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Influence of the Cyp1B1 L432V gene polymorphism and exposure to tobacco smoke on Cyp1B1 mRNA expression in human leucocytes.

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Running title:

Cyp1B1 L432V gene polymorphism and Cyp1B1 mRNA expression

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Abbreviations:

PCB	Polychlorinated biphenyls
PAH	Polycyclic aromatic hydrocarbons
TCDD	Tetrachlorodibenzo-p-dioxin
DMOS	Dimethyl sulfoxide
[Tm; dF; dT; T]	Fluorescence data were converted to derivative melting curves by plotting the negative derivative of the fluorescence with respect to temperature vs. temperature [-(dF/dT) vs. T] and show two different melting maxima (Tm), one for each allele.
PCR	Polymerase Chain Reaction
AhR	Aromatic hydrocarbon receptor
Cyp1B1	Cytochrome P450 1B1

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Abstract

Cytochrome P450 1B1 (CYP1B1), a phase I enzyme, is involved in the activation of a broad spectrum of procarcinogens. An association of the Cyp1B1 Leu432Val polymorphism with diverse types of cancer as well as an impact on the catalytic activity of the enzyme have been described. To demonstrate the functional impact of the allelic variant Cyp1B1*3 we investigated the quantitative Cyp1B1 mRNA expression in a population of smokers non-smokers and ex-smokers and determined their genotypes. Detection of the Leu432Val polymorphism in exon 3 of the Cyp1B1 gene was performed by rapid capillary PCR with melting curve analysis. For quantitative comparison of Cyp1B1 mRNA levels real-time PCR was performed using SYBR-green fluorescence in a LightCycler® System. Calculations of expression were made with the $2^{-\Delta\Delta CT}$ method. In comparing relative Cyp1B1 mRNA expression, highly significant differences between the two homozygote genotypes *1/*1 and *3/*3 (0.185±0.027 n=118 vs. 0.071±0.013 n=56; p=0.000) as well as between the heterozygote genotype *1/*3 and the homozygote genotype *3/*3 (0.178±0.025 n=171 vs. 0.071±0.013 n=56; p=0.000) were revealed. Significant differences between the genotypes were also detected within the subgroup smokers, non-smokers and ex-smokers. No significant differences were determined in comparing the relative Cyp1B1 mRNA expression with regards to tobacco smoke exposure. Our results suggest that genotypes carrying the C-allele (*1/*1 and *1/*3) at Cyp1B1 Leu432Val polymorphism have a significantly higher Cyp1B1 mRNA expression compared to the genotype without the C-allele (*3/*3). Gene expression of Cyp1B1 mRNA cannot be used as a biomarker for exposure of tobacco smoke.

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Introduction

To have a carcinogenic effect most environmental compounds require a metabolic initiation to reactivate electrophilic intermediates by the cytochrome P450 mono-oxygenase system (CYP) (Bartsch et al., 2000). Cytochrome P450 1B1 (CYP1B1) is a phase I enzyme which belongs to a multigene super family of monomeric mixed-function monooxygenases (Sutter et al., 1994). It is involved in the activation of a broad spectrum of procarcinogens, for example components of tobacco smoke such as polycyclic aromatic hydrocarbons (PAHs). The expression of the Cyp1B1 gene is induced through the aromatic hydrocarbon receptor (AhR), which is activated by PAHs (Furukawa et al., 2004). Since Cyp1B1 expression can easily be determined in peripheral blood lymphocytes, it is often promoted as a biomarker of exposure to environmental AhR agonists (van Duursen et al., 2005b).

CYP1B1 is expressed in many normal human tissues including peripheral blood cells (Hanaoka et al., 2002). Additionally Cyp1B1 is found to be over expressed in a variety of human tumours (Murray et al., 1997).

Molecular epidemiology can have an important impact when analyzing the complexity of gene-environment interactions at the molecular level. While gene mutations of high penetration often result in a high risk for cancer (Caporaso and Goldstein, 1995), polymorphisms of genes with low penetration (such as Cyp1B1), are thought to predispose the individual risk if there is exposure to a certain chemical (Wunsch Filho and Zago, 2005). Although Cyp1B1 expression is frequently investigated as a biomarker, the polymorphisms are seldom considered. Several genetic polymorphisms have been identified in the Cyp1B1 gene. For example, the Leu432Val polymorphism was reported to be associated with a higher catalytic activity of the enzyme (Aklillu et al., 2002) (Li et al., 2000). This increase in catalytic activity may be caused through changes in the tertiary or quaternary structure of the Cyp1B1 protein, as the Leu432Val polymorphism is located near a catalytically important heme-binding domain in the Cyp1B1 gene (Sissung et al., 2006). Furthermore, the Cyp1B1*3 haplotype was associated with an increased Cyp1B1 mRNA expression in lymphocytes when treated in vitro with TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) (Landi et al., 2005). For Cyp1B1 allele nomenclature refer to the home page of the Human Cytochrome P450 (CYP) Allele Nomenclature

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Committee (www.cypalleles.ki.se/). The Cyp1B1 4326C>G polymorphism causes an amino acid exchange (rs1056836). The C-allele codes for Leu432 and the G-allele codes for Val432 (Table 1). An association of this polymorphism with the incidence of head and neck squamous cancer (Ko et al., 2001), lung cancer susceptibility (Wenzlaff et al., 2005), renal cancer (Sasaki et al., 2004) and colorectal cancer (Fritsche et al., 1999) has been reported. The ability to identify individuals at highest risk cancer is important for cancer control strategies. The fact that 20% of lung cancer cases occur in non-smokers is often attributed to a combination of genetic factors (Gorlova et al., 2007).

Therefore, we investigate the functional impact of the allelic variant Cyp1B1*3 on quantitative Cyp1B1 mRNA expression in a population of smokers, non-smokers and ex-smokers in relation to their Cyp1B1 genotypes.

