A comprehensive contribution of genes for aryl hydrocarbon receptor signaling pathway to hypertension susceptibility

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Objective **The present study was designed to investigate whether genetic polymorphisms of the aryl hydrocarbon receptor (AHR) signaling pathway are involved in the molecular basis of essential hypertension (EH).**

Methods **A total of 2160 unrelated Russian individuals comprising 1341 EH patients and 819 healthy controls were recruited into the study. Seven common AHR pathway single-nucleotide polymorphisms (SNPs) such as rs2066853, rs2292596, rs2228099, rs1048943, rs762551, rs1056836, and rs1800566 were genotyped by TaqMan-based allele discrimination assays.**

Results **We found that SNP rs2228099 of *ARNT* is associated with an increased risk of EH (odds ratio = 1.20 95% confidence interval: 1.01–1.44, $P = 0.043$) in a dominant genetic model, whereas polymorphism rs762551 of *CYP1A2* showed an association with a decreased risk of disease in a recessive genetic model (odds ratio = 0.68, 95% confidence interval: 0.52–0.89, $P = 0.006$). A log-likelihood ratio test enabled identification of epistatic interaction effects on EH susceptibility for all SNPs. MB-MDR analysis showed that cigarette smoking, rs1048943, rs762551, rs1056836, and rs2228099 were significant contributing factors in 19, 18, 13, 13, and 11 interaction models, respectively. The best MDR model associated with EH risk included rs1048943, rs762551, rs1056836, and cigarette smoking (cross-validation consistency 100%, prediction error 45.7%, $P_{permutation} < 0.0001$). The mRNA expression and in-silico function prediction analyses have confirmed a regulatory potential for a majority of SNPs associated with EH susceptibility.**

Conclusion **Our pilot study was the first to show that gene–gene and gene–environment interactions in the AHR signaling pathway represent important determinants for the development of EH, and the pathway may become an attractive target for a pharmacological intervention in hypertensive patients in the future. *Pharmacogenetics and Genomics* 27:57–69**

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Keywords: aryl hydrocarbon receptor signaling pathway, essential hypertension, gene–gene interaction, gene–smoking interaction, genetic polymorphism, multifactor dimensionality reduction

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Introduction

Hypertension is a major health burden because of its high prevalence and associated increased rates of morbidity, mortality, and disability from cardiovascular disease and stroke worldwide [1,2]. It has been estimated that almost 28% of the world's adult population has uncontrolled hypertension [3] and the global burden of disease will increase to more than 1.5 billion by 2025 [4]. In most cases, the etiology of hypertension remains unclear, which is the reason for the definition of the disease as essential hypertension (EH). The mechanisms involved in the regulation of blood pressure (BP) in human populations are complex and are likely modulated by tight interactions between genetic and environmental factors, suggesting a multifactorial nature of hypertension [5,6].

Genome-wide association scans and candidate gene studies have successfully identified a number of common genetic variants influencing BP variation and hypertension susceptibility in ethnically diverse populations [7–10]. Despite the progress in hypertension genomics, the difficult task remains in the bridging of genetic findings into the clinic. Such a translation, on the one hand, takes considerable time to move from a identified gene target to an approved marketed drug; on the other, the effect sizes of genome-wide association scans identified BP loci are relatively small and the advantage of their utilization in the clinical practice is not clear [11]. Although adequate drug treatment and control of hypertension result in reduced morbidity and mortality [12,13], the

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findings obtained by pharmacogenomic studies of antihypertensive drugs are also far from being utilized in the clinic [14–16]. Thus, much of the heritability of BP, hypertension, and efficacy of antihypertensive treatment remain unexplained, highlighting the need for further identification of major genetic and environmental factors responsible for the global epidemic of the disease.

A huge number of studies have shown a positive relationship between incident hypertension and ambient air pollution [17–24]. A rapidly growing body of evidences suggests that airborne polycyclic aromatic hydrocarbons (PAHs) may represent an important group of organic toxic chemicals with a potentially causative role for hypertension [25–29]. PAHs are a group of pollutants prevalent widely in the environment, formed during incomplete combustion of organic materials such as coal and petroleum product combustion, cigarette smoking, food cooking, and industrial activities [30]. Individuals exposed to PAHs defend themselves against intracellular damage by activating the transcription of genes involving in the aryl hydrocarbon receptor (AHR) signal transduction cascade that defends the host, removing and metabolizing the toxicant [31,32]. Figure 1 summarizes the organization and functions of the AHR signaling pathway. AHR is a ligand-dependent transcription factor that regulates the induction of the phase I and II xenobiotics-metabolizing enzymes (XMEs), and thus mediating most of the toxic and carcinogenic effects of PAHs as well as polyhalogenated hydrocarbons (dioxins, furans) and polychlorinated biphenyls [37,38]. The basic helix–loop–helix proteins AHR, aryl hydrocarbon receptor nuclear translocator (ARNT) and aryl hydrocarbon receptor repressor (AHRR), and regulated XMEs represent the AHR signaling pathway, the adaptive xenobiotic stress system that recognizes putatively toxic compounds and triggers their detoxification and elimination [39].

Several common single-nucleotide polymorphisms (SNPs) in genes involved in the AHR signaling pathway have been identified and shown to determine interindividual differences in the ability to activate and detoxify PAHs [40–44]. A single study, which investigated two SNPs of the AHR pathway, observed that an interaction between the *CYP1A1* T3801C and *AHR* G1661A polymorphisms is associated with BP [45]. A few studies in humans have shown an association between genes encoding AHR regulated XMEs such as *CYP1A1*, *CYP1A2*, and *CYP1B1* and hypertension susceptibility [46–48]. To our knowledge, no studies have been carried out so far to investigate a comprehensive contribution of AHR pathway genes toward the development of EH. Therefore, the aim of our pilot study was to investigate whether common polymorphisms of the AHR signaling pathway are comprehensively involved in the molecular basis of EH.

Methods

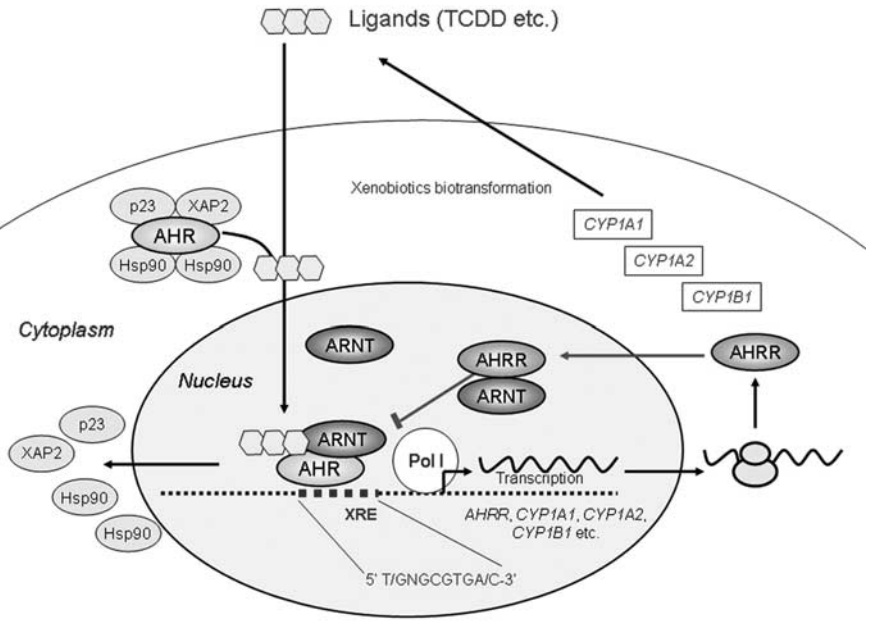
Study population

The study protocol was approved by the Ethical Review Committee of Kursk State Medical University and written informed consent was obtained from each participant before enrollment. A total of 2160 unrelated individuals of Russian origin from of the Central Russia (predominantly from Kursk region) comprising 1341 EH patients and 819 healthy individuals with normal BP were recruited at Cardiology Clinics of Kursk Regional Clinical Hospital and Neurology Clinics of Kursk Emergency Medicine Hospital as described previously [49,50]. EH was diagnosed by qualified cardiologists. Patients were defined as hypertensive according to WHO criteria or if they had a history of receiving any antihypertensive drug. All EH patients had no clinical signs, symptoms, and laboratory findings suggestive of secondary hypertension. Study patients completed a questionnaire on conventional demographic characteristics and also smoking status, which was considered a measure of individual exposure to PAHs. The baseline characteristics of the study participants are shown in Table 1. EH patients were matched to healthy controls in terms of sex and age ($P > 0.05$).

