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16

Electrocardiogram (ECG) Abnormality Among Residents in Arseniasis-Endemic and Non-Endemic Areas of Southwestern Taiwan – A Study of Gene-Gene and Gene-Environment Interactions

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1. Introduction

Natural occurrence of arsenic in groundwater is found in the Americas, European, Western Africa, and Asia including Taiwan, Japan, southern Thailand and China where in some areas, drinking water supplies are primarily based on groundwater resources [2]. For general population in southwestern coast Taiwan, the major arsenic exposure resource is the ingestion of arsenic contaminated groundwater. The residents have used high-arsenic contaminated well water for drinking and cooking for many decades since early 1910s. The tap water supply system was implemented in the early 1960s, however artesian well water has not been used for drinking or cooking until mid-1970s [3].

Because arsenic toxicity operates in a highly nonlinear manner and different levels of exposure measurements applied result in large discrepancy across studies and made it difficult to come up a reliable dose-response relationship for arsenic hazard. There is a long-standing observation of individual variability in susceptibility to arsenic toxicity [4] and this variation may be partly due to differences in age and sex distribution across areas, and also individual arsenic metabolism capabilities [5,6]. Inter-individual differences in the speciation and amounts of arsenic metabolites have been reported among subjects chronically exposed to arsenic [7] and significant genetic determinants of arsenic metabolism was supported by epidemiological study [8]. Toenail and hair arsenic has been reported to provided an integrated measure of internal arsenic exposure [9]. However epidemiologic studies showed that external contamination lead to overestimation of internal dose and urinary arsenic concentration seems to be a better marker than concentrations in drinking water[10]. Growing epidemiological evidence also suggests that some factors such as age, sex and genetic susceptibility are related to its metabolism and can be important predictors for arsenic-related health hazard [11,12].

1.1 Nature history of cardiovascular disease (CVD)

Heart disease or cardiovascular disease is defined as the class of disease that involved the cardiac or blood vessels including arteries and veins. Although the term technically refers to

any disease that affects the cardiovascular system, it is usually to refer to those related to atherosclerosis and arterial disease since they shared similar conditions of causes, mechanisms and treatments[13]. The primary underlying disease process that leads to atherosclerosis is the deposition of lipid on the arterial surface progress to form plaques that reduced blood flow and induced blood clots that blocked flow entirely [14].

Most countries face high and increasing rates of cardiovascular disease. In United States, mortality from heart and hypertensive diseases was greater than mortality from neoplasm. In recent years, cardiovascular risk in women has been increasing and has killed more women than breast cancer [15]. The estimated age-adjusted mortalities of cardiovascular disease in US is 152.1 per 100,000 in year 2002 and is 48.3 in Taiwan 2005 [16]. By the time that heart problems are detected, the underlying causes, atherosclerosis, is usually quite advanced, have progressed for decades [17]. Therefore increased emphasis on preventing atherosclerosis by modifying risk factors is remained important.

1.2 Arsenic-related CVD

Chronic arsenic exposure can lead to hyperkeratosis and loss of skin pigmentation as well as cancers of the skin, bladder, and lung. The international Agency for Research on Cancer [18] and the U.S. EPA [19] have classified arsenic as a group 1 and group A carcinogen based on human evidence. However, the mechanism of action for iAs-induced carcinogenicity is not known [20]. Cardiovascular death is the major cause of mortality worldwide, and a small increased risk may imply a large quantity of excess mortality [21]. Arsenic has also been shown to be a major risk factor of an unique form of peripheral vascular disease, Blackfoot disease (BFD) [22,23], especially in southwest Taiwan. Although the etiology of BFD development is still unclear, the dose-response relationships between arsenic and the prevalence of cardiovascular diseases have been documented including atherosclerosis, peripheral vascular disease (PVD), ischemic heart disease (IHD), hypertension, and cerebrovascular disease [21]. IHD is a disease characterized by reduced blood supply to heart muscle, usually due to atherosclerosis of the coronary arteries. Its risk increases with age, smoking, hypercholesterolemia, diabetes, hypertension, and is more common in men and those who have close relatives with ischemic heart disease. Standard diagnosis of IHD including electrocardiogram, blood tests with cardiac enzymes, history and physical examinations.

Although both population-based and occupational studies had shown that long-term exposure to inorganic arsenic has significant toxic effects on the cardiovascular system and the maximum arsenic contamination level in drinking water has been lowered from 0.05 to 0.01 ppm by US Environmental Protection Agency in 2006. The allowable limit for arsenic in drinking water is 0.025 for Canada [24] and 0.05 ppm for Bengal, India, and Bangladesh [25]. , the long-term association with chronic exposure to arsenic remains unclear and there is still epidemiological evidence needed for developing regulatory guidelines [26,27].

