were detected in six samples (data not shown). These results indicate that at least a portion of fetal RNA in maternal plasma exists in the cell-free form. This observation is consistent with the recent finding that tumor-derived RNA can be detected in the circulation of cancer patients *(11, 12)*.

Our data demonstrate that fetal RNA can be detected in maternal plasma. The detection rates of plasma fetal RNA in early and late pregnancies were 22% and 63%, respectively. The detection rate of fetal RNA in early pregnancy cases was lower than that in late pregnancy cases, suggesting that the concentration of plasma fetal RNA is lower in early pregnancy. This observation is similar to our previous finding that the concentration of fetal DNA in maternal plasma increases with gestation *(13)*. We also realized that the detection rate of plasma fetal RNA in this study is lower than that of plasma fetal DNA *(1)*. It is possible that fetal RNA is more susceptible to degradation in maternal blood. As a result, the amount of fetal RNA in plasma is much lower than plasma fetal DNA. This is supported by the fact that Y-specific DNA was detected in all plasma samples from women carrying male fetuses in this study (data not shown). To improve the sensitivity of maternal plasma fetal RNA detection, we are now developing a highly sensitive real-time quantitative RT-PCR assay for this purpose.

In conclusion, we have shown for the first time that fetal RNA can be detected in maternal plasma, and our data provide a novel means of noninvasive prenatal diagnosis. Plasma fetal DNA analysis can provide data on the presence and concentration of fetal genetic material in the maternal circulation. Plasma fetal RNA analysis, in addition, can provide valuable information regarding the gene expression patterns of fetal tissues. For example, abnormal pregnancies, such as those with preeclampsia, often are associated with abnormal gene expression patterns in fetal tissues *(14)*. Thus, with the development of further RNA markers, maternal plasma RNA analysis may allow the noninvasive monitoring of fetal gene expression in a multitude of physiological and pathological conditions.

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***CYP3A4-V* Polymorphism Detection by PCR-Restriction Fragment Length Polymorphism Analysis and Its Allelic Frequency among 199 Dutch Caucasians,** *Ron H.N. van Schaik,[1*] Saskia N. de Wildt,[2] Nienke M. van Iperen,[2] Andre G. Uitterlinden,[3,4] John N. van den Anker,[2] and Jan Lindemans[1]* (Departments of [1] Clinical Chemistry, [2] Pediatrics, [3] Internal Medicine, and [4] Epidemiology & Biostatistics, University Hospital Rotterdam, PO Box 2040, 3000 CA Rotterdam, The Netherlands; * author for correspondence: fax 31-10-4367894, e-mail vanschaik@ckcl.azr.nl)

Interindividual variation in drug metabolism is a complicating factor in pharmacotherapy. Enzymes of the cytochrome P450 system are involved in the metabolism of several endogenous substrates and a broad range of foreign compounds, such as drugs, environmental pollutants, and carcinogens *(1)*. The cytochrome P450 enzyme family consists of several subfamilies, with CYP3A4 being the most abundant P450 enzyme in human liver *(2)*. CYP3A4 is involved in the metabolism of >50% of all drugs used in humans *(3, 4)*. Interindividual differences in CYP3A4 expression may account for the observed interindividual differences in pharmacokinetics of drugs metabolized by this enzyme *(4–6)*. Variations in CYP3A4 expression may be caused by factors inhibiting or stimulating transcription and/or translation (e.g., concomitant drug administration) and by genetic polymorphisms.

In a recent study, an A(−290)G substitution was described in the 5′ regulatory region of the *CYP3A4* gene *(7, 8)*. This allele was termed *CYP3A4-V* but was recently also referred to as *CYP3A4*1B (9)*. The allelic frequency, determined by conformation-sensitive gel electrophoresis,

was estimated as 9.6% among US Caucasians *(7)*. In 94 healthy unrelated male volunteers, 3.2% were found to be homozygous and 12.8% heterozygous for the *CYP3A4-V* allele *(7)*. In a recent publication, the allelic frequency was shown to display major interethnic differences, ranging from 0.0% among Chinese-Americans to 54.6% among black Americans *(10, 11)*. The *CYP3A4-V* allele has recently been associated with higher clinical stage and grade of prostate cancer *(11–13)*.