Genetic analysis

Whole-blood samples were collected by venipuncture from all study participants in EDTA-coated tubes and maintained at − 20°C until processed. Genomic DNA was isolated from thawed blood samples using a standard phenol/chloroform procedure. Candidate genes involved in the AHR signaling pathway were selected on the basis of their involvement in the pathway using the KEGG Pathway (*http://www.genome.jp/kegg/pathway.html*), the Reactome Pathway (*http://www.reactome.org*), and PharmGKB (*http://www.pharmgkb.org*) databases. The selected AHR pathway genes included *AHR* (gene ID 196), *ARNT* (gene ID 405), *AHRR* (gene ID 57491), *CYP1A1* (cytochrome P450 family 1 subfamily A member 1, gene ID 1543), *CYP1A2* (cytochrome P450 family 1 subfamily A member 2, gene ID 1544), *CYP1B1* (cytochrome P450 family 2 subfamily A member 1, gene ID 1545), and *NQO1* [NAD(P)H dehydrogenase, quinone 1, gene ID 1728]. Most commonly studied and potentially functional SNPs in these genes (minor allele frequency > 5%) such as *AHR* R554K (rs2066853), *AHRR* P185A (rs2292596), *ARNT* 567C > G (rs2228099), *CYP1A1* I462V (rs1048943), *CYP1A2*–163C > A (rs762551), *CYP1B1*, V432L (rs1056836), and *NQO1* P187S (rs1800566) were selected for the study. Detailed information on the biological function of the genes and their polymorphisms is present in Supplementary Table 1 (Supplemental digital content 1, *http://links.lww.com/FPC/B134*). The polymorphisms were genotyped by TaqMan-based allele discrimination assays on the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA) on the basis of the protocols published in the literature (sequences of TaqMan-probes and primers sets with references used in this study are listed in Supplementary Table 2, Supplemental digital content 2, *http://links.lww.com/FPC/B135*). The average call rate for genotyping was 97.6%. As quality controls, about 5% of the samples were selected randomly in a blinded manner to case–control status and their repeated genotyping yielded 100% reproducibility.

Fig. 1



The organization of the AHR signaling pathway and regulated xenobiotic-metabolizing enzymes [32–36]. The ligand binding to and following activation of AHR is the initial step in the mode of action for a variety of biological and toxicological responses to TCDD and dioxin-like compounds of the environment. AHR recognizes the presence of xenobiotics in the cytoplasm, and then acts to induce XMEs to facilitate the elimination of the foreign compounds. The main genes of the AHR pathway include the ligand-binding receptor *AHR*, the AHR nuclear translocator (*ARNT*), and the AHR regulator (*AHRR*). Normally, AHR exists in a dormant state within the cytoplasm in association with proteins Hsp90, XAP2, and p23, which help to correctly fold and stabilize the AHR and prevent inappropriate trafficking to the nucleus. Upon ligand binding, AHR in the complex is activated by a conformation change and migrates to the nucleus, where it forms a heterodimer with ARNT, thereby forming a protein complex capable of binding to DNA. The AHR-ARNT complex binds to the xenobiotic response element (XRE) motifs in enhancers of target genes, thereby inducing the transcription of XMEs such as *CYP1A1*, *CYP1A2*, *CYP1B1*, and others. AHRR, sharing structural similarities with AHR and ARNT, may compete with the AHR to bind XRE. The AHRR–ARNT heterodimer is capable of binding with XRE, but without transactivate gene expression. However, AHRR may enhance the release of AHR–ARNT complex from the XRE sequence, resulting in inhibition of AHR-mediated signal transduction and, therefore, protecting against XME-mediated cardiotoxicity. AHR, aryl hydrocarbon receptor; Pol I, DNA polymerase I; TCDD, 2,3,7,8, tetrachlorodibenzo-p-dioxin; XME, xenobiotics-metabolizing enzyme.

Table 1 Baseline characteristics of the study groups

Baseline characteristics	Controls ($n = 819$) [n (%)]	Essential hypertension patients ($n = 1341$) [n (%)]	P-value
Age (mean ± SD)	56.2 ± 8.9	56.4 ± 10.2	0.63
Male	393 (49.1)	675 (52.9)	0.09
BMI (mean ± SD) (kg/m^2)	27.1 ± 7.4	27.7 ± 6.8	0.06
Antihypertensive medication use	–	979 (73.0)	–
Positive family history of hypertension	422 (57.3)	717 (64.0)	**0.003***
Number of smokers (ever/never)	314 (39.0)	407 (31.5)	**0.001***

*Bold values indicate statistically significant difference between the groups.

To evaluate genotype–phenotype correlations, we used the genotype and mRNA expression data available for 60 HapMap European individuals and the SNPexp v1.2 online tool (*http://app3.titan.uio.no/biotools/tool.php?app = snpexp*). The functionality of selected SNPs was also assessed *in silico* using the SNP Function Prediction tool developed by Xu and Taylor [51] and available online at the SNPinfo Web Server (*https://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm*).

Data analysis

The sample size for the study groups was estimated using statistical power calculations for a χ^2-test on the basis of allele and genotype frequencies of AHR pathway SNPs in European populations. An association analysis of the AHR pathway SNPs with EH risk could detect a difference of 4–7% in the genotype distributions between the cases and controls assuming 77–97% power and a 5% type I error ($\alpha = 0.05$) on the basis of the sample sizes of 1341 hypertensives and 819 healthy individuals.

Allele frequencies were estimated using the gene-counting method and the χ^2-test was used to identify significant departures from Hardy–Weinberg equilibrium (HWE). The distribution of alleles was analyzed by 2×2 contingency tables, and the distributions of the genotypes

and their combinations between patients and controls were evaluated by logistic regression analysis. Categorical variables such sex, smoking status, and family history of hypertension were also compared using the χ^2-test. These statistics were calculated using STATISTICA software for Windows 10.0 (StatSoft Inc., Tulsa, Oklahoma, USA). The association between genotypes and EH risk was determined by multiple logistic regression analysis to calculate odds ratios (ORs) with 95% confidence intervals (CIs) and adjusted for age and sex. Pairwise gene–gene interactions were evaluated using the log-likelihood ratio test (LRT) assuming codominant, dominant, recessive, and overdominant models and adjusted for age and sex. The calculations were carried out using the SNPassoc package for R [52].