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Methods

Subjects

The study population consisted of a total of 365 Caucasians. Volunteers were personnel of the Universitätsklinikum Giessen und Marburg GmbH, students of the Justus-Liebig-Universität Giessen as well as patients recruited at the Policlinic of the Institute for Arbeits- and Sozialmedizin Giessen, Germany.

All subjects included in this study were interviewed using a questionnaire to obtain information on lifestyle including smoking habits, alcohol consumption, indoor air pollution (coal heating and cooking), eating habits, lifetime occupational history, and disease history. The group composed 271 males (mean age 56 years; min: 20y max: 91y) and 93 females (mean age 40 years; min: 16y max: 82y). The subgroup smokers (N=107) was defined as people who smoke daily, non-smokers (N=103) were defined as people who had never smoked and ex-smokers (N=155) were defined as people who had quit smoking at least 3 months ago.

The ethics committees of the university hospital, Giessen, Germany, approved the study.

Real-time PCR and polymorphism detection.

Blood samples (ca. 3 ml) were obtained from all subjects. Genomic DNA was isolated from whole blood using the VersageneTM DNA Purification Kit (Gentra Systems, Minneapolis, USA). Detection of the Leu432Val polymorphism in exon3 of the Cyp1B1 gene was performed by rapid capillary PCR, with melting curve analysis, using fluorescence-labelled hybridisation probes in a LightCycler® System (Bruning et al., 1999). Both negative and positive controls were included in each PCR reaction. TIB MOLBIOL, Berlin, Germany, synthesized the PCR primers as well as the fluorescent-labelled detection probes. The primers Cyp1B1-for (5`-GAAATAAGAATTTTGCTCACTTGC-3`) and Cyp1B1-rev (5`-CTTAGAAAGTTCTTCGCCAATG-3`) amplify a 441-bp fragment of the Cyp 1B1 gene that includes codon 432 containing the sequences CTG or GTG, coding for leucin or valin, respectively. The fluorescein-labeled probe (ATGACCCACTGAAGTGACCTAACCC-FL) and the LC-Red640-labeled probe

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(LC640-AACTTTGATCCAGCTCGATTCTTGGACAA-PH) bind to nucleotides 8123-8147 and 8152-8180 within the human Cyp1B1 gene (GenBank entry U56438) respectively. Therefore, the expected melting curve in case of a C-allele will be higher than in case of a G-allele. The samples homozygous for the C-allele (Cyp1B1 *1/*1) contain a single peak and a melting temperature of 63°C, and samples homozygous for the G-allele (Cyp1B1 *3/*3) show a single peak at 55°C. Heterozygous (Cyp1B1 *1/*3) samples give rise to biphasic melting curves (Fig.1). In the following the homozygous genotype Cyp1B1 *1/*1" is denoted "*1/*1", the heterozygous genotype Cyp1B1 *1/*3 is denoted "*1/*3" and the homozygous genotype Cyp1B1 *3/*3 is denoted "*3/*3" (Fig.1).

The reaction mixture comprised 1x FastStart DNA Master Hybridization Probes, (Roche Diagnostics, Mannheim, Germany), primers Cyp1B1-for (0.55 µM) and Cyp1B1-rev (1.1 µM), sensor (0.275 µM), anchor (0.275 µM), $MgCl_2$ (3 mM), 25 ng of genomic DNA in a 10 µl reaction mixture. PCR contamination was checked by the inclusion of a negative control. PCRs were initially denatured at 95°C for 10 min and subjected to 50 cycles of amplification with 5 sec of denaturing at 95°C, 20 sec of annealing at 56°C, and 35 sec of extension at 72°C. The melting curve was achieved by 2 sec at 95°C, 30 sec at 40°C and 1 sec at 90°C 0.1°C/s.

RNA extraction and reverse transcription

White blood cells (WBC) were isolated from 10 ml peripheral blood using Ficoll® (GE Healthcare) as described by the manufacturer. Total RNA was isolated from WBCs using a commercial RNA isolation reagent (TRI Reagent®, Sigma) according to the manufacturer's instructions. Isolated RNA was resuspended in 10 µl of RNAse-free water. Each sample was treated twice with 2 µl RNAse-free DNAse 1 unit/µl (Qiagen) for 10 min at 37°C to eliminate remaining DNA. The prepared RNA was reverse-transcribed to synthesize cDNA. RT reactions were performed for 1h at 37°C in a final volume of 10 µl using 1 µg RNA, 500 ng of Oligo(dT)15 (Promega), 10 mMol/L dNTPs (Fermentas), 8 units of RNAsin (Promega) and 3 units of AMV Reverse Transcriptase and 1x AMV Reverse Transcriptase Reaction buffer (Promega).

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Real-time polymerase chain reaction (PCR)

For quantitative comparison of Cyp1B1 mRNA levels real-time PCR was performed using SYBR-green fluorescence in a LightCycler® System (Roche Diagnostic GmbH). After optimization of PCR conditions, amplification efficiency was tested in standard curves using serial cDNA dilutions. The correlation coefficient had to be above 0.9 and the slope around −3.5. Amplification specificity was checked using melting curves. Both negative and positive controls were included in each PCR reaction. All assays were carried out three times as independent PCR runs for each cDNA sample. Gene expression was always related to expression of beta-2-microglobulin (B2M) as housekeeping gene, which is known to be a good choice for normalization of leucocytes expression levels (Vandesompele et al., 2002). Calculations of expression was performed with the $2^{-\Delta\Delta CT}$ method according to Pfaffl et al. (Pfaffl, 2001). The gene expression levels were rounded to three decimal places. The sequence of the specific primers for CYP1B1 and B2M were CYP1B1for (5'-AACCGCAACTTCAGCAACTT-3'), CYP1B1rev (5'-GAGGATAAAGGCGTCCATCA-3'), and B2Mfor (5'-ACTGAATTCACCCCCACTGA-3'), B2Mrev (5'-CCTCCATGATGATGCTTACA-3'). PCR reactions were carried out in a final volume of 20 µl using 1x Absolut™ QPCR SYBR Green Capillary Mixes (Abgene), 300 nM of B2M primers or 600 nM of Cyp1B1 primers and 2 µl cDNA. The PCR conditions for Cyp1B1 were as follows: Initial denaturation 15 min at 95°C, touch down PCR 2 cycles of 95°C for 10 sec, 67°C for 10 sec, 72°C for 25 sec; 2 cycles of 95°C for 10 sec, 65°C for 10 sec, 72°C for 25 sec; 2 cycles of 95°C for 10 sec, 63°C for 10 sec, 72°C for 25 sec; 45 cycles of 95°C for 10 sec, 61°C for 10 sec, 72°C for 25 sec. The PCR conditions for B2M were as follows: Initial denaturation 15 min at 95°C, PCR 55 cycles of 95°C for 10 sec, 63°C for 10 sec, 72°C for 10 sec.

