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New perspectives on the impact of cytochrome P450 3A expression for pediatric pharmacology

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Advances in the basic and clinical sciences of drug actions and safety have been applied almost exclusively to the largest demographic patient group – adults. Metabolism-dependent drug clearance is not only a primary determinant for obtaining efficacious drug exposure, but could also demonstrate clear age-dependence. These concepts are exemplified by the three major human cytochrome P450 (CYP) 3A enzymes: CYP3A4, CYP3A5 and CYP3A7. Recent preclinical and clinical studies show CYP3A7 is the most abundant CYP3A enzyme in fetal liver, with a gradual shift towards CYP3A4 expression throughout childhood. However, the polymorphic nature and regulatory intricacies of CYP3A5 and CYP3A7 expression could cause an underappreciated contribution to interindividual variability in drug response and safety.

Over forty years ago, the lack of information on drug safety and efficacy in the pediatric population, coupled with the numerous obstacles and scientific uncertainties when testing drugs for pediatric use, led a prominent pediatrician to categorize infants and children as 'therapeutic orphans' [1]. However, in the past decade, the convergence of two factors has led to progress regarding practical drug-dosage guidelines and better definitions of the pharmacogenetic contributions directed at age-related differences in drug response. First, the FDA Modernization Act (1997) and the Best Pharmaceuticals for Children Act (2002) have established the clinical trial priorities and manufacturers' incentives for the evaluation of generic and branded drugs for pediatric use [2]. Whether measured by the number of completed clinical trials or the expanded labeling of drugs for pediatric populations, these initiatives have produced a dramatic improvement in the art of pediatric clinical pharmacology (statistics available at www.fda.gov). The second factor is the development of the field of pharmacogenetics and its application in medical practice [3]. Although the sequencing of the human genome was not completed until 2003, this milestone was predated by several significant advances, translating the genetic basis for disease (or drug response) to pharmacology. Examples from the field of clinical oncology include the work of Weinshilboum [4], linking a genetic polymorphism of the thiopurine methyltransferase gene to clinically significant variations in the efficacy and toxicity of thiopurine drugs, such as 6-mercaptopurine. Genetic variations have also been exploited as targets for drug therapy, as in the example of women who have mutations in the gene for an epidermal growth factor receptor (HER2) who are at increased risk of developing aggressive forms of breast cancer [5]. Fortunately, these patients have shown encouraging responses to treatment with a monoclonal antibody that targets this same receptor (trastuzumab, Herceptin®) [6]. Together, these advances allow for a greater understanding of the roles of pharmacogenetics, environment and age-related changes in pediatric pharmacology.

Variability in drug response could have pharmacokinetic and/or pharmacodynamic determinants. For the purpose of this article, only the actions of the body effecting drug absorption, metabolism and excretion (pharmacokinetics) will be considered. Several reviews have closely examined the practice of establishing a pediatric drug dosage by starting with the recommended adult dosage and then scaling it down, in view of the physiological changes with pharmacokinetic processes [7,8]. Analyzing pharmacokinetic parameters for 45 common drugs showed that the half-lives of the drugs were generally 3–9 times longer in neonates, compared with half-lives in adults; however, there are exceptions where drugs in infants actually have shorter half-lives. In addition, this range was found to: exceed that which could be attributed to the normal interindividual variability of biological processes; not

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be influenced by parameters such as body composition and protein binding that can alter drug half-lives; be significantly larger for neonates with drugs primarily cleared by oxidative metabolism [7].

General considerations of cytochrome-P450-dependent metabolism

As the primary catalyst of metabolism-based drug clearance, the P450 enzyme family has been extensively studied from structural, functional and regulatory perspectives. Although there are many P450 gene families distributed throughout the human body, gene families numbered 1, 2 and 3 provide the majority of P450s involved in therapeutic drug metabolism. In addition, these enzymes are located predominantly in the liver and intestine, and have a broad range of substrates; attributes that create a significant barrier to the uptake of orally administered drugs before subsequent distribution to the systemic circulation. Given that the human cytochrome P450 (CYP) 3A subfamily comprises the majority of the total CYP content in these organs and has been found to be responsible for the metabolism of approximately one-half of the drugs prescribed today [9,10], understanding the activity, regulation and developmental changes in the expression of individual CYP3A forms is crucial for improving drug dosing – leading to optimal safety and efficacy in children.