As the dimensionality of the data is known to be the central problem in statistical analysis for gene–gene interactions in common disease [53,54], we used the multifactor dimensionality reduction method (MDR) [55–57], a data-mining bioinformatic approach, to investigate high-order gene–gene and gene–environment interactions in EH. We applied the model-based multifactor dimensionality reduction method (MB-MDR) [58] implemented in the mbmdr package for R [59] in our dataset. It is an extension of the popular MDR method, which enables the measurement of the association between multilocus genotypes and the phenotype, and provides a set of statistically significant interactions instead of a single best model. In the first step of the MB-MDR algorithm, association tests of each multilocus genotype combination and environment risk factor (cigarette smoking) with EH risk are performed using logistic regression. Then, each multilocus genotype was assigned to three risk categories: high, low, and no risk, respectively. In the second step of the algorithm, the association of pooled genotypes in low-risk and high-risk categories was evaluated through logistic regression analysis. Wald statistics were used to explore the significance of results and the interaction models were ranked by adjusted *P*-values in the third step. Then, the most significant high-order interaction between the predictors and EH risk was considered the best model and adjusted for multiple testing through 1000 permutations. Finally, we selected the best most promising interaction for further evaluation by the conventional MDR analysis (MDR 3.0.2, *http://sourceforge.net/projects/mdr/*) to assess the model's cross-validation consistency and prediction error. Permutation testing was used to test the significance of the reported measure of prediction accuracy and cross-validation consistency. A generalized linear model was used for the genotype–phenotype correlation analysis to evaluate the differences in the relative mRNA expression levels among carriers with different genotypes.

Results

Association analysis of AHR pathway SNPs with EH

A percentage of a positive family history of hypertension was significantly greater in EH patients versus healthy controls (Table 1). In contrast, the number of smokers was greater among the controls than the EH patients. No differences were found between the groups in other demographic characteristics. A departure from HWE was observed for rs2066853 in both cases ($P = 0.03$) and controls ($P = 0.03$) and also for rs762551 in controls ($P = 0.05$). The rest of the SNPs were in agreement with HWE in the study groups.

Table 2 shows the genotype and allele frequencies of AHR pathway SNPs. Allele and genotype frequencies in the studied groups were compatible with those reported in European populations. SNP rs2228099 showed an association with an increased risk of EH (OR = 1.20, 95% CI: 1.01–1.44, $P = 0.043$) in a dominant model, whereas polymorphism rs762551 was associated with a decreased disease risk in a recessive genetic model (OR = 0.68, 95% CI: 0.52–0.89, $P = 0.006$). The association of rs762551 with EH risk remained significant after correction for multiple testing ($P_{\text{correction}} = 0.05$). In the meantime, the association of SNP rs2228099 with EH did not reach statistical significance after correction for multiple testing ($P_{\text{correction}} = 0.39$).

Epistatic interactions between AHR pathway SNPs and the risk of EH

To assess gene–gene interactions determining hypertension susceptibility, first, we explored associations between AHR pathway pairwise genotype combinations and disease risk. The analysis identified 14 combinations of AHR pathway genotypes associated with EH risk at *P* less than or equal to 0.05. Supplementary Table 3 (Supplemental digital content 3, *http://links.lww.com/FPC/B136*) shows the overall genotype combinations associated with EH risk. Figure 2 summarizes plots of AHR pathway genotype combinations associated significantly with EH risk. Carriers of the 462IV *CYP1A1* × 432VL *CYP1B1* genotypes (Fig. 2a) had a significantly decreased risk of hypertension compared with carriers of the rest genotypes (OR = 0.49, 95% CI: 0.22–0.75, $P = 0.001$), showing an epistatic interaction between the loci at an overdominant genetic model. An overdominant model of the gene–gene interaction was also observed for the *CYP1A2* and *ARNT* loci (Fig. 2b). Genotype combination *NQO1* 187PP × *ARNT* 567CG (Fig. 2c) was associated with an increased EH risk (OR = 1.23, 95% CI: 1.01–1.50, $P = 0.04$), whereas the *NQO1* 187PP × *ARNT* 567CC/CG genotype combination showed an association with decreased disease risk (OR = 0.81, 95% CI: 0.67–0.98, $P = 0.03$).

In addition, the LRT was performed to look for epistatic interaction effects between AHR pathway SNPs on hypertension susceptibility. As can be seen from Table 3, SNPs rs2228099 and rs762551 showed significant individual effects on the risk of EH. The analysis identified epistatic interactions between *ARNT* and *CYP1A2* (overdominant model, $P_{\text{interaction}} = 0.008$), *CYP1A1* and *CYP1B1* (dominant model, $P_{\text{interaction}} = 0.001$), *AHR* and

Table 2 Genotype and allele frequencies of AHR pathway genes in EH patients and controls

Gene, polymorphism	Genotype, allele	Controls ($n=819$) [n (%)][a]	EH patients ($n=1341$) [n (%)][a]	P-value OR (95% CI)[b]	P-value OR_{adj} (95% CI)[c]
AHR, R554K (rs2066853)	554RR	658 (80.6)	1044 (79.0)	0.63	0.64
	554RK	142 (17.4)	252 (19.1)	1.12 (0.89–1.40)	1.12 (0.89–1.40)
	554KK	16 (2.0)	26 (2.0)	1.02 (0.55–1.92)	1.00 (0.53–1.88)
	554K	0.107	0.114	0.50	–
				1.07 (0.88–1.31)	
AHRR, P185A (rs2292596)	185PP	254 (31.4)	403 (31.2)	0.75	0.76
	185PA	408 (50.4)	636 (49.3)	0.98 (0.80–1.20)	0.98 (0.81–1.20)
	185AA	147 (18.2)	251 (19.5)	1.08 (0.83–1.39)	1.08 (0.83–1.39)
	185A	0.565	0.558	0.69	–
				0.98 (0.86–1.11)	
ARNT, 567C > G (rs2228099)	567CC	344 (43.2)	501 (38.8)	0.14	0.12
	567CG	351 (44.0)	618 (47.9)	1.21 (1.00–1.46)	1.21 (1.00–1.47)
	567GG	102 (12.8)	172 (13.3)	1.16 (0.87–1.53)	1.17 (0.88–1.55)
	567G	0.348	0.373	0.11	–
				1.11 (0.98–1.27)	
CYP1A1, I462V (rs1048943)	462II	691 (85.6)	1145 (86.6)	0.82	0.83
	462IV	112 (13.9)	171 (12.9)	0.92 (0.71–1.19)	0.92 (0.71–1.19)
	462VV	4 (0.5)	6 (0.5)	0.91 (0.25–3.22)	0.97 (0.27–3.46)
	462V	0.074	0.069	0.50	–
				0.92 (0.72–1.17)	
CYP1A2, −163C > A (rs762551)	−163AA	387 (47.3)	635 (47.6)	0.02	**0.015**
	−163AC	322 (39.3)	571 (42.8)	1.08 (0.90–1.30)	1.09 (0.90–1.31)
	−163CC	110 (13.4)	128 (9.6)	0.71 (0.53–0.94)	0.71 (0.53–0.94)
	154C	0.330	0.311	0.19	–
				0.92 (0.80–1.05)	
CYP1B1, V432L (rs1056836)	432VV	278 (33.9)	424 (31.8)	0.56	0.60
	432VL	390 (47.6)	651 (48.8)	1.09 (0.90–1.33)	1.09 (0.90–1.33)
	432LL	151 (18.4)	260 (19.5)	1.13 (0.88–1.45)	1.12 (0.87–1.44)
	432L	0.422	0.439	0.30	–
				1.07 (0.94–1.21)	
NQO1, P187 S (rs1800566)	187PP	506 (63.0)	836 (62.7)	0.51	0.51
	187PS	252 (31.4)	437 (32.8)	1.05 (0.87–1.27)	1.04 (0.86–1.26)
	187SS	45 (5.6)	61 (4.6)	0.82 (0.55–1.22)	0.82 (0.55–1.22)
	187S	0.212	0.208	0.75	–
				0.98 (0.84–1.14)	

AHR, aryl hydrocarbon receptor; CI, confidence interval; EH, essential hypertension; OR, odds ratio.
[a]Absolute number and percentage of individuals with a particular genotype.
[b]OR with 95% CIs (codominant genetic model).
[c]OR with 95% CIs adjusted for age and sex.
Statistically significant P-value with 2 *d.f.* is represented in bold.