1.3 Genetic factors associated with arsenic metabolism and CVD

Although the mechanism of action for iAs-induced CVD hazards is not known [20]. Proposed mechanisms include arsenic metabolism, pathogenesis of atherosclerosis and oxidative stress [28-30]. More than one of these mechanisms may occur, and some may work together.

1.4 Arsenic metabolism genes

In general, the distribution of arsenic in human urine is 10-30% iAs, 10-20% MMA(V), and 60-70% DMA(V) [6]. However, some populations showed significant variation of arsenic

methylation levels in urine [31] which suggests that there are genetic factors in the regulation of the enzymes that metabolize arsenic, which may lead to difference in toxicity related to arsenic exposure. Not until recently have genes encoding enzymes that are responsible for arsenic metabolism been cloned and characterized. These genes include AS3MT and GSTO. The AS3MT gene directly encodes a cytosolic enzyme, arsenic methyltransferase, which catalyzes the multi-step process to convert inorganic arsenic to monomethyl arsenical (MMA) and dimethyl arsenical (DMA) [32]. Glutathione S-transferases (GSTs) are Phase II detoxification enzymes that catalyze the conjugation of reduced glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic compounds [33]. A subfamily of GSTs, GST omega class,was shown to be identical with human monomethylarsonic acid (MMAV) reductase that is the rate-limiting enzyme for biotransformation of inorganic arsenic. Polymorphisms of GSTO genes were shown associated with the intracellular thiol status and arsenic biotransformation efficiency of the cell [34].

1.5 Atherosclerosis- and CVD-related genes

High-density lipoprotein (HDL) is postulated to prevent the development of atherosclerosis by inhibiting the oxidation of low-density lipoprotein (LDL). Human paraoxonase (PON1) is a serum esterase/lactonase transported on HDL particles which is considered the major determinant of the antioxidant action of HDL [35]. Both in vitro studies and animal studies using PON1-knockout mice have shown that PON1 can prevent both HDL and LDL oxidation and therefore a protective enzyme against the development of atherosclerosis [36-38]. Our previous data also showed significant synergistic effects of genetic variations in the PON gene cluster and chronic arsenic exposure on electrocardiogram abnormality [39].

1.6 Other genes may linked to arsenic-related CVD

A recent review article also pointed out that some other genes that associated with arsenic toxicity and altered gene expression in humans including genes involved in stress response, DNA damage response and apoptosis related genes, cell cycle regulatory genes and cell signaling and altered growth factor [40]. Evidence from experimental studies had also suggested that arsenic increases the production of reactive oxygen species (ROS) [41-43]. The induction of oxidative stress by arsenic may influence gene expression, inflammatory responses, and endothelial nitric oxide homeostasis [44], which play an important role in maintaining vascular tone. Genes involved in endogenous defenses against ROS thus may modify arsenic's effect. Genetic material is constantly being subjected to insult from a wide range of DNA damage agents and this damage is controlled by the action of DNA repair enzymes.

1.7 Arsenic-related early effect-biomarkers for CVD

Preclinical or subclinical disease was defined as the pathological changes in the heart and arteries that develop early in the course of cardiovascular disease before symptoms or morbid events occur. They were developed before the evaluation of any of the risk factors or the determination of the subsequent incidence and mortality and thus are unbiased. Persons with subclinical disease, regardless of whether other risk factors are present, are at greater risk of future cardiovascular events than are those without subclinical disease [45]. Although standard methods of cardiac risk assessment from the physical examination, laboratory tests, and treadmill exercise testing are often used clinically in cardiovascular disease stratification [46], evaluation of subclinical disease often not taken into account.

Epidemiology studies have showed that the population attributable fractions to CHD of subclinical disease was 36.8% and 42.5% for men and women respectively, which is much higher than for most of the known risk factors or combination of risk factors and further documents the importance of subclinical disease as a contributor to subsequent incident clinical disease [45]. Among coronary artery disease (CAD) patients, the preoperative electrocardiogram (ECG) is shown to be predictive of long-term outcome independent of clinical findings and perioperative ischemia [47]. Moreover, unrecognized silent myocardial infarction as diagnosed by electrographic changes is a major risk factor for subsequent myocardial infarction and coronary disease deaths [48], and it is also a useful additional tool for differentiating the x-lined form of hereditary cardiac myopathies [49]. Subclinical disease and clinical disease shared similar risk factors and thus aggressive interventions to prevent clinical disease should be oriented to individuals with subclinical disease [50].

Various ECG abnormalities have been observed among cases of acute arsenic poisoning and in acute promyelocytic leukemia patients treated with arsenic trioxide. Individuals exposed to excess arsenic through drinking water showed some of the ECG abnormalities [51]. QT prolongation and dispersion have been implicated in the genesis of ventricular arrhythmia and directly predictors of cardiovascular and all-cause mortality [52,53]. The gradient relationship of chronic arsenic poisoning and prolonged QT interval and increased QT dispersion has been reported recently [54,55] and arsenic-induced QT dispersion was associated with atherosclerosis disease and predicted cardiovascular mortality. However, evidence was based on risk assessment on subjects with previous exposure to high arsenic level and biomarkers for methylation metabolism were not considered. Besides, the accuracy and reproducibility of ECG reading including QT dispersion measurement have been restricted by difficulties with reliable determination of T-waves offset. Further study with a standardize measurement of ECG reading is warranted for a reliable assessment of ECG abnormality.