There are four functional isoforms in the CYP3A subfamily (CYP3A4, CYP3A5, CYP3A7 and CYP3A43), and the corresponding genes for these subfamilies are located on chromosome 7 [11,12]. CYP3A43 is only expressed at very low levels in the liver, and therefore does not contribute to total CYP3A activity [13]. Although the identification of the CYP3A4, CYP3A5, and CYP3A7 forms is unequivocal from a gene-sequence perspective, differentiating between (and characterizing) these enzymes at the protein and/or functional level has been plagued by technical problems for the past 20 years [14–16]. More specifically, available antibodies have, historically, been unable to distinguish between individual CYP3A forms or were mistakenly identified as 'specific' because of the inability to separate the highly related proteins by conventional electrophoretic techniques [17]. Thus, conclusions on tissue CYP3A content determined by immunochemistry, particularly in fetal and neonatal tissue, have not been able to be linked to individual CYP3A forms and, instead, have had to refer to 'total' CYP3A content [18]. Similarly, substrate probes and chemical inhibitors are largely form-selective rather than specific, leaving the most definitive and reliable activity determinations for CYP3A forms to studies using expressed enzyme that originated from the specific CYP3A gene. Even here, however, caution in experimental design and data interpretation is required. The *in vitro* determination of CYP3A-dependent enzyme activity, whether intended for induction–evaluation analysis, drug-interaction assessment or kinetic analysis, must also consider potential complications caused by the phenomena of protein and/or substrate interactions leading to atypical kinetics [19]. Several groups have invoked the presence of multiple substrate binding domains as the reason for CYP3A4 activation [13,20,21], and these results have been used to derive comparative pharmacophore models of the CYP3A enzymes [22]. This field continues to pose a conundrum for the drug industry regarding the pursuit of accurate predictions of CYP3A drug interactions, and is the subject of intense research by leading protein crystallographers and biochemists.

Technical problems have also been encountered with attempts to differentiate among CYP3A forms *in vivo*. Because of the importance of validating *in vitro* methods with *in vivo* data, and the limitations of obtaining human biopsy tissue for determinations of CYP3A enzyme activity or protein levels, several laboratories have pursued the design and use of CYP3A form-selective probes to assess enzyme activity *in vivo* [23,24]. Among the complications of this approach are: the influence of hepatic versus intestinal CYP3A activity; the resultant sensitivity to the route of probe-drug administration; the interference caused by the co-located drug transporter P-glycoprotein [25,26]. *In vivo* CYP3A phenotyping has also been useful for evaluating the ability of a drug or a xenobiotic to produce enzyme inhibition or induction. The finding that the antifungal drug ketoconazole can act as a general CYP3A inhibitor and dramatically alter the pharmacokinetic profile of a co-administered CYP3A substrate has now been widely recognized and adopted by pharmaceutical companies as a standard clinical drug–drug interaction protocol [27]. For analysis of induction, tools such as the ^{14}C-erythromycin breath test or midazolam clearance have been successful in linking the ingestion of drugs and nutraceuticals, such as rifampin or St John's Wort, to the increased CYP3A-dependent metabolism of drugs such as cyclosporine and antivirals [28,29].