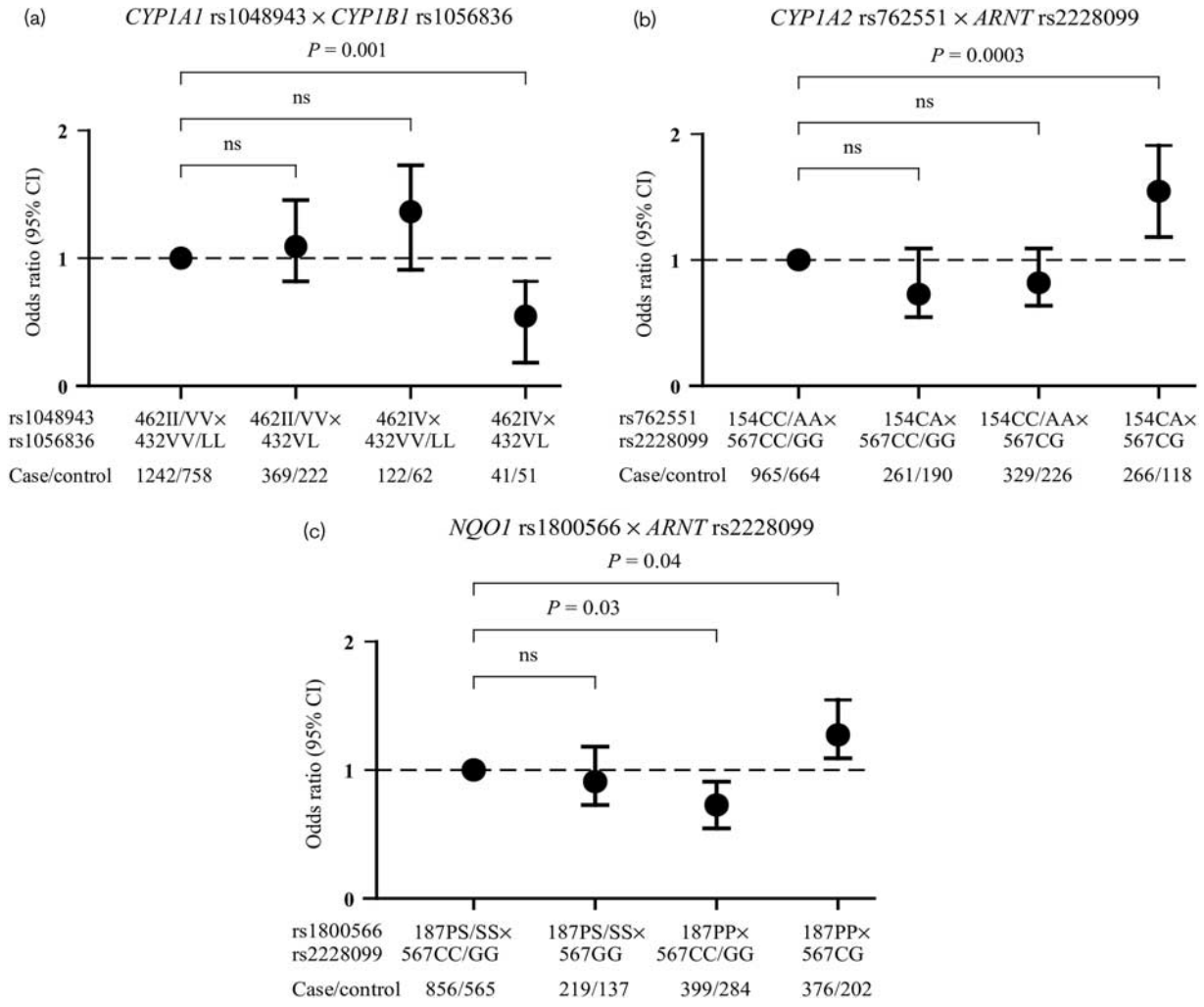
NQO1 (recessive model, $P_{interaction}=0.004$), *ARNT* and *NQO1* (overdominant model, $P_{interaction}=0.013$), and *AHRR* and *CYP1A1* (recessive model, $P_{interaction}=0.041$).

High-order gene–gene and gene–environment interactions in hypertension susceptibility

The MB-MDR method was applied to the dataset to investigate high-order gene–gene and gene–environment interactions contributing toward hypertension. Two-order, three-order, and four-order interaction models among seven SNPs and smoking status were analyzed and then adjusted for age and sex. Table 4 shows the best AHR pathway gene–gene and gene–smoking interactions associated significantly with the risk of hypertension. Cigarette smoking, rs1048943, rs762551, rs1056836, and rs2228099 were included as significant contributing factors in 19, 18, 13, 13, and 11 interaction models, respectively (a detailed list of all 27 interaction models is shown in Supplementary Table 4, Supplemental digital content 4, *http://links.lww.com/FPC/B137*).

High-order gene–gene and gene–environment interactions obtained by the conventional MDR method are shown in Table 5 and Fig. 3. The best interaction model associated with the risk of EH included rs1048943 (*CYP1A1*), rs762551 (*CYP1A2*), rs1056836 (*CYP1B1*), and cigarette smoking (Wald statistic = 31.51, $P_{permutation}<0.001$). The interaction between rs1048943, rs762551, rs1056836, and cigarette smoking showed the highest cross-validation consistency (100%) and the lowest prediction error (45.7%). The dendrogram (Fig. 3b) shows a complex pattern of gene–gene and gene–environment interactions determining EH susceptibility. Cigarette smoking and rs762551 had the highest degree of redundancy in their interactions and were also found to interact with rs1056836 in the same manner, but to a lesser degree. In the interaction graph (Fig. 3c), cigarette smoking and rs762551 eliminated 0.48 and 0.36% of class entropy, respectively, thereby having the largest univariate effects. A substantial percentage of entropy (0.11%) was explained by rs762551 and smoking, indicating a redundant (antagonistic) interaction between them. SNPs rs1048943 and rs1056836 showed relatively small percentages of entropy when considered independently (0.03 and 0.11%, respectively), whereas a large percentage of entropy was explained by their interactions with smoking and rs762551.

Fig. 2



Plots of interactions between AHR pathway genotypes associated with essential hypertension at different genetic models. (a) *CYP1A1* × *CYP1B1* interactions; (b) *CYP1A2* × *ARNT* interactions; (c) *NQO1* × *ARNT* interactions. AHR, aryl hydrocarbon receptor; CI, confidence interval; ns, not significant.

Then we carried out an MB-MDR analysis stratified by smoking status, which enabled identification of specific combinations of SNPs influencing disease susceptibility in exposed and unexposed individuals. Table 6 shows the best models of gene–gene interactions associated with EH in cigarette smokers and nonsmokers. Detailed data on genotype combinations associated with EH risk in smokers and nonsmokers are shown in Supplementary Table 5 (Supplemental digital content 5, *http://links.lww.com/FPC/B138*). There are considerable differences in gene–gene interactions between smokers and nonsmokers, suggesting that exposure to PAHs is an important factor modifying the association between AHR pathway genes and hypertension susceptibility.