In this study, a simple and specific PCR-restriction fragment length polymorphism (RFLP) procedure was developed that can be used in routine screening procedures to detect the *CYP3A4-V* polymorphism. EDTA-whole blood was collected from 199 healthy Caucasian volunteers after informed consent. The study was approved by the Medical Ethical Committee of the University Hospital Rotterdam. Genomic DNA was isolated from 300 μL of blood, using the GenomicPrep Blood DNA Isolation reagent set (Amersham Pharmacia Biotech). DNA yields were estimated by measuring the absorbance at 260 nm (A_{260}). A total of ~50 ng of genomic DNA was used in a PCR volume of 50 μL. The PCR mixture contained 1× buffer (10 mmol/L Tris-HCl, pH 8.3, 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 0.01 g/L gelatin; Perkin-Elmer), 0.2 mmol/L each dNTP (Roche), 1.25 U of AmpliTaq Gold (Perkin-Elmer), and 40 pmol each of forward primer [5′-GGA CAG CCA TAG AGA CAA <u>CT</u>G CA-3′; positions −315 to −291 *(8)*] and reverse primer [5′-CTT TCC TGC CCT GCA CAG-3′; positions +22 to +5 *(8)*]. The underlined nucleotides are mismatches with the *CYP3A4* sequence, creating a *Pst*I restriction site in the *CYP3A4-V* allele PCR product. The 3′ end of the forward primer lies in the nifedipine-responsive element, a sequence that is characteristic for *CYP3A4* and is absent in the homologous *CYP3A7* sequence, ensuring specific amplification of *CYP3A4* *(8)*. The PCR conditions were as follows: 7 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C; and finally 7 min at 72 °C. A 334-bp fragment was amplified using the *CYP3A4-V* primers described (Fig. 1, lane 0). PCR product (15 μL) was digested with 10 U of *Pst*I (Roche) for 1.5 h at 37 °C and analyzed on a 3% agarose/Tris-borate-EDTA gel with ethidium bromide staining. The fragments produced were 220, 81, and 33 bp for the wild-type sequence (Fig. 1, lane wt/wt) and 199, 81, 33, and 21 bp for the *CYP3A4-V* allele (Fig. 1, lane V/V). Two internal *Pst*I sites in this fragment serve as an internal control for digestion. In heterozygous samples, both the 220- and the 199-bp fragments are present (Fig. 1, lane wt/V).

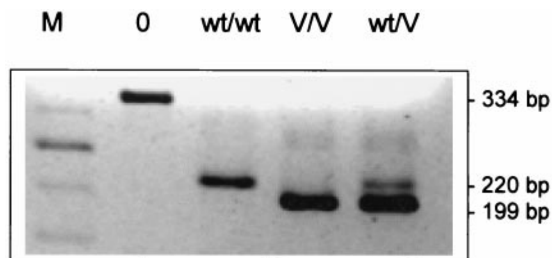

Fig. 1. PCR-RFLP for the *CYP3A4-V* allele.

Lane M, base pair marker (50-bp ladder); *lane 0*, undigested PCR product. *Lanes wt/wt*, *V/V*, and *wt/V*, *Pst*I-digested PCR products for wild-type, homozygous, and heterozygous *CYP3A4-V* DNA samples, respectively. The 81-, 33-, and 21-bp bands are not visible. Analysis on a 3% agarose/Tris-borate-EDTA gel. Figure is printed negative.

The DNA sequences of the nifedipine-responsive elements of these controls were verified by direct sequencing on an automated ABI 310 capillary sequencer (Perkin-Elmer), using the Big Dye Terminator Cycle Sequencing method (Perkin-Elmer) with forward primer 5′-AAC AGG GGT GGA AAC ACA AT-3′ and reverse primer 5′-CTT TCC TGC CCT GCA CAG-3′ *(7)*. The PCR profile was as follows: 7 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C; and finally, 7 min at 72 °C. Interestingly, in heterozygous samples, the 220-bp wild-type allele-derived band was usually less intense than the 199-bp *CYP3A4-V* allele-derived band. Because the only difference between the wild-type and the *CYP3A4-V* sequence is the A(−290)G substitution, we assume that the first base added by the Taq polymerase (an A for the wild-type sequence and a G for the *CYP3A4-V* sequence) increases the stability of the forward primer-genomic DNA complex for the *CYP3A4-V* allele more than for the wild-type sequence. We tried to minimize this effect by having a forward primer synthesized minus the 3′ A base, making the stability of the primer-DNA complex less dependent on the first base to be added by the Taq polymerase. PCR-RFLP with this alternative forward primer in combination with the described reverse primer yielded 220- and 199-bp bands of equal intensity (results not shown). However, the overall efficiency of the PCR reaction was dramatically decreased, probably because the two mismatches with the *CYP3A4* sequence were now too close to the 3′ end of the primer. Application of this alternative primer therefore needed 45 cycles of PCR and 25 μL of PCR product for digestion and subsequent detection on gel.