Certainly a noninvasive probe of CYP3A activity, or a suitable drug with an established safety record that could be given at a subpharmacological dose, would be ideal for pediatric studies. The latter criterion has been met and routinely applied in clinical practice for the polymorphically expressed CYP2D6. Low doses of dextromethorphan, an over-the-counter cough syrup ingredient, can be given to infants or children and the urinary levels of the CYP2D6-dependent O-demethyl metabolite can be determined from diaper urine samples [30]. For CYP3A, the search for a noninvasive probe has largely centered on the CYP-dependent metabolism of the endogenous steroid cortisol (reviewed in [31]). The fact that CYP3A4 and CYP3A5 were the primary catalysts of 6β-hydroxycortisol formation was demonstrated by *in vivo* studies showing that changes in the urinary ratio of metabolite:parent were in accord with the administration of known CYP3A inducers. However, this assay has not been found to correlate with hepatic CYP3A4 enzyme levels, a finding that might be related to the expression of CYP3A5 in the kidney or the diurnal and stress-related changes in endogenous cortisol production [32]. As discussed later in this review, in neonates the utility of *in vivo* probes for assessing changes in the developmental expression of individual CYP3A forms is limited by logistical and ethical issues; and in older children by the dominance of CYP3A4 levels and the resultant lack of sensitivity of these probes to CYP3A5 and CYP3A7.

Current understanding of CYP3A expression

One approach aimed at understanding the genetic and developmental dependence of individual CYP3A forms is considering cases where the technical advances (previously discussed) have dramatically changed or gradually reinforced certain hypotheses and conclusions relating to the regulation and function of individual CYP3A forms. For example, CYP3A4 has historically and accurately been described as the major adult-human hepatic CYP3A form, although early reports probably overestimated

CYP3A4 content because of antibody cross-reactivity with other CYP3A forms. As previously alluded to, there is clear evidence that at least some fraction of the interindividual variability of CYP3A4 levels in adults can be linked to differences in dietary components and drug administration (reviewed by [33,34]). In addition, despite speculation that the large interindividual variability in CYP3A4 protein levels and/or activity has a genetic basis that is traceable to the coding region [35], to date, there are no clear data supporting this theory. CYP3A4-inducible expression is mediated by the pregnane X receptor (PXR) and/or the steroid and xenobiotic receptor (SXR), however PXR knockout mice show similar constitutive hepatic CYP3A levels to those in the wild-type (wt) [36]. However, with respect to fetal regulation of CYP3A4 and CYP3A7, Matsunaga *et al.* [37] found that induction was mediated largely by the glucocorticoid receptor, rather than by PXR. Regarding the constitutive expression of CYP3A4, there is an increasing weight of data that implicate regulatory polymorphisms translated via transcription factors that control hepatic and intestinal CYP3A4 expression [38]. It is also worth noting that there are clinical and/or preclinical data that suggest changes in endogenous factors related to maturation (such as growth hormone, triiodothyronine or 1,25-dihydroxy vitamin D) could play a role in constitutive CYP3A4 expression (reviewed in [39]). Clearly, this topic has important implications, because it would be logical to assume that knowledge of the birth-associated chemical mediators and biochemical events that initiate the increased expression of CYP3A4 could indicate which transcription factors are important for determining constitutive enzyme expression.

A frequent generalization in human CYP3A literature is that there is exclusive expression of CYP3A4 in adults and CYP3A7 in the fetus, and the associated change in phenotype occurs in the first few weeks after birth. Again, various limitations in historical experimental approaches have contributed to a concept that lacks detail and a true understanding of more-recently described exceptions. For example, the small number of fetal or pediatric liver samples studied has resulted in either a wide-range of age categories or a lack of statistical analysis [18,40,41]. In addition, the compromised quality of tissue samples, caused by extended postmortem intervals, has been a confounding factor in the measurement of enzyme activity and mRNA levels. However, more-recent data have been generated using substantially larger numbers of fetal and pediatric liver samples, procured under defined acceptance criteria and carefully assessed to ensure biological quality [42–44]. Finally, these investigators applied novel methodologies to these improved sample sets to distinguish between CYP3A4 and CYP3A7.