Genotype–phenotype correlation analysis in AHR pathway genes

Data on both mRNA expression levels and genotypes for AHR pathway gene polymorphisms were available from 60 HapMap individuals of European descent. The levels of *ARNT* mRNA were correlated with the rs2228099 locus ($P = 0.0003$, Fig. 4c). *AHRR* mRNA expression levels showed an increased trend for rs2292596 ($P = 0.007$, Fig. 4b). An increased trend was also observed in *CYP1B1* mRNA expression levels and rs1056836 ($P = 0.01$, Fig. 4f). A board-line correlation ($P = 0.08$) of *NQO1* mRNA expression levels occurred with the rs1800566 (Fig. 4g). No significant correlations were found between both *AHR* and *CYP1A2* expression levels and SNPs rs2066853 and rs762551, respectively (Fig. 4a and e). Moreover, an in-silico functional analysis carried out using the SNP Function Prediction tool has confirmed a regulatory potential for the *ARNT*, *AHRR*, and *CYP1B1* loci (Supplementary Table 6, Supplemental digital content 6, *http://links.lww.com/FPC/B139*). The *CYP1A1* and *NQO1* loci also showed a regulatory potential with a possibly damaging effect on rs1800566. An SNP

Table 3 Epistatic interactions between AHR pathway SNPs in EH

SNPs	Genetic models	*AHR* rs2066853	*AHRR* rs2292596	*ARNT* rs2228099	*CYP1A1* rs1048943	*CYP1A2* rs762551	*CYP1B1* rs1056836	*NQO1* rs1800566
AHR rs2066853	odominant	0.644	0.746	0.838	0.320	0.483	0.874	**0.017**
	Dominant	0.379	0.738	0.577	0.476	0.863	0.753	0.166
	Recessive	0.944	0.643	0.281	–	0.886	–	**0.004***
	Overdominant	0.348	0.346	0.706	0.468	0.589	0.433	0.065
AHRR rs2292596	odominant	0.802	0.759	0.970	0.255	0.257	0.587	0.466
	Dominant	0.967	0.930	0.606	0.960	0.383	0.494	0.102
	Recessive	0.992	0.468	0.966	**0.041**	0.105	0.252	0.279
	Overdominant	0.647	0.626	0.674	0.523	0.320	0.699	0.319
ARNT rs2228099	odominant	0.824	0.750	0.124	0.584	**0.041**	0.696	**0.041**
	Dominant	0.605	0.753	**0.043**	0.161	**0.012**	0.287	0.409
	Recessive	0.874	0.949	0.702	0.805	0.134	0.500	0.157
	Overdominant	0.545	0.752	0.083	0.496	**0.008***	0.829	**0.013***
CYP1A1 rs1048943	odominant	0.760	0.736	0.807	0.833	0.944	**0.002**	0.530
	Dominant	0.470	0.923	0.521	0.549	0.795	**0.001***	0.380
	Recessive	0.999	0.977	0.973	0.974	0.416	0.210	0.641
	Overdominant	0.459	0.465	0.501	0.547	0.983	**0.003**	0.267
CYP1A2 rs762551	odominant	0.672	0.665	0.122	0.798	**0.014**	0.247	0.120
	Dominant	0.929	0.999	0.915	0.701	0.912	0.418	0.969
	Recessive	0.936	0.414	0.679	0.927	**0.005**	0.058	0.411
	Overdominant	0.375	0.518	0.080	0.552	0.097	0.980	0.879
CYP1B1 rs1056836	odominant	0.705	0.808	0.448	0.855	0.510	0.599	0.864
	Dominant	0.435	0.908	0.207	0.574	0.861	0.321	0.778
	Recessive	0.987	0.582	0.678	0.970	0.457	0.600	0.597
	Overdominant	0.607	0.712	0.467	0.678	0.648	0.605	0.451
NQO1 rs1800566	odominant	0.599	0.753	0.500	0.748	0.550	0.640	0.502
	Dominant	0.941	0.967	0.967	0.882	0.965	0.893	0.915
	Recessive	0.909	0.497	0.697	0.690	0.333	0.697	0.278
	Overdominant	0.561	0.563	0.554	0.624	0.590	0.564	0.539

Gene–gene interactions are evaluated using SNPassoc package for R [52].
The upper part of the matrix includes the *P*-values for epistatic interactions evaluated using the log-likelihood ratio (LRT) test. The diagonal includes the *P*-values from LRT for the crude effect of each SNP. The lower triangle includes the *P*-values from LRT comparing the two-SNP additive likelihood with the best of the single-SNP models. *P*-values are adjusted for age and sex.
AHR, aryl hydrocarbon receptor; EH, essential hypertension; SNP, single-nucleotide polymorphism.
Statistically significant *P*-values for SNP–SNP interactions are in bold.
*Most significant *P*-values for a particular model are indicated in bold.

Table 4 Best gene–gene and gene–smoking interactions associated significantly with the risk of EH[a]

G × G/G × E interaction models		NH	β H	WH	NL	β L	WL	P_{perm}
Two-order interaction models								
1	*CYP1A1* rs1048943 × smoking	1	0.263	7.79	2	-0.342	12.28	0.003
2	*CYP1A2* rs762551 × smoking	2	0.323	11.83	2	−0.390	12.08	0.003
3	*CYP1A2* rs762551 × *ARNT* rs2228099	1	0.422	12.53	1	−0.598	7.66	0.011
Three-order interaction models								
1	*CYP1A1* rs1048943 × *CYP1B1* rs1056836 × smoking	2	0.277	8.10	2	−0.608	22.12	< 0.001
2	*CYP1A1* rs1048943 × *CYP1A2* rs762551 × *CYP1B1* rs1056836	0	NA	NA	4	−0.636	21.26	0.001
3	*ARNT* rs2228099 × *NQO1* rs1800566 × smoking	1	0.264	4.42	4	−0.567	19.04	0.003
Four-order interaction models								
1	*CYP1A1* rs1048943 × *CYP1A2* rs762551 × *CYP1B1* rs1056836 × smoking	2	0.345	7.49	4	−0.896	31.51	< 0.001
2	*ARNT* rs2228099 × *CYP1A1* rs1048943 × *CYP1B1* rs1056836 × smoking	1	0.537	5.02	4	−0.617	22.07	0.007
3	*ARNT* rs2228099 × *CYP1A1* rs1048943 × *NQO1* rs1800566 × smoking	1	0.274	4.21	3	−0.845	19.15	0.008

Models are obtained using the model-based multifactor dimensionality reduction method, MB-MDR package for R [59].
β H, regression coefficient for high-risk exposition in the step 2 analysis; β L, regression coefficient for low-risk exposition in the step 2 analysis; EH, essential hypertension; NA, not available; NH, number of significant high-risk genotypes in the interaction; NL, number of significant low-risk genotypes in the interaction; P_{perm}, permutation *P*-value for the interaction model. The models were adjusted for age and sex; WH, Wald statistic for the high-risk category; WL, Wald statistic for the low-risk category.
[a]The full list of statistically significant models for gene–gene and gene–smoking interactions is presented in Supplementary Table 1 (Supplemental digital content 1, *http://links.lww.com/FPC/B134*).

rs762551 is located at binding sites for transcription factors such as, for instance, general transcription factor IIIA (V$AP2ALPHA_01) and paired box gene 2 (V $PAX2_01), suggesting a functional significance of the *CYP1A2* polymorphism.