All measurements were made without information about the origin of the samples and were performed in triplicate. After amplification reaction products were controlled and separated on 2% agarose gels, stained with SYBR Safe, and photographed under UV illumination.

Statistical analysis

Smokers were considered current smokers at the time of blood withdrawal. Ex-smokers were all individuals who were previously smokers but had quit smoking at

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least 3 months before blood withdrawal. Information was collected on the usual number of cigarettes smoked per day, the age at which the subject started smoking and, if the person was an ex-smoker, the age and time at which the subject stopped smoking. One pack-year was defined as smoking 20 cigarettes daily over one year. All statistical analyses were performed using the statistical software package, SSPS 15.0 (SPSS Inc., Chicago, IL, USA). Allelic and genotype frequencies were obtained by direct counting. Hardy–Weinberg equilibrium was assessed by a χ^2 test with 1 degree of freedom. Results are expressed as mean ± standard error of the mean (SE). Means in different subgroups were analysed by Student's t-test, by one-way ANOVAs, followed by Duncan- and Student-Neumann-Keuls post hoc analysis and Mann-Whitney-U-test. A value of $P < 0.05$ was regarded as significant

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Results

Allele frequencies

To determine if our studied population does not deviate from Hardy-Weinberg equilibrium and if allele frequencies were within other published frequencies, we investigated allele and genotype frequencies in the total population as well as in all subgroups.

In the total population the allele frequency for q(Cyp1B1 *3) was 0.415. This allele frequency is similar to other observed frequencies in various healthy Caucasian populations and did not significantly deviate from the Hardy-Weinberg equilibrium (P = 0.1 = 1-α). The allele frequencies of Cyp1B1 *3 were not statistically different within the subgroups smokers q(Cyp1B1 *3) = 0.43, non-smokers q(Cyp1B1 *3) = 0.40 and ex-smokers q(Cyp1B1 *3) = 0.42, and they did not deviate from the Hardy-Weinberg equilibrium (P = 0.1 = 1-α). Allele frequencies were also not different within the male q(Cyp1B1 *3) = 0.415 or female q(Cyp1B1 *3) = 0.414 subgroups and they did not deviate from the Hardy-Weinberg equilibrium males (P = 0.1 = 1-α) and females (P = 0.1 = 1-α).

Cyp1B1 mRNA expression

To investigate the functional impact of the Cyp1B1 allelic variants the quantitative Cyp1B1 mRNA expression among the different genotypes for Cyp1B1 Leu432Val polymorphism (*1/*1, *1/*3; *3/*3) was analysed. Comparing the means ± standard error of the relative Cyp1B1 mRNA expression revealed significant differences between the two homozygote genotypes *1/*1 and *3/*3 (0.185±0.027; n=118 vs. 0.071±0.013; n=56; p=0.000) as well as between the heterozygote genotype *1/*3 and *3/*3 (0.178±0,025; n=171 vs. 0.071±0.013; n=56; p=0.000). This suggests that genotypes carrying the C-allele (*1/*1 and *1/*3) have a significantly higher Cyp1B1 mRNA expression compared to the genotype without the C-allele (*3/*3) (Fig.2).

To further investigate the functional impact of the Cyp1B1 allelic variants and to exclude a tobacco smoke influence, the quantitative Cyp1B1 mRNA expression was analysed within the subgroups smokers, non-smokers and ex-smokers. In each subgroup the relative Cyp1B1 mRNA expression was compared between the three different genotypes *1/*1, *1/*3 and *3/*3. A significant difference of the means ±

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standard error between the homozygote genotypes *1/*1 and *3/*3 was detected within the subgroup smokers (0.169±0,043; n=33 vs. 0.056±0,016; n=19; p=0.020) and non-smokers (0.219±0,063; n=34 vs. 0.064±0,019; n=14; p=0.025) respectively. Significant differences of the means ± standard error between the heterozygote genotype *1/*3 and the homozygote genotype *3/*3 was revealed within the subgroups smokers (0.205±0.058; n=50 vs. 0.056±0.016; n=19; p=0.018) and ex-smokers (0.181±0.084; n=72 vs. 0.084±0.027; n=24; p=0.024), respectively (Fig.3). These findings confirm that the genotypes containing a C-allele have a significantly higher Cyp1B1 mRNA expression compared to the genotype without a C-allele.

To determine if Cyp1B1 is activated in leucocytes by components of tobacco smoke, we analysed the quantitative mRNA expression of Cyp1B1 in relation to smoking habits. Comparing the means and the standard error of the relative Cyp1B1 mRNA expression between smokers (0.167±0,033; n=101), non-smokers (0.191±0.037; n=98) and ex-smokers (0.149±0.017; n=148) did not reveal any significant differences (Fig.4).