Recently, another PCR-RFLP procedure was described for detection of the *CYP3A4-V* allele, which is based on creating a *Mbo*II restriction site *(14)*. This procedure makes use of nested PCR, which is more laborious and has an increased risk of sample contamination. Moreover, the PCR products for the *CYP3A4-V* allele cannot be digested, which means that this assay lacks an important internal control on digestion efficiency.

We screened 199 unselected and unrelated healthy Caucasians for the *CYP3A4-V* allele, applying the described PCR-RFLP and using the wt/wt, wt/V, and V/V samples described above as controls. We identified 21 *CYP3A4-V* alleles, giving an allelic frequency of 5.3%. In total, 21 individuals were heterozygous for this allele, giving a heterozygote frequency of 10.6%. DNA sequencing of all heterozygous individuals confirmed the A(−290)G substitution in all cases; no additional mutations were detected in the nifedipine-responsive element. As a control, 10 wild-type samples were also sequenced. The observed allelic frequency was significantly different (P <0.05, Fisher exact test) from the reported allelic frequency of 9.6% (18 *CYP3A4-V* alleles in 94 individuals)

by Rebbeck et al. *(7)* for US Caucasians. In that specific study on 94 healthy volunteers, 3.2% appeared to be homozygous for this mutation. We did not find any *CYP3A4-V* homozygotes among the 199 individuals studied. The allele and genotype frequencies were in Hardy-Weinberg equilibrium ($P = 0.432$); the absence of homozygotes in our study population of 199 individuals is consistent with a Hardy-Weinberg distribution. In Swedish Caucasians, 3 *CYP3A4-V* alleles were found recently among 39 individuals studied, giving an allelic frequency of 3.8% *(15)*, whereas Sata et al. *(9)* reported an allelic frequency of 4.2% in 59 white subjects. These data are more in agreement with our results.

CYP3A4 is the most abundant form of the cytochrome P450 enzyme family present in human liver and is involved in the metabolism of many drugs *(3, 4, 16)*. The recently described A(−290)G genetic polymorphism in the 5′ regulatory region potentially alters the transcription efficiency and thus the overall enzymatic activity of CYP3A4. Initially, the effect of this mutation on transcription was believed to be a decrease, based on the clinical presentation of prostate cancer *(7)* and drug-induced leukemia *(12)*. Later experiments, in which protein expression and enzymatic activity in liver samples were compared, suggested that the *CYP3A4-V* mutation had no effect on transcription *(14)*. This was supported by experiments on the 6β-hydroxylation of testosterone in three microsomal liver samples from individuals heterozygous for the *CYP3A4-V* allele *(15)*, although this conclusion was later questioned by others *(17)*. Experiments in which the promoter region of *CYP3A4* was fused to the luciferase reporter gene, followed by expression of these constructs in HepG2 and MCF7 cells, indicated that the *CYP3A4-V* polymorphism increases *CYP3A4* transcription compared with the *CYP3A4* wild-type allele *(18)*. Further studies are needed to show that the *CYP3A4-V* polymorphism will lead to increased CYP3A4 enzymatic activity not only in cell culture systems but also in individuals.

In conclusion, we have described and validated a fast and simple PCR-RFLP analysis that can be applied for specific screening for the *CYP3A4-V* allele. This assay could greatly facilitate studies on the effect of this polymorphism in endogenous processes, environmental susceptibility to cancer, and individual ability to metabolize drugs.

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Phagocytosis and Oxidative Burst: Reference Values for Flow Cytometric Assays Independent of Age, *Andreas Lun,[1*] Markus Schmitt,[2] and Harald Renz[3]* ([1] Institute of Laboratory Medicine and Pathobiochemistry and [2] Clinic of Pediatric Pneumology and Immunology, University Hospital, Charité, Campus Virchow-Klinikum of the Humboldt-University, Augustenburger Platz 1, 13353 Berlin, Germany; [3] Department of Clinical Chemistry and Molecular Diagnostics, Clinic of the Philipps University Marburg, Baldingerstrasse, 35033 Marburg, Germany; * author for correspondence: fax 49-30-45069900, e-mail andreas.lun@charite.de)

The main function of neutrophils is to provide a front line of defense against invasive bacteria. Disturbances in the functioning of neutrophils lead to repeated and life-threatening infections caused by bacteria and fungi *(1, 2)*. Pathological neutrophil functions are detected as perma-