Discussion

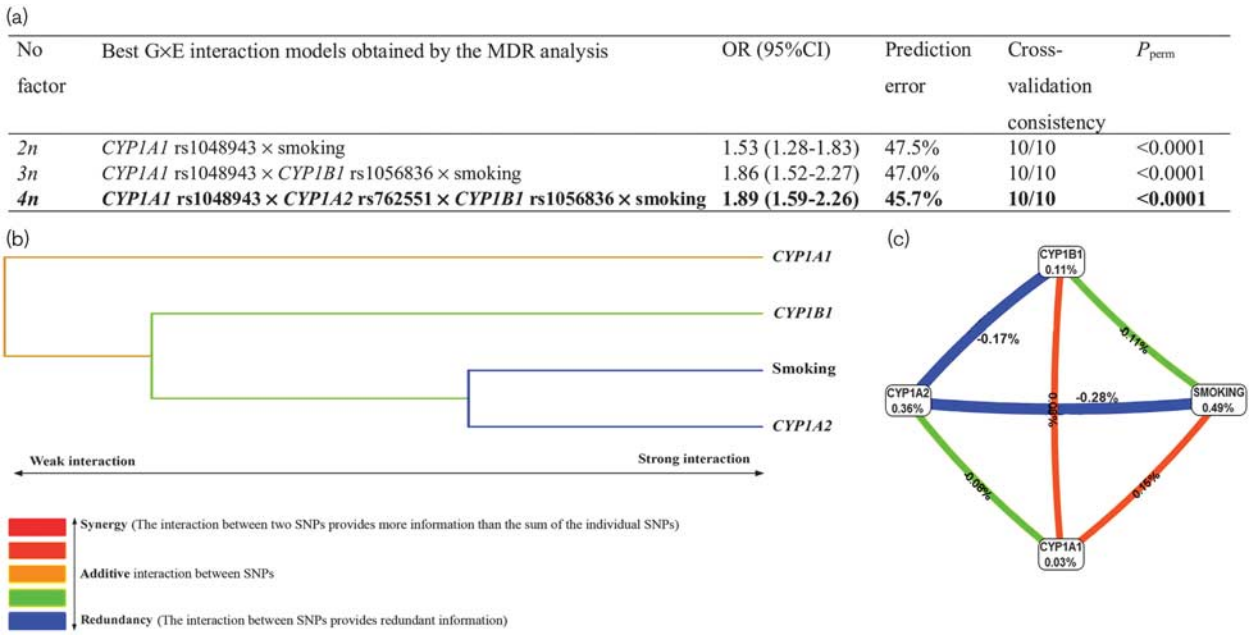
In our pilot study, we investigated whether common polymorphisms of the AHR signaling pathway, an inherited determinant for PAH-mediated cardiovascular toxicity, are comprehensively involved in the molecular

Table 5 Cross-validation statistics for best models of gene–gene and gene–smoking interactions in EH

G × G/G × E interaction models	OR (95% CI)	Testing balanced accuracy	Cross-validation consistency	P_{perm}
Two-order interaction models				
1 *CYP1A1* rs1048943 × smoking	1.53 (1.28–1.83)	0.543	10/10	< 0.0001
2 *CYP1A2* rs762551 × smoking	1.46 (1.23–1.74)	0.539	10/10	< 0.0001
3 *CYP1A2* rs762551 × *ARNT* rs2228099	1.38 (1.16–1.63)	0.513	10/10	0.0003
Three-order interaction models				
1 *CYP1A1* rs1048943 × *CYP1B1* rs1056836 × smoking	1.86 (1.52–2.27)	0.530	10/10	< 0.0001
2 *CYP1A1* rs1048943 × *CYP1A2* rs762551 × *CYP1B1* rs1056836	2.18 (1.70–2.79)	0.503	10/10	< 0.0001
3 *ARNT* rs2228099 × *NQO1* rs1800566 × smoking	1.66 (1.40–1.97)	0.529	10/10	< 0.0001
Four-order interaction models				
1 *CYP1A1* rs1048943 × *CYP1A2* rs762551 × *CYP1B1* rs1056836 × smoking	1.89 (1.59–2.26)	0.525	10/10	< 0.0001
2 *ARNT* rs2228099 × *CYP1A1* rs1048943 × *CYP1B1* rs1056836 × smoking	1.94 (1.63–2.31)	0.512	10/10	< 0.0001
3 *ARNT* rs2228099 × *CYP1A1* rs1048943 × *NQO1* rs1800566 × smoking	1.85 (1.56–2.20)	0.515	10/10	< 0.0001

Models are obtained using the multifactor dimensionality reduction method, version 3.0.2.
CI, confidence interval; OR, odds ratio; P_{perm}, permutation *P*-value for the interaction model.

Fig. 3



High-order gene–gene (G × G) and gene–environment (G × E) interaction analyses for the AHR pathway SNPs in essential hypertension (data obtained by Multifactor Dimensionality Reduction package, version 3.0.2). (a) Cross-validation statistics for the best G × E interaction models underlying essential hypertension susceptibility. The best four-order interaction model with the maximum cross-validation consistency and the minimum prediction error is indicated in bold. (b) Interaction dendrogram. The lines comprise a spectrum of lines representing a continuum from synergy to redundancy of G × G and G × E interactions with a variable strength. Brown lines represent the midway point between synergy and redundancy (additive interaction). On the redundancy end of the spectrum, the highest degree is represented by blue, with a lesser degree represented by green. The synergy lines range from red, representing a high degree of synergism (not present in the dendrogram), to orange, representing a lower degree of synergism. (c) Interaction entropy graph. Each SNP is shown in a rectangle box with the percent of entropy (main effect). Two-way G × G and G × E interactions are shown as color lines accompanied by a percent of entropy (interaction effect). CI, confidence interval; MDR, multifactor dimensionality reduction; OR, odds ratio; P_{perm}, permutation *P*-value for the interaction model; SNP, single-nucleotide polymorphism.

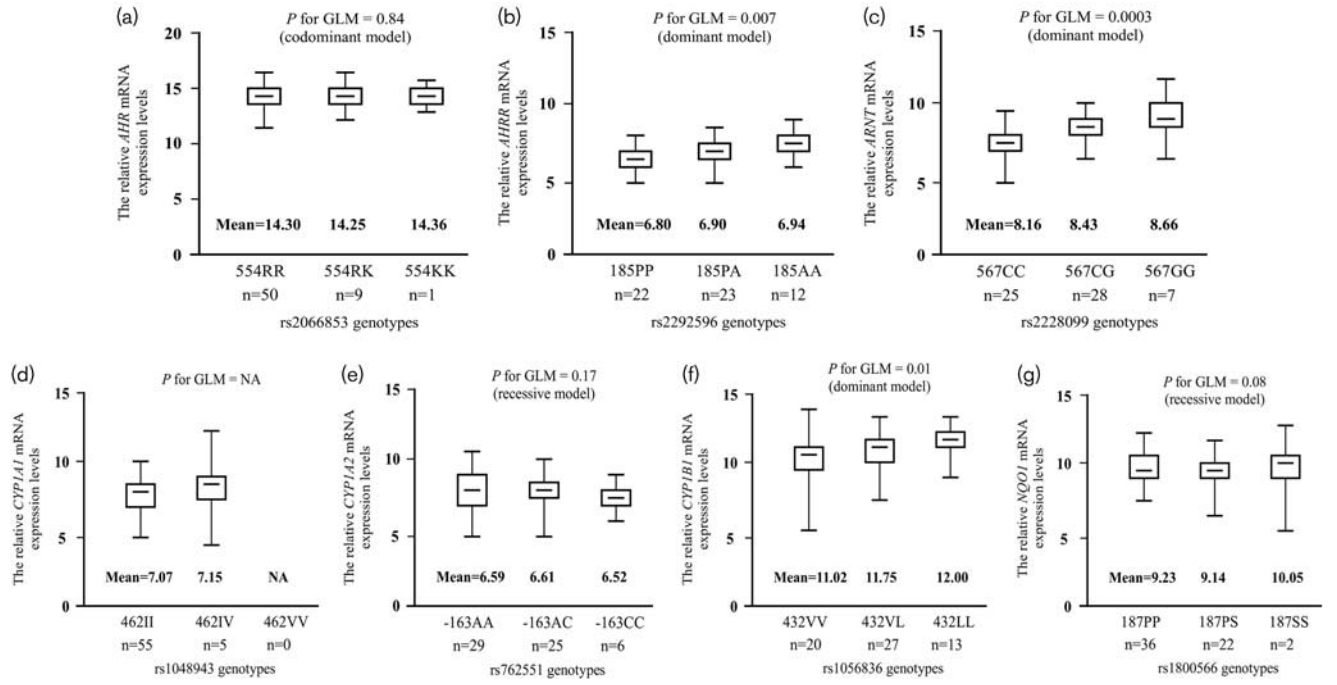
basis of EH. The study showed for the first time that polymorphic genes for the AHR pathway are important determinants of genetic susceptibility to EH. We found that SNP rs762551 of *CYP1A2* is associated with a decreased risk of EH. CYP1A2 is a PAHs-induced cytochrome P450 enzyme metabolizing xenobiotics such as PAHs, caffeine, aflatoxin B1, and acetaminophen [33]. Polymorphism rs762551 is known to influence caffeine metabolism and has been found to be associated with the risk of myocardial infarction [60], BP variation, and hypertension [61]. This polymorphism is known to be in a linkage disequilibrium (LD) with an SNP rs1378942