The results of these studies indicate that fetal CYP3A7 protein levels are higher than all previously published estimates, and account for the majority of total hepatic P450 content [42–44]. Specifically, Leeder *et al.* [44] found average levels of 235 pmol mg^{-1} CYP3A7 and CYP3A4 per milligram of microsomal protein in 51 fetal liver samples. These conclusions on protein expression are supported by the finding of CYP3A7 mRNA levels that were 1000-fold higher than corresponding CYP3A4 mRNA levels, and that 49 out of 51 samples showed testosterone hydroxylase activity patterns that were consistent with exclusive CYP3A7 expression. Studies from the author's work utilized ratios of dehydroepiandrosterone metabolites generated by expressed CYP3A4 and CYP3A7 and fitted to a multiple regression model

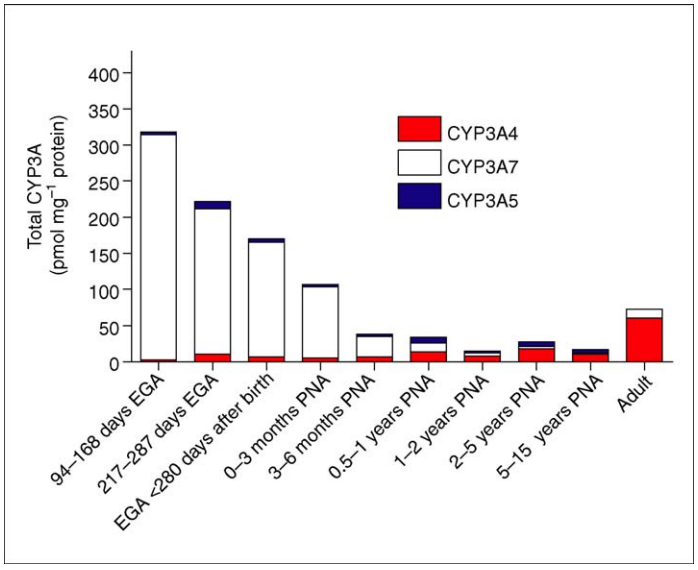

FIGURE 1

Levels of cytochrome P450 (CYP) 3A4, CYP3A5, and CYP3A7 protein as a function of age. CYP3A5 protein levels (blue bars) were determined by immunoquantitation. CYP3A4 (red bars) and CYP3A7 (open bars) protein levels were determined from the relative amounts of hydroxylated dehydroepiandrosterone (DHEA) metabolites generated from microsomal incubations and subsequent calculations by multivariate regression analysis (reproduced with author and journal permission from Ref. [43]). Abbreviations: EGA, estimated gestational age; PNA, postnatal age.

to calculate protein levels in 77 fetal and pediatric liver samples [43] (Figure 1). For CYP3A7 expression, the first major finding identified average CYP3A7 levels of 311 and 201 pmol mg^{-1} for the second- and third-trimester fetal samples, respectively. These levels are 2–3-fold higher than previous estimates [18] and, although total P450 content was not determined in these samples, strongly suggest that CYP3A7 represents the major component of fetal hepatic P450 content. In addition, CYP3A7 content fell throughout gestation and during the first year after birth, but remained greater than the average CYP3A4 levels until at least six months postnatal. Thus, CYP3A4 levels appear to be slower to develop in children than previously reported [45]. Liver samples taken from the 5–15 year age-group had, on average, CYP3A4 levels that were only 25% of the average CYP3A4 value obtained from the adult samples. An important (and admittedly provocative) question raised by these data is whether the physiological and hormonal changes of adolescence trigger a phase of increased CYP3A4 expression that has been largely overshadowed by the focus on CYP3A4 changes associated with parturition.