Furthermore, the quantitative Cyp1B1 mRNA expression was analysed within the different genotypes (*1/*1, *1/*3, *3/*3). Means ± standard errors were compared between smokers, non-smokers and ex-smokers. In accordance with the results in the total population, no significant differences in Cyp1B1 mRNA expression between smokers, non-smokers and ex-smokers were determined (Fig.5).

Cyp1B1 plays a crucial role in metabolising xenobiotics such as PAHs, but it is also responsible for hormone metabolism. Cyp1B1 is known to be the most catalytically efficient E2 hydroxylase (Spink et al., 1998). Therefore, we divided the total population into males (n=271) and females (n=93). Within these two groups quantitative mRNA expression of Cyp1B1 was determined and compared between the different genotypes for Cyp1B1 Leu432Val (*1/*1, *1/*3, *3/*3). Significant differences were only observed within the male group. Here the relative Cyp1B1 mRNA expression was significantly higher in C-allele carrying homozygote and heterozygote genotypes than in the homozygote genotype without a C-allele (0.139±0.022; n=90 vs. 0.062±0.010; n=44; p=0.000 and 0.138±0.020; n=124 vs. 0.062±0.010; n=44; p=0.026 respectively). The higher Cyp1B1 mRNA expression in females compared to males is worth noting (Fig.6).

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Discussion

In this study we demonstrate an influence of the Cyp1B1 Leu432Val polymorphism on its Cyp1B1 mRNA expression in human lymphocytes in relation to interindividual differences by PAH induction through tobacco smoke. Therefore, we investigated the quantitative mRNA expression and the Leu432Val genotypes of Cyp1B1 in 365 Caucasian volunteers. The allele frequencies for the studied genotypes did not deviate from the Hardy-Weinberg equilibrium, and allele frequencies were similar to other published frequencies (Bailey et al., 1998; Bruning et al., 1999; Tang et al., 2000; Ko et al., 2001).

We found significant differences in comparing the relative Cyp1B1 mRNA expression between the different Cyp1B1 genotypes, suggesting that genotypes carrying the wild type C-allele (Cyp1B1*1 allele) have a significantly higher Cyp1B1 mRNA expression compared to the genotype without the C-allele. These findings were also confirmed when we divided the population into the subpopulations smokers, non-smokers and ex-smokers.

Hanaoka et al. 2002 found a significant correlation between Cyp1B1 mRNA expression and urinary 1-hydroxypyrene (1-OHP) in subjects with the Cyp1B1 432 Leu/Leu wild type genotype (Cyp1B1*1/*1) (Hanaoka et al., 2002). On the other hand, Hu et al. 2006 found a significantly higher mRNA expression in Taiwanese municipal waste incinerator workers carrying the Cyp1B1*3 allele (n=9) compared to workers carrying the wild type allele (n=34). This finding was confirmed in workers exposed to high levels of PAHs/dioxin only. Workers exposed to medium levels or controls, showed a insignificantly lower Cyp1B1 mRNA expression when carrying the Cyp1B1*3 allele (n=15 vs. n=48) (Hu et al., 2006). However, van Duursen et al (2005) found no statistically significant effects of Cyp1B1 L432V polymorphism on mRNA expression levels when studying human blood lymphocytes of ten non-smoking females (van Duursen et al., 2005b). Also, no statistically significant differences were found in Cyp1B1 mRNA expression when comparing the Cyp1B1 L432V polymorphism of 114 individuals living in a PCB-polluted area in Slovakia (van Duursen et al., 2005a). In contrast, subjects heterozygous or homozygous for the Cyp1B1*3 allele showed a higher TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin)-induced Cyp1B1 mRNA expression in mitogen-treated lymphocytes than subjects

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homozygous for the Cyp1B1*1 allele (Landi et al., 2005). The low sample size in some studies as well as the different distribution of the Cyp1B1 PM among Chinese and Caucasians (Tang et al., 2000) should be considered. The fact that mitogens cause lymphocytes to proliferate, which occurs concomitantly with an activation of several cell-signalling pathways and increases in gene transcription, should also be mentioned. Even when housekeeping genes are used for normalisation, expression levels of Cyp1B1 can be higher in mitogen-stimulated cells than in non-stimulated cells (van Duursen et al., 2005b). The genetic impact on the expression pattern of Cyp1B1 is only one contributing factor, especially since post-transcriptional mechanisms have been suggested to be involved in regulation of Cyp1B1 (McFadyen et al., 2003). Although the consequences of our findings are not yet clear, further studies need to consider the Cyp1B1 Leu432Val polymorphism when investigating gene as well as protein expression of Cyp1B1.

We did not find any significant differences in comparing the Cyp1B1 expression of smokers, non-smokers and ex-smokers. Furthermore, we did not find any differences in Cyp1B1 expression of smokers, non-smokers and ex-smokers when we divided the population into subpopulations by their different Cyp1B1 genotypes. Therefore, we conclude that Cyp1B1 gene expression levels in lymphocytes are not suitable to use as biomarkers of exposure to environmental or tobacco smoke PAHs. Nevertheless, a higher expression of Cyp1B1 was observed in bronchial epithelial cells of smokers than in non-smokers (Kim et al., 2004) (Willey et al., 1997).

Blood lymphocytes do not function as a target organ. Accordingly, Dassi et al. 1998 estimated Cyp1B1 mRNA levels in blood monocytes from 25 smokers and 50 non-smokers without finding any differences between the two groups (Dassi et al., 1998). In a group of volunteers, no significant association between self-reported PAH exposure and Cyp1B1 mRNA expression was detected (Tuominen et al., 2003). Additionally, Lin et al. 2003 did not find any significant differences in gene expression level between Taiwanese male smokers (n=12) and non-smokers (n=10) (Lin et al., 2003). For other AhR agonists, similar results were obtained in diverse studies.