Table 6 Best models of gene–gene interactions associated with EH stratified by cigarette smoking

G × G interaction models		NH	β H	WH	NL	β L	WL	P_{perm}
Two-order, three-order, and four-order interaction models in smokers								
1	*CYP1A1* rs1048943 × *CYP1B1* rs1056836	1	0.434	5.98	2	−0.499	9.93	0.014
2	*ARNT* rs2228099 × *CYP1A1* rs1048943	0	NA	NA	1	−0.952	8.20	0.03
3	*ARNT* rs2228099 × *CYP1A2* rs762551	0	NA	NA	1	−1.232	8.25	0.05
4	*CYP1A1* rs1048943 × *CYP1A2* rs762551 × *CYP1B1* rs1056836	1	0.440	3.08	4	−0.795	20.02	< 0.002
5	*ARNT* rs2228099 × *CYP1A1* rs1048943 × *CYP1A2* rs762551	0	NA	NA	2	−1.520	15.04	0.004
6	*CYP1A1* rs1048943 × *CYP1B1* rs1056836 × *NQO1* rs1800566	1	0.364	3.04	3	−1.344	14.77	0.01
7	*AHR* rs2066853 × *CYP1A1* rs1048943 × *CYP1A2* rs762551 × *CYP1B1* rs1056836	1	0.507	3.75	3	−1.637	19.81	0.004
8	*ARNT* rs2228099 × *CYP1A1* rs1048943 × *CYP1A2* rs762551 × *CYP1B1* rs1056836	0	NA	NA	3	−1.988	16.02	0.03
9	*ARNT* rs2228099 × *AHRR* rs2292536 × *CYP1A1* rs1048943 × *NQO1* rs1800566	1	0.631	4.401	4	−0.951	15.25	0.034
Two-order, three-order, and four-order interaction models in nonsmokers								
1	*ARNT* rs2228099 × *CYP1A2* rs762551	1	0.460	8.45	1	−0.254	2.83	0.043
2	*CYP1A2* rs762551 × *NQO1* rs1800566	0	NA	NA	2	−0.658	8.61	0.049
3	*ARNT* rs2228099 × *CYP1A2* rs762551 × *CYP1B1* rs1056836	3	0.580	10.45	3	−0.826	12.39	0.096
4	*ARNT* rs2228099 × *AHRR* rs2292536 × *CYP1B1* rs1056836	0	NA	NA	3	−0.697	11.80	0.126
5	*AHR* rs2066853 × *ARNT* rs2228099 × *AHRR* rs2292536 × *CYP1B1* rs1056836	0	NA	NA	5	−0.847	21.41	0.016
6	*ARNT* rs2228099 × *CYP1A2* rs762551 × *CYP1B1* rs1056836 × *NQO1* rs1800566	2	1.216	7.41	6	−0.769	22.00	0.022
7	*AHR* rs2066853 × *ARNT* rs2228099 × *CYP1A2* rs762551 × *CYP1B1* rs1056836	2	0.772	6.27	5	−0.786	19.84	0.04

Models are obtained using the model-based multifactor dimensionality reduction method.
β H, regression coefficient for high-risk exposition in the step 2 analysis; β L, regression coefficient for low-risk exposition in the step 2 analysis; EH, essential hypertension; NA, not available; NH, number of significant high-risk genotypes in the interaction; NL, number of significant low-risk genotypes in the interaction; P_{perm}, permutation *P*-value for the interaction model; WH, Wald statistic for the high-risk category; WL, Wald statistic for the low-risk category.

Fig. 4



The relative expression levels of AHR pathway genes mRNA by different genotypes in 60 HapMap individuals of European descent. Expression profiles were analyzed by the HumanWG-6 Expression BeadChip. The effects of SNPs such as (a) *AHR* rs2066853, (b) *AHRR* rs2292596, (c) *ARNT* rs2228099, (d) *CYP1A1* rs1048943, (e) *CYP1A2* rs762551, (f) *CYP1B1* rs1056836, and (g) *NQO1* rs1800566 on mRNA levels of corresponding genes are evaluated by generalized linear models (GLMs). Absence of carriers for 462VV CYP1A1 genotypes in this HapMap sample did not enable the evaluation of the correlation analysis for rs1048943. NA, not available.

($r^2 = 0.63$, HapMap CEU), located in the gene cluster including the *CYP1A2* gene, showed the strongest association ($P = 1 \times 10^{-23}$) with diastolic BP in a sample of 34 433 patients of European ancestry [46]. Furthermore, the relationship between rs762551 and hypertension risk was shown in the study of Guessous *et al.* [47], who observed that this negative association occurred in nonsmokers and is modified by reported caffeine intake. Thus, the present study provided additional evidence that *CYP1A2* is an important susceptibility gene for EH.

The present study was the first to show that polymorphism rs2228099 of the *ARNT* gene could be a novel susceptibility gene to hypertension. Although association of the SNP with the risk of EH did not reach statistical significance after correction for multiple testing, rs2228099 in combinations with other AHR pathway SNPs showed combined effects on EH risk. Like AHR, the AHR translocator is a member of the basic helix–loop–helix transcription protein superfamily, which is necessary for dimerization with AHR [37,39]. ARNT associates with ligand-bound AHR to form a protein complex for binding to the xenobiotic response element in enhancers of target genes such as those encoding XMEs as well as genes associated with oxidative stress, fat metabolism and transport, and cell proliferation [62]. Besides participation in the AHR signaling, ARNT is also known as hypoxia-inducible factor-1β, a transcriptional factor for vascular endothelial cells that regulates genes involved in response to hypoxia [63], a pathological process that plays a role in the pathogenesis of hypertension. SNP rs2228099 represents a synonymous change Val–Val (C > G) at codon 189 in exon 7 of the *ARNT* gene. No functional information is available for this polymorphism in dbSNP (*http://www.ncbi.nlm.nih.gov/projects/SNP/*). Although this silent SNP is not accompanied by the amino acid change in the ARNT protein, genotypes G and GG are associated with increased expression of ARNT mRNA compared with genotype CC, as it has been shown by the genotype–phenotype correlation analysis in our study. It can be assumed that the carriers with genotypes G and GG of *ARNT* may have favorable conditions for chronic and persistent activation of the AHR-ARNT complex resulting in the induction of XMEs. It cannot be excluded that this polymorphism is in LD with another yet unidentified functional SNP of *ARNT* that could be related to BP variation and/or hypertension risk. For instance, SNP rs2228099 is in strong LD with rs12410394, which has been found to be associated with the risk of colorectal neoplasia [64], the finding pointing out, on the one hand, the functionality of this SNP, and on the other, the link of this locus with PAHs-related cancer susceptibility.