The unequivocal demonstration of significant hepatic CYP3A7 expression in a portion of the adult population is also a relatively recent finding [42,46]. Interethnic differences in the frequency of expression of adult CYP3A7 are likely, however the report by Sim *et al.* [42] estimates the frequency to be ~10% in the Caucasian population. For this population, hepatic CYP3A7 protein expression ranges from ~20–100% of CYP3A4 levels in the same liver sample [42,43]. CYP3A7 protein expression in adults can be attributed, at least in part, to the frequency of the CYP3A7*1C allele – where a mutation has caused a portion of the CYP3A4 promoter to be incorporated into the corresponding CYP3A7 promoter [47]. Whether this sequence-swap has direct implications for birth-

associated change in expression of CYP3A4 and CYP3A7 in the majority of individuals remains to be studied. As for the implications of CYP3A7 expression in drug metabolism, to date, the limited studies have found that this enzyme is less prone to inhibition (i.e. higher IC_{50} values are seen) and is often significantly less efficient than CYP3A4 (higher K_m and/or lower V_{max}) at metabolizing the same substrate [22,48]. There is, by comparison, more-compelling data for the role of CYP3A7 in fetal steroid metabolism and homeostasis, based on the specific and efficient hydroxylation of the estradiol precursor dehydroepiandrosterone [49]. Given the ethical and logistical limitations that all but preclude a detailed study (aimed at understanding the expression of CYP3A4 and CYP3A7) of clinical drug metabolism in infants, a subpopulation of adults can serve as scientific surrogates for children. That is, closely examining adult clinical trial participants for adverse drug responses that might be associated with CYP3A7 genotype(s) could provide a path forward for understanding CYP3A expression in children.

In contrast to the dramatic developmental shift observed in the expression of CYP3A4 and CYP3A7, more-recent data suggest that genotype plays a bigger role in age-related CYP3A5 expression [43,47]. From a historical perspective, the initial characterization of the CYP3A5 protein was soon followed by data suggesting that this protein could be expressed at higher levels in children compared with levels seen in adults [50]. Once again, low sample numbers (n = 17 samples from 0–19 years), necessitating broad age categories, negated additional statistical analyses. Over the past decade, several laboratories have described the expression of hepatic CYP3A5 to be measurable in 25–40% of the population [51,52]. Significant extrahepatic levels of CYP3A5 have also been documented, with the enzyme contributing to the clearance of drugs during intestinal absorption (first-pass metabolism), or in association with renal drug-excretion [51,53]. Interindividual differences in CYP3A5 expression have been traced to a single nucleotide polymorphism in intron 3, designated CYP3A5*3 [47]. This mutation creates a consensus splice site, resulting in a small amount of mRNA that is properly spliced and a larger amount of improperly spliced mRNA that encodes a truncated protein. Thus, individuals that are homozygous for the CYP3A5*1 allele generally express high levels of protein, heterozygotes (CYP3A5*1 and CYP3A5*3) express lesser amounts and CYP3A5*3 homozygotes express little or no enzyme.

For individuals with at least a single CYP3A5*1 allele, hepatic protein levels of CYP3A5 can equal those of CYP3A4 [54]. This work, by Lin *et al.* [54] (Figure 2), illustrates CYP3A5 genotypes and phenotypes across a set of Caucasian liver samples (largely adult, with a median donor age of 46 years). Allele frequency also shows a dramatic ethnic component regarding frequencies for CYP3A5*1: ~15% for Caucasians; 20–30% for Asians; and ~45% for African Americans [47], and genotype generally corresponds to phenotype – as measured by the metabolism of midazolam *in vitro*. Studies have also indicated co-regulation between CYP3A4 and CYP3A5, in that the variability of hepatic CYP3A5 protein correlated with CYP3A4 protein levels in individuals genotyped as CYP3A5*1 and CYP3A5*3 heterozygotes [54]. These data, combined with the study by Burk *et al.* [55] demonstrating the potential for inducing CYP3A5 by either the PXR or constitutive androstane receptor (CAR), support the CYP3A argument for co-regulation. Induction can also explain the large variability in CYP3A5 expression among heterozygotes, and a potential for 'phenotype masking' of the CYP3A5*1 allele.