When investigating cultured lymphocytes of 121 subjects from Seveso, Italy, accidentally exposed to TCDD no association between Cyp1B1 mRNA expression and TCDD plasma levels was detected (Landi et al., 2003). Furthermore, the concentration of dioxin or dioxin-like compounds necessary to evoke an in vitro effect on Cyp1B1 expression are much higher than the human blood levels found in vivo

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(van Duursen et al., 2005b). Within a population of Slovakians from a region polluted with polychlorinated biphenyls, there was no significant correlation between PCB levels and Cyp1B1 mRNA expression (van Duursen et al., 2005a). On the other hand, Hanaoka et al. 2002 found significantly higher Cyp1B1 mRNA levels in peripheral blood of Chinese coke oven workers exposed to PAHs at high levels (n=9) compared to workers exposed to lower PAH levels (n=16) and controls (n=13) (Hanaoka et al., 2002). Also, a significantly higher mRNA expression of Cyp1B1 was found in leucocytes of Taiwanese municipal waste incinerator workers (n=77) frequently exposed to PAHs and dioxins compared to controls (n=35) (Hu et al., 2006). A possible explanation for the contrasting findings is that occupational and environmental exposure may differ in magnitude, chemical composition and frequency. Moreover, the studies differ in sample size, time period between exposure and measurement of RNA as well as exposure registration.

In our study there was a higher mRNA expression of Cyp1B1 in females compared to males. The low sample size of females we investigated in this study should be considered for these results. Lin et al. 2003 reported similar results however, when comparing DMSO-treated lymphocytes from female non-smokers with DMSO-treated lymphocytes from male non-smokers (Lin et al., 2003). Additionally, in Taiwanese females the mean Cyp1B1 mRNA expression was insignificantly higher than in males (Hu et al., 2006). Several epidemiological studies have indicated that female smokers are at higher risk of lung cancer than male smokers (Engeland, 1996; Zang and Wynder, 1996; Prescott et al., 1997; Prescott et al., 1998a; Prescott et al., 1998b). It is possible that the higher Cyp1B1 expression in females could account for their elevated lung cancer risk. These findings require further investigation.

In conclusion, we demonstrated in this study that the Cyp1B1 *3/*3 genotype is associated with a decreased Cyp1B1 mRNA expression compared to the Cyp1B1 *1/*1 and Cyp1B1 *1/*3 genotypes. We also observed a different Cyp1B1 mRNA expression between females and males. However, we did not find any statistically significant effect of tobacco smoke on Cyp1B1 mRNA expression in leucocytes.

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Footnotes

* Some of the results are included in the thesis of B. Hadzaad

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Legends for figures:

Fig.1: Melting curve analysis of the Cyp1B1 Leu432Val polymorphism using LightCycler®. Fluorescence data were converted to derivative melting curves by plotting the negative derivative of the fluorescence with respect to temperature vs. temperature [K(dF/dT) vs. T] and show two different melting maxima (Tm), one for each allele. Melting maxima for the Cyp1B1 Leu432Val polymorphism were 55 °C for the G/G genotype (Cyp1B1 *3/*3), 55 °C and 63 °C for the heterozygous C/G genotype (Cyp1B1 *1/*3) and 63 °C for the homozygous C/C genotype (Cyp1B1 *1/*1).

Fig.2: Quantitative mRNA expression of Cyp1B1 in leucocytes was determined among the different genotypes for Cyp1B1 Leu432Val. Significant differences in Cyp1B1 expression between the subgroups *1/*1 and *3/*3 as well as between the subgroups *1/*3 and *3/*3 were observed. Therefore, genotypes containing the C-allele had a significantly higher Cyp 1B1 mRNA expression compared to the genotype without a C-allele. Data are shown as means ± S.E., *= $p<0.05$

Fig.3: Quantitative mRNA expression of Cyp1B1 was determined among the different genotypes for Cyp1B1 Leu432Val within the subgroups smokers, non-smokers and ex-smokers. Significant differences were observed between the genotypes *1/*1 and *3/*3 within the subgroups smokers and non-smokers as well as between the genotypes *1/*3 and *3/*3 within the subgroups non-smokers and ex-smokers. Therefore, genotypes containing the C-allele had a significantly higher Cyp 1B1 mRNA expression within the subgroups. Data are shown as means ± S.E., *= $p<0.05$

Fig.4: Quantitative mRNA expression of Cyp1B1 in leucocytes was determined in a group of smokers (n=101), non-smokers (n=98) and ex-smokers (n=148). No significant difference in Cyp1B1 gene expression between the subgroups was observed. Data are shown as means ± S.E., *= $p<0.05$

Fig.5: Quantitative mRNA expression of Cyp1B1 was determined for the different smoking habits within the subgroups of different genotypes for Cyp1B1 Leu432Val. No significant differences were observed within the different genotypes *1/*1, *1/*3 and *3/*3 concerning smoking habits. Data are shown as means ± S.E., *= $p<0.05$

Fig.6: Quantitative mRNA expression of Cyp1B1 was determined among the different genotypes for Cyp1B1 Leu432Val within the subgroups males (M) and females (F). Significant differences were only observed within the subgroup males. Here the Cyp1B1 expression was significantly higher in C-allele-carrying genotypes (*1/*1, *1/*3) than in the genotype without a C-allele (*3/*3). Worth noting is the higher Cyp1B1 mRNA expression in females compared to males. Data are shown as means ± S.E., *= $p<0.05$

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Table 1: The Cyp1B1 Leu432Val polymorphism

	Homozygote wild type	**Heterozygote**	**Homozygote mutant**
Genotypes	Cyp1B1 *1/*1	Cyp1B1 *1/*3	Cyp1B1 *3/*3
Nucleotides	C/C	C/G	G/G
Amino acids	Leu/Leu	Leu/Val	Val/Val