The present study did not observe an association between EH and the polymorphism of AHR, the main player and initiator of the signaling cascade. This was not surprising as AHR, like many such proteins, induced as part of the stress response to environmental toxicants, is evolutionarily conserved, and any functional alterations in the AHR cascade appear to be critical to the evolution, at least for humans. Apparently, a relatively low rate of mutations and functional polymorphisms in the *AHR* gene [32,40] confers advantages in the bridging between AHR and its regulated XMEs in maintaining the optimal setting of the host for adaptive responses to PAHs and other chemical compounds in the constantly changing environment.

The MB-MDR method provided additional evidences indicating that (a) the integrated function of the AHR pathway genes may promote a coordinated metabolism of PAH xenobiotics and (b) the AHR signaling pathway loci and their related XMEs are collectively involved in the molecular basis of EH. A majority of the modeled gene–gene interactions associated with EH risk comprise genes such as *ARNT*, *CYP1A1*, *CYP1A2*, *CYP1B1*, and *NQO1*, findings consistent with the results obtained in the previous stages of our study. The analysis for gene–gene interactions carried out using the SNPassoc package enabled the identification of SNPs exerting significant effects on disease risk only in combinations. Overall, 27 statistically two-order, three-order, and four-order interaction models have been identified to influence the risk of EH. In particular, significant gene–gene interactions were found between *CYP1A1* and *CYP1B1*, *AHR* and *NQO1*, *ARNT* and *NQO1*, *ARNT* and *CYP1A2*, and *AHRR* and *CYP1A1*. These findings point to epistasis; the effect of one gene may not be disclosed if the effect of another gene is not considered [57]. Interactions between the loci suggest that the gene–gene effect on disease risk may be driven by a true interaction, rather than by the main effect from each gene alone. Notably, AHR pathway SNPs showed complex hierarchic interactions, as identified by the MDR method (Fig. 3). The observed gene–gene interactions make mechanistic sense because these genes may be collectively involved in the pathogenesis of EH through the same detoxification pathway.

It is known that cigarette smoking is a model of chronic AHR activation in humans [65]. Notably, a majority of interaction models identified by MDR included cigarette smoking as a covariate, indicating an importance of gene–environment interactions for the penetration of hypertension phenotype. The best gene–smoking MB-MDR interaction model associated with EH risk comprised cigarette smoking and *CYP1A1*, *CYP1A2*, *and CYP1B1*. Furthermore, MDR analysis stratified by smoking status enabled the identification of specific SNPs combinations influencing hypertension susceptibility in exposed and unexposed individuals. Interactions between *ARNT*, *CYP1A1*, *CYP1A2*, and *CYP1B1* were significantly associated with disease susceptibility in smokers, whereas *ARNT*, *AHRR*, *CYP1B1*, and *NQO1* gene polymorphisms contributed toward the disease in nonsmokers. Differences in the spectrum of interacted genes between smokers and nonsmokers apparently reflect that the molecular mechanisms by which AHR pathway SNPs contribute toward hypertension may be distinguished considerably depending on whether the individual is exposed or not exposed to PAHs. It is permissible to assume that the mechanisms of hypertension in smoker individuals are related to an enhanced metabolic activation of PAHs by the CYP1 family of enzymes such as 1A1, 1A2, and 1B1. For instance, a carriage of common 'high-risk genotype' combinations such as *CYP1A1* 462II × *CYP1B1* 432VV and

AHR 554RR×*CYP1A1* 462II×*CYP1A2* 164AA×*CYP1B1* 432VV (Supplementary Table 5, Supplemental digital content 5, *http://links.lww.com/FPC/B138*, A) in cigarette smokers could promote xenobiotics' toxification (the conversion of a chemical compound into a more toxic form than a parent molecule). In contrast, a carriage of common genotype combinations, for example, *ARNT* 567CC×*CYP1A2* 164CC, *CYP1A1* 462II×*CYP1A2* 164CC×*CYP1B1* 432VL or *ARNT* 567CC×*CYP1A1* 462II×*CYP1A2* 164CC (Supplementary Table 5, Supplemental digital content 5, *http://links.lww.com/FPC/B138*, A) is associated with a decreased EH risk. This association may be explained by decreased activation of the AHR cascade and CYP1A2 induction in PAH-exposed individuals ('low-activity genotypes' 567CC and 164CC are associated with decreased mRNA levels of *ARNT* and *CYP1A2*, respectively).

The allele 462Val of the *CYP1A1* gene is known to be associated with a significant increase in the enzyme activity and induction [66]. In this context, it is unclear why genotype 462IV showed a protective effect against EH risk even in the carriers of 'high-risk genotypes' such as *CYP1A2* 164AA or *CYP1B1* 432VV. It should be noted that a similar 'protective effect' of allele 462Ile *CYP1A1* was found against the risk of lung cancer [67,68]. An interesting finding is that nonsmoker individuals with the most common genotype combination, that is, *ARNT* 567CG×*CYP1A2* 164AC, were at a higher risk of EH. Apparently, a carriage of these 'high-activity genotypes' (Fig. 4 and Supplementary Table 6, Supplemental digital content 6, *http://links.lww.com/FPC/B139*) promotes an enhanced activation of the AHR cascade and, therefore, the increased risk of hypertension could be related to CYP1A2-mediated cardiovascular toxicity because of an exposure to the background levels of PAHs present in the environment. In nonsmokers, the protective effects of genotype combinations *AHRR* 185PA×*ARNT* 567CC×*CYP1B1* 432VV and *AHRR* 185PA×*ARNT* 567GG×*CYP1B1* 432LL against hypertension risk can be explained by the fact that 'the high-activity' of genotype *CYP1B1* 432VV could be compensated by 'the low-activity' of genotype *ARNT* 567CC and vice versa, thus decreasing AHR pathway activation and associated cardiovascular toxicity.

The present study has some limitations. A majority of the associations of AHR pathway SNPs with hypertension susceptibility were not strong, thereby showing small-to-modest effects of these genes on disease phenotype. The study focused only on major XMEs genes regulated by the pathway, whereas genes under transcriptional regulation from the AHR-ARNT heterodimer also include at least *GSTA2*, *UGT1A1*, *UGT1A6*, and *NFE2L2* [35,69]. Because not all AHR pathway SNPs were selected for this study, our findings do not allow any definitive conclusion to be made as yet on the comprehensive contribution of the genes toward hypertension susceptibility. It is safe to assume that the simultaneous examination of all tag-SNPs within these genes may provide more comprehensive genetic profiling of the AHR pathway in EH. Therefore, the hypothesis that AHR pathway genes are collectively involved in the molecular basis of EH requires further confirmation in other studies. Nevertheless, on the basis of the study findings, it is plausible to assume that individuals with increased activity of the AHR cascade and enhanced toxification of xenobiotics are at increased risk for EH related to PAH exposure. Undoubtedly, a complete understanding of the causative role of environmental PAHs in the development of hypertension will require more experimental and clinical studies to answer the question of whether the toxicogenomic mechanisms are an important part of disease pathogenesis.

Although the exact role of AHR signaling in the regulation of BP remains to be elucidated, undoubtedly, the pathway could serve as a target in the treatment and prevention of hypertension and related diseases. In particular, pharmacological approaches that antagonize the AHR signaling pathway with a focus on the adverse effects of toxic AHR-ligands could decrease cardiovascular toxicity and benefit patients with hypertension and associated diseases. For instance, Resveratrol, a dietary antioxidant supplement with a natural substance, would be a potential candidate as a means of prevention of AHR-mediated toxicity of smoking and environmental pollution on a widespread scale [65]. Further ecological and pharmacological genomics studies are required to provide deeper insights into the roles of the AHR pathway genes in responses to environmental xenobiotics and will identify effective therapeutic options for the management of hypertension at population and individual levels.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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