 4 5 6 7
*4/*4
wt/*4
wt/wt
ln(activity CYP2D6)
1 2 3 4 5 6 7
*10/*10
wt/*10
wt/wt
ln(activity CYP2D6)
1 2 3 4 5 6 7
wt/*41
wt/wt
ln(activity CYP2C9)
5 6 7 8
wt/*2
wt/wt
ln(activity CYP2C9)
5 6 7 8
wt/*3
wt/wt
ln(activity CYP2C19)
0 2 4 6 8
*2/*2
wt/*2
wt/wt
ln(activity CYP2C19)
0 2 4 6 8
wt/wt
wt/*17
*17/*17

Figure 5


ln(activity CYP2E1)
5
6
7
8
1.5
2.5
3.5
ln(abundance CYP2E1)

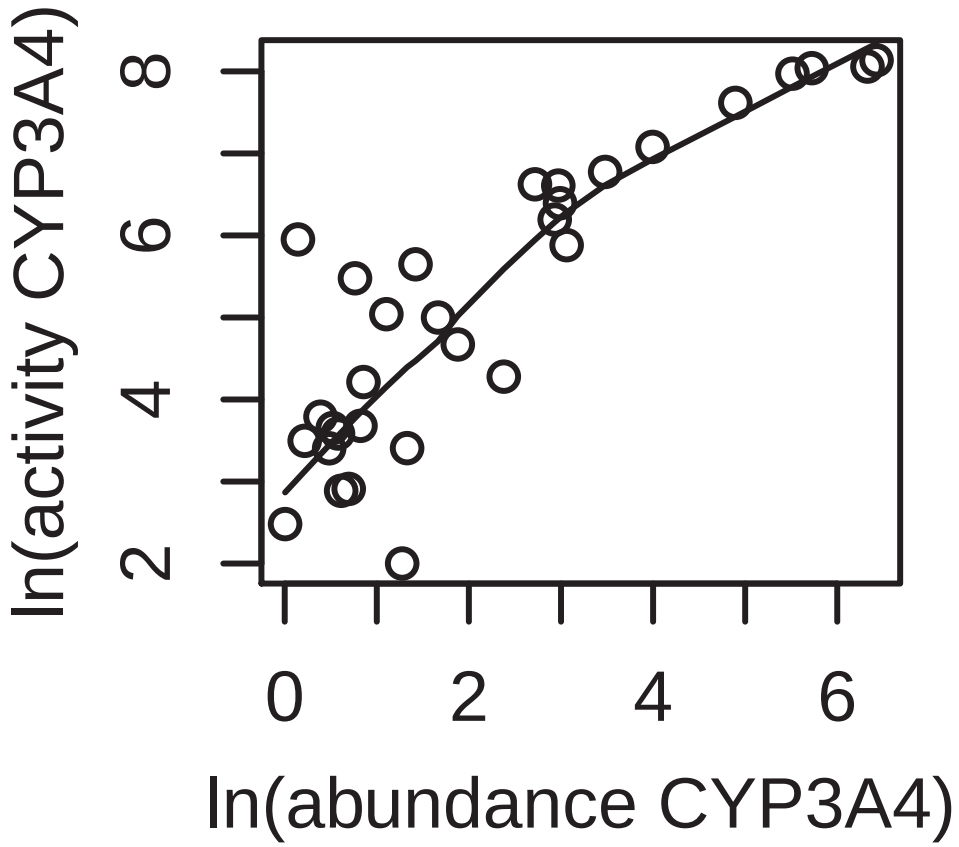

ln(activity CYP3A4)
2
4
6
8
0
2
4
6
ln(abundance CYP3A4)

Figure 6

ln(activity CYP1A2)
PELD
ln(activity CYP2C9)
PELD
ln(activity CYP2C19)
PELD
ln(activity CYP2D6)
PELD
ln(activity CYP2E1)
PELD
ln(activity CYP3A4)
PELD