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Fig. 1

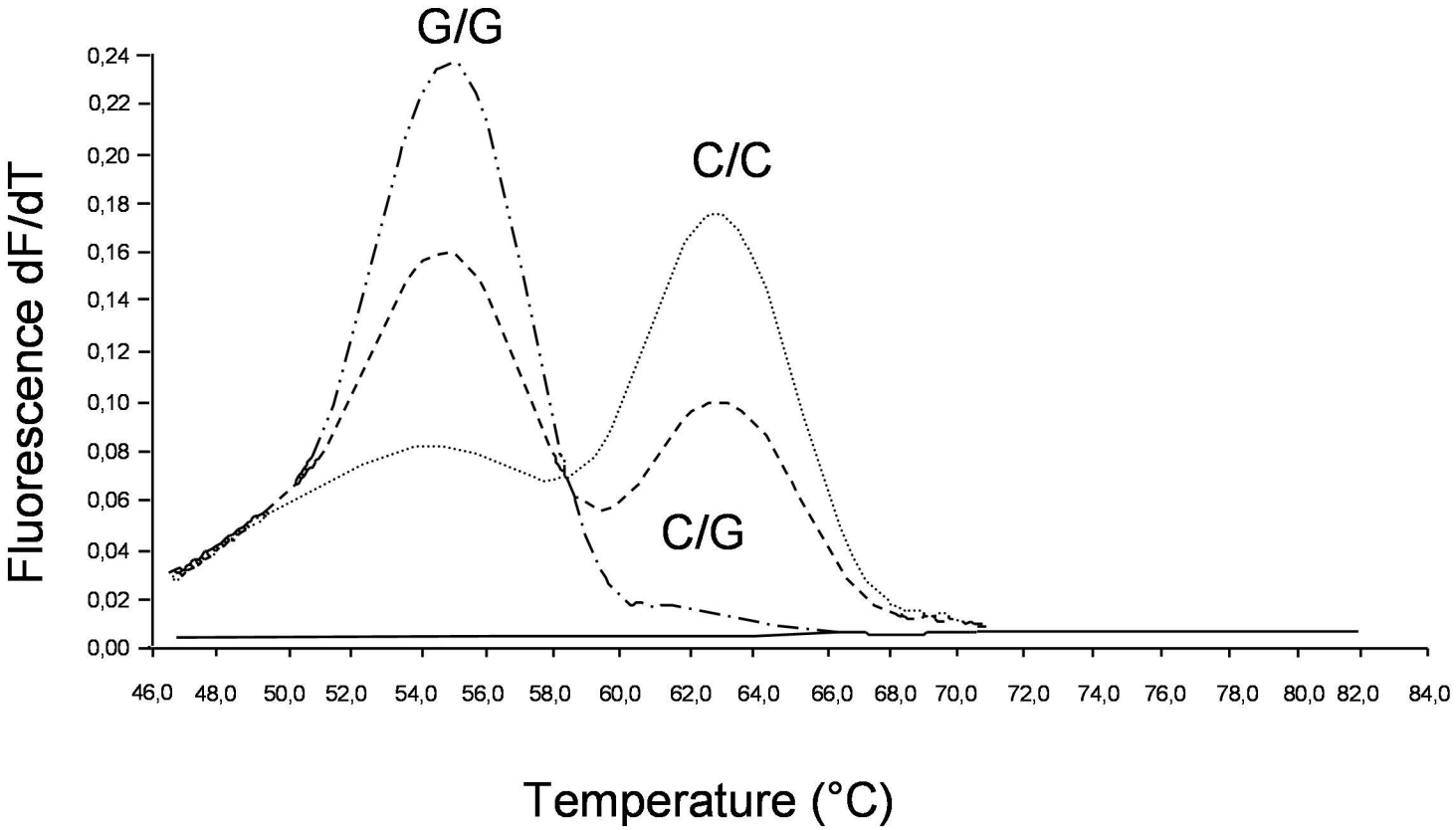

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Fig. 2

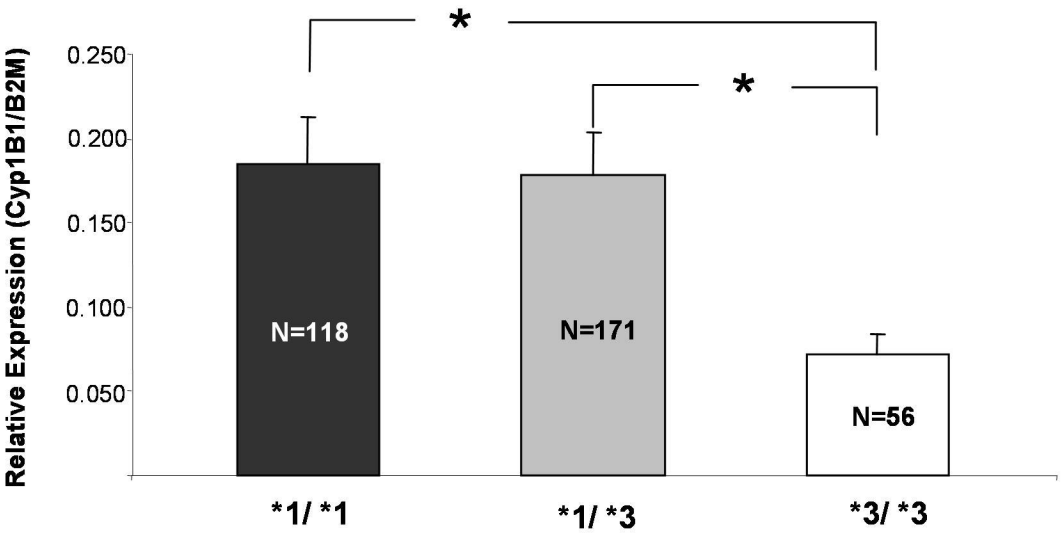