Studies on the developmental expression of CYP3A5 are very limited but they do suggest that variability is largely independent of age. Hakkola *et al.* [16] were able to identify CYP3A5 protein in a small percentage of fetal liver samples, however the analysis was very limited ($n = 9$ samples) and gestational status and age ranges were not reported. Our recent analysis of 212 fetal and pediatric liver samples found that CYP3A5 protein levels were highly variable, but generally independent of age. Advantages with this analysis were a broad developmental and ethnic range for the sample donors and a highly sensitive immunodetection method (sensitive to 0.1 pmol mg^{-1} of protein). However, the disadvantage was the lack of genotype data to confirm the presence of the CYP3A5*3 allele in the 46% of samples where protein could not be detected or used to assess interindividual variability in protein levels for individuals with the same genotype. Finally, a trend toward higher CYP3A5 protein levels in African American donors compared with Caucasian donors was observed, consistent with the ethnic influence observed for adults [47,56].

Examples of clinical impact

Although the full spectrum of clinical studies addressing CYP3A ontogeny is beyond the scope of this review, several examples highlight the complexity of translating genotype and *in vitro* analyses into patient care. One of the most extensively investigated topics is the metabolic clearance of midazolam (a short-acting benzodiazepine) by neonates and children compared with adults. The plasma clearance of midazolam follows the pattern: adults > full-term neonates > pre-term neonates [57]. Given the limited involvement of CYP3A7 in midazolam metabolism, its developmental pattern appears to follow the developmental pattern of CYP3A4. However, at least one report suggests that, like the *in vitro* analysis of CYP3A4 expression depicted in Figure 1, infants do not necessarily display a rapid increase in midazolam clearance during the first weeks and months postpartum [58]. The role of CYP3A5 might also need to be considered, because research on the interaction between different CYP3As in the intestine and liver has led one group to the conclusion that individuals with at least one CYP3A5*1 allele will metabolize midazolam more rapidly than CYP3A5*3 homozygotes [54].

Cisapride, a treatment for gastroesophogeal reflux disease, provided another example of clinical pediatric investigations with CYP3A involvement. Cisapride is primarily cleared by CYP3A4 metabolism [59] and, given the link between plasma drug-levels and serious cardiac arrhythmia (QTc elongation), an understanding of CYP3A4 development and the implications for drug dosing are crucial for achieving efficacy without toxicity. Kearns *et al.* [60] found that the metabolic clearance of cisapride was developmentally dependent in neonates and infants, and that postconceptional age correlated with drug elimination. A final example is the use of HIV protease inhibitors as standard therapy for infants born to infected mothers [61,62]. Given the potential of a wide-range of drugs for the induction or inhibition of fetal CYP3A forms based on maternal ingestion, and that combinations or cocktails of protease inhibitors could be used for infants, the complexity of this issue is beyond the scope of this review. It should be noted, however, that the topics discussed in this article on the subject of