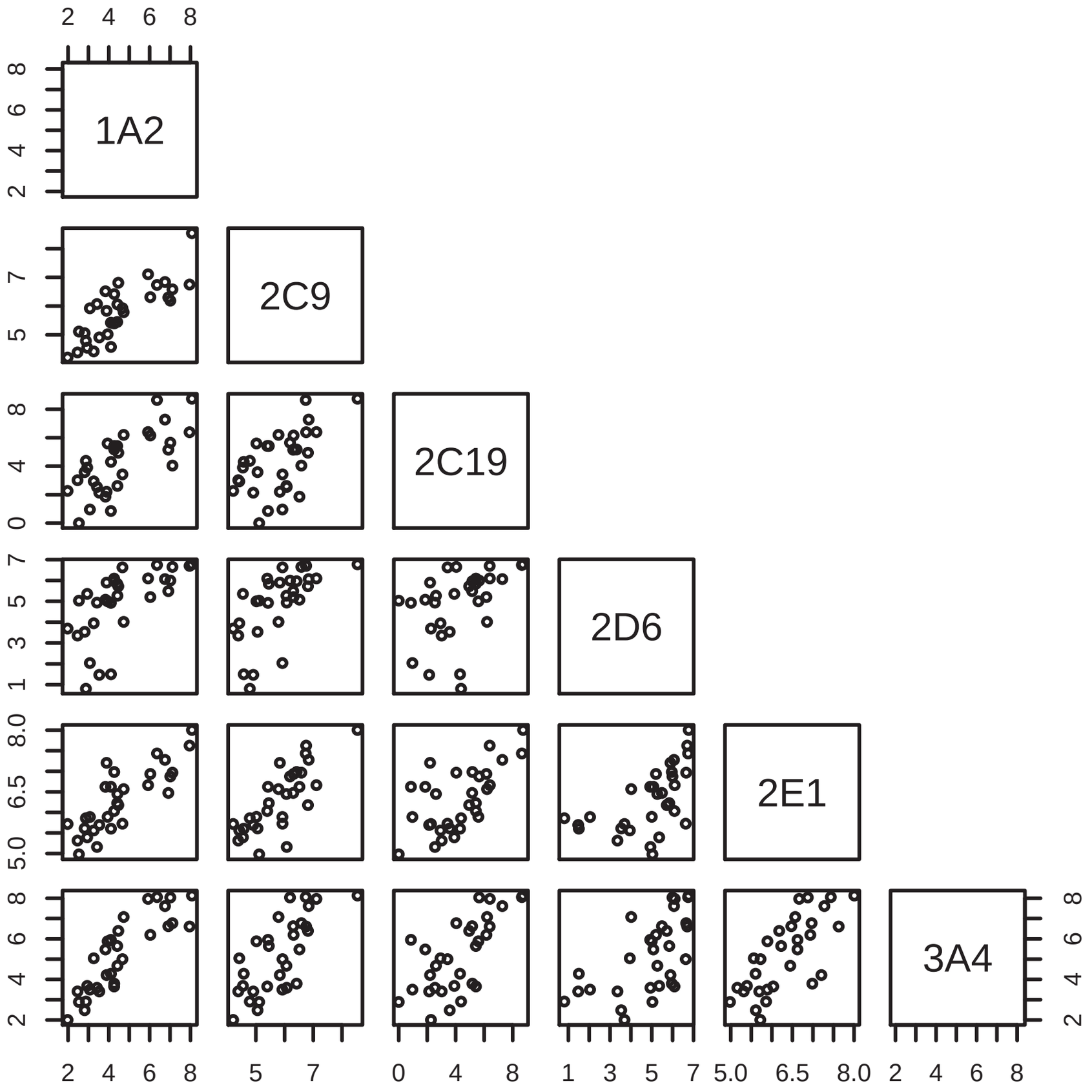


Figure 7