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Fig. 3

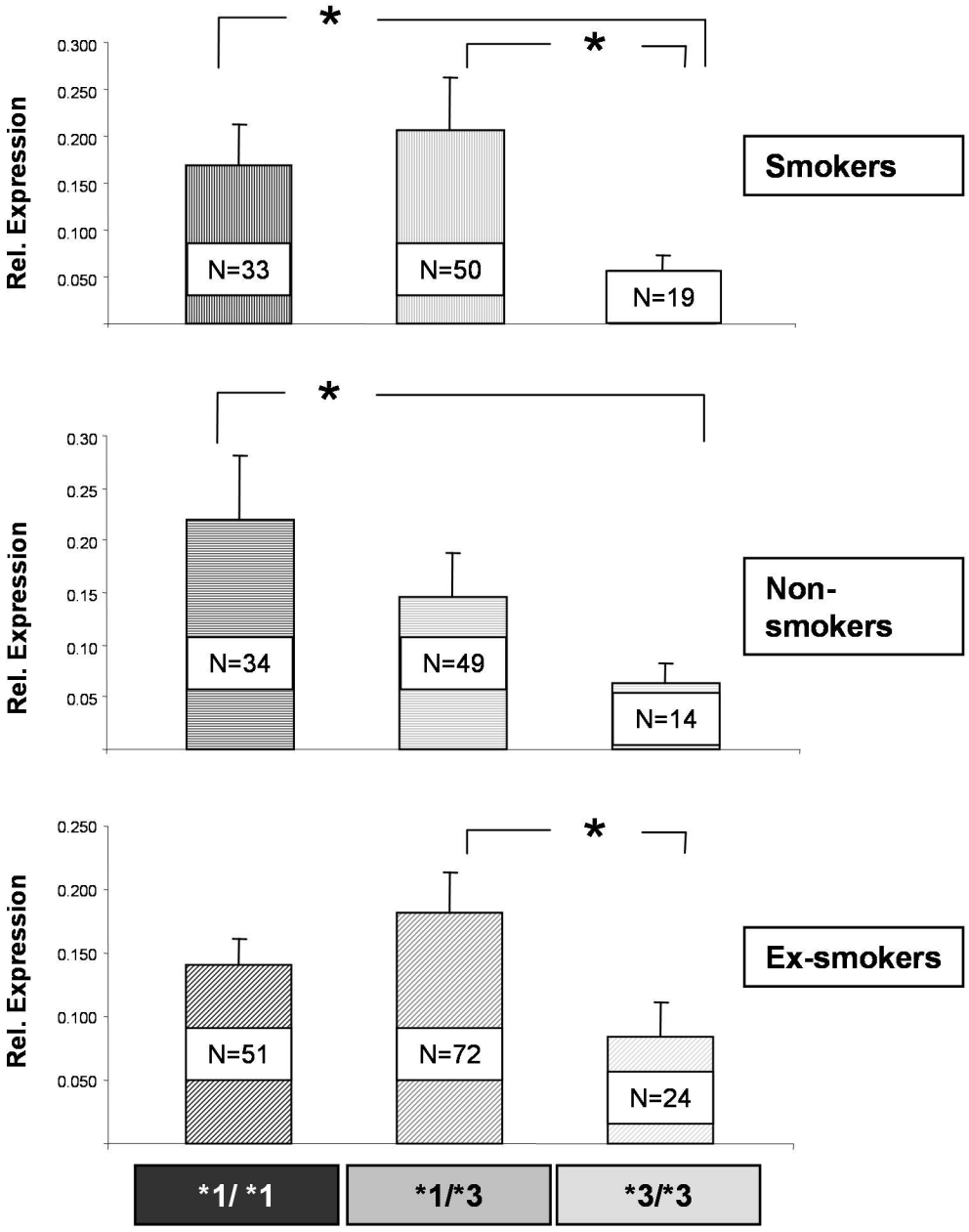

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Fig. 4

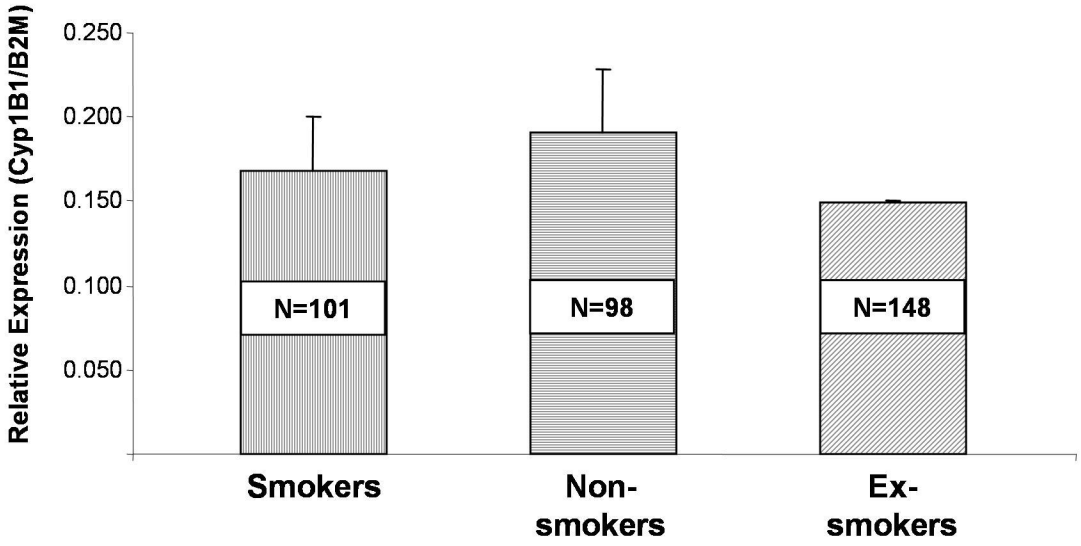