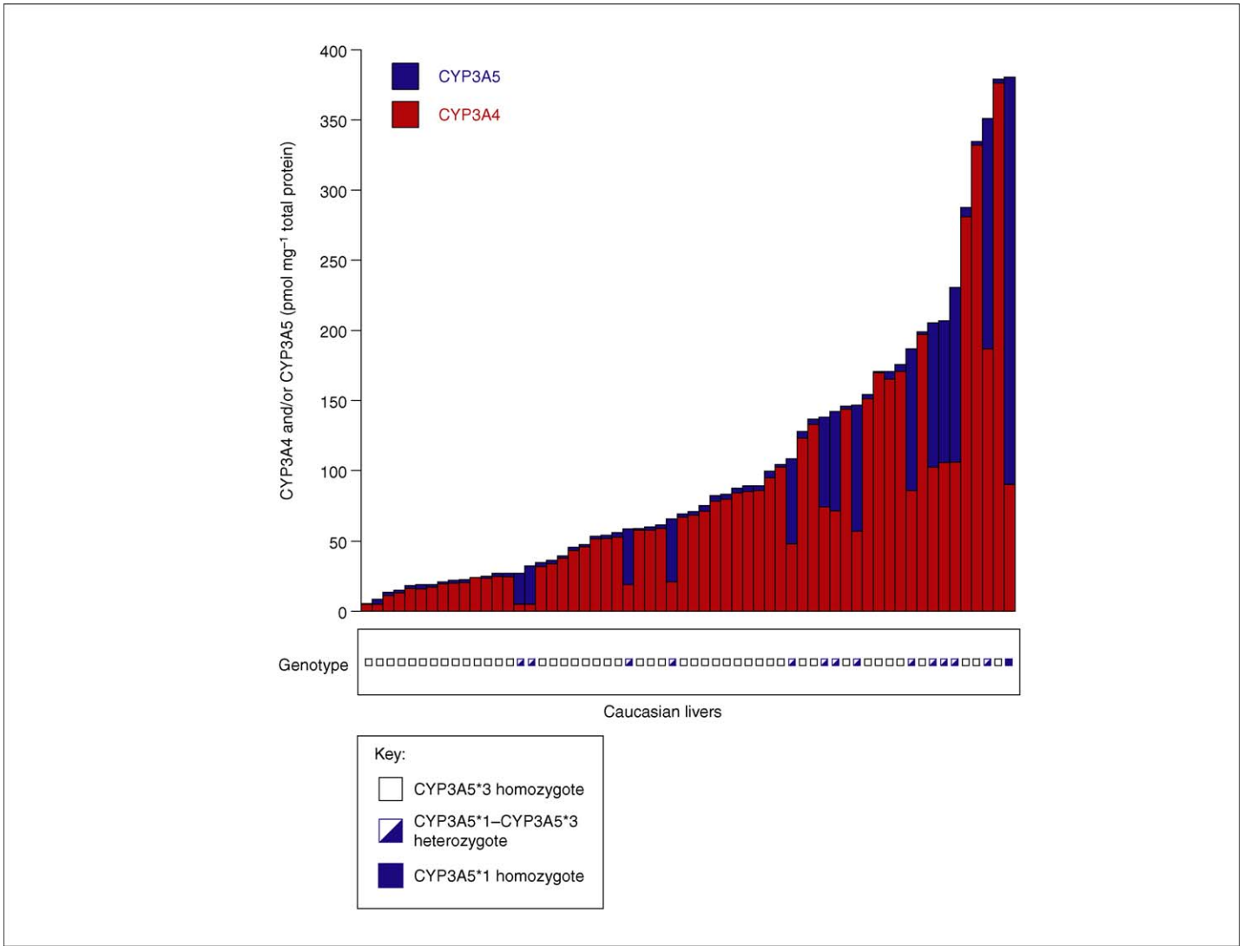

FIGURE 2

Cytochrome P450 (CYP) 3A5 genotype and CYP3A4 and CYP3A5 protein content as a fraction of total CYP3A in livers. All liver samples that were analyzed in this experiment were from Caucasian donors (reproduced with author and journal permission, Ref. [54]).

developmental changes in CYP3A7 and CYP3A5 genotypes have clearly been advanced from basic research to clinical trials for better antiviral therapies [63].

Conclusions and future directions

In the past five years alone, important advances have been made in understanding the contribution of genetics and development with regard to interindividual variability in drug metabolism. Although this review specifically focuses on the CYP3A subfamily, similar ontogeny analyses for other P450s and other drug-metabolizing enzyme families have recently been published (or are in progress). Because this information has coincided with the emergence of new requirements regarding pediatric drug labeling, a logical direction for future studies has become apparent. The clinical application of pharmacogenetics in deciphering the true influence of age in drug metabolism in children should provide better detail for the practice of pediatric pharmacology and, thus, help to eliminate the labeling of children as 'therapeutic orphans'.

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