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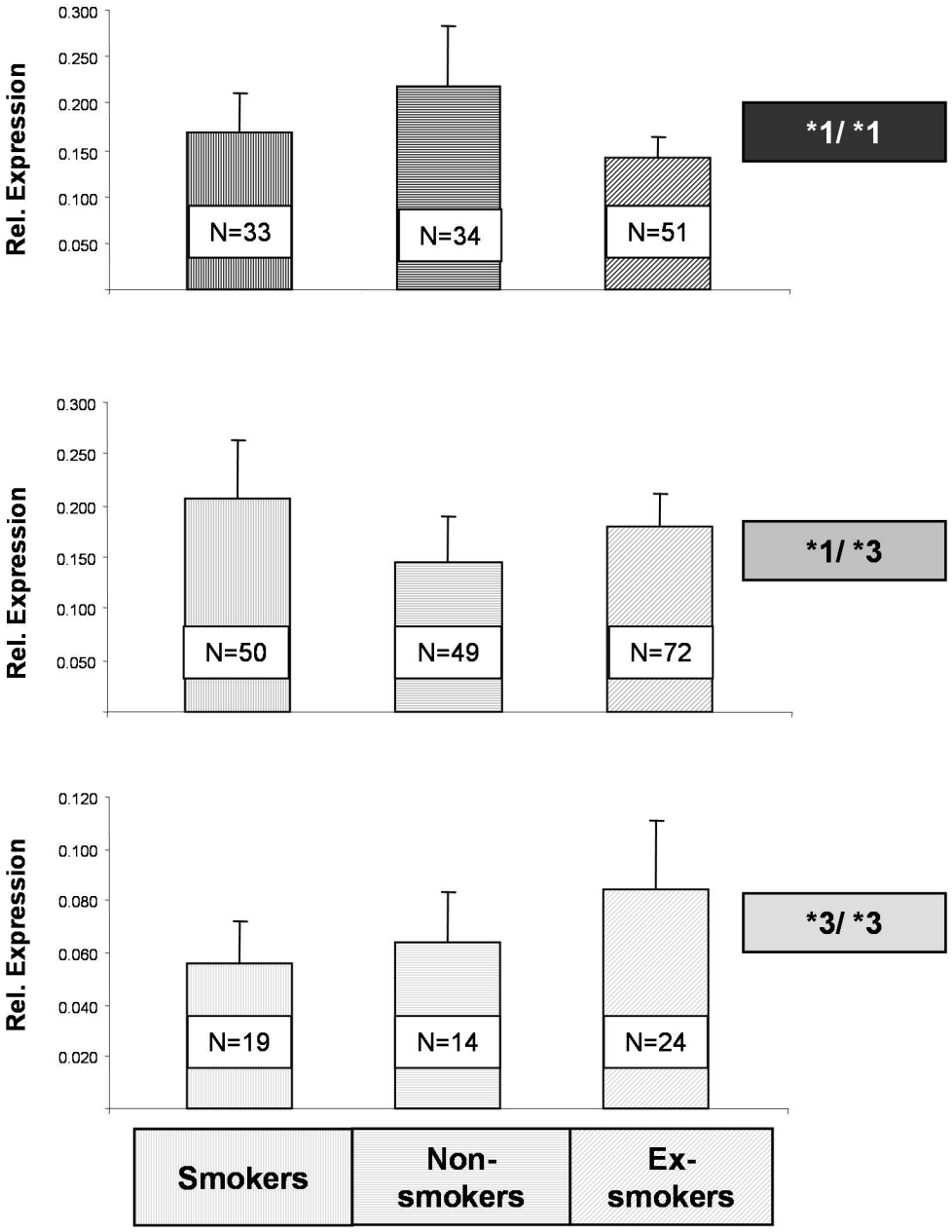
Fig. 5



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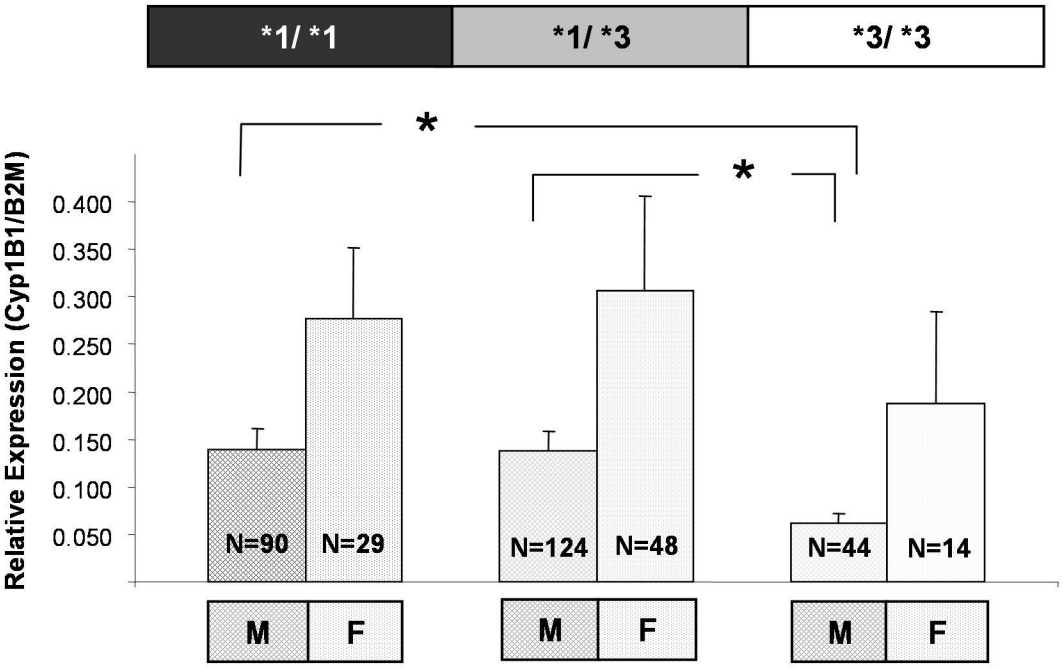
Fig. 6