Although an association between chronic arsenic exposure and CVD has been found in many studies, nearly all of these studies were limited by use of cross-sectional data, and longitudinal evidence by follow-up study was still limited. Besides, majority of previous studies were focus on the clinical arsenic-related cardiovascular disease, instead of the manifest of preclinical or subclinical detections. Morbidity and mortality from peripheral vascular disease, ischemic heart disease, and cerebral infarction are relative late clinical manifestations of chronic arsenic damage. These health effects may be the consequence of the interactions between predisposing and precipitating factors for cardiovascular diseases. The risk assessment based on these late cardiovascular events may be underestimated due to competing causes of death and the correctness in the diagnosis of the sudden death from cardiovascular diseases. Studies based on subclinical finding including ECG abnormality are needed to detect the early sign of chronic poisoning.

Furthermore, the variation in distribution of arsenic in human urine across areas [31] suggested that there are genetic factors in the regulation of the enzymes that metabolize arsenic, which may lead to difference in toxicity related to arsenic exposure. Association studies based on genetic polymorphisms have not provided consensus data that could generate a viable hypothesis on the molecular mechanism that determines the genetic basis of arsenic toxicity. The major objective of this study is to investigate the joint contribution of genetic factors including PON1, AS3MT, and GSTO gene families and the long-term arsenic

impacts on cardiovascular disease through measuring ECG abnormality as subclinical phenotypes and to evaluate whether the arsenic methylation patterns modifies the association between cumulative arsenic exposure and the risk of CVD.

2. Materials and methods

2.1 Study area and population

The study included a community-based cohort from previous arseniasis-endemic area in southwestern Taiwan and a non-exposed population recruited from documented non-endemic area in the same county with similar age, gender contribution and ecological status in 2002. The arseniasis-endemic area included Homei, Fusin and Hsinming villages in Putai Township on the southwestern coast of Taiwan which had been described previously [56-58]. In short, residents in the study area consumed high-arsenic contaminated well water for decades since the 1910s because of the high salinity in shallow village wells [23]. The arsenic concentration of artesian well water measured in the early 1960s was from 0.035 to 1.14 ppm, with a median of 0.78 ppm [59,60]. An estimated total daily amount of arsenic ingested by local residents was as high as 1 mg, mainly from drinking water [61]. A tap water supply system was implemented in the area in the early 1960s and the entire arseniasis-endemic area has been supplied with municipal water since the early 1970s. The arsenic concentration of tap water supplied in the study area was less than 0.01 ppm [62]. The original cohort established in 1989 including 1571 residents and 1081 subjects provided informed consents and enrolled in the study cohort. In 1993, 732 residents from the villages had a 12-lead baseline Electrocardiogram (ECG) recorded. In 2002, after an average follow up period of eight years, 216 out of 380 subjects recruited provided a second ECG recording; 141 of them provided blood and urine specimens without an ECG recording; 229 were dead and their mortality determined through linkage with the national database; and the remaining 146 were lost to follow-up. Among the 121 residents with normal baseline ECGs, 42 developed an ECG abnormality at follow up. The non-exposed area was Chiali Township where the arsenic concentration of well water was very low according to the results of surveys conducted in 1960s and 1970s[60,63]. Climate, ethnic background (Han Chinese), urbanization degree and socioeconomic status were similar between Putai and Chiali. Frequency matching by age strata and gender were conducted for recruitment of resident and a total of 303 subjects were recruited.

2.2 Measurement of arsenic exposure

Arsenic level of well water for this study area was measured by the National Taiwan University group[60]. The water-contained arsenic recovery efficiencies were 95 percent or greater and were obtained using a PerkinElmer UV-VIS Spectrophotometer incorporating with Klett-Summerson Colorimeter. Detail validations of the water arsenic levels have been presented previously[57,64]. For villages which used more than one artesian well as a source of potable water, the medial levels of water arsenic contamination across those wells were assigned. The arsenic levels in artesian well water in this study area have been reported to be stable[65]. An index of cumulative arsenic exposure (micrograms per liter-years) were defined as the summation of products derived by multiplying the arsenic concentration (in micrograms per liter) in well water by duration of water consumption (in years) during

consecutive periods of living in the defacement villages. Both cumulative arsenic exposure and average arsenic concentrations in drinking water were calculated only for subjects who had complete information on arsenic exposure from drinking water throughout his or her lifetime.

2.3 Questionnaire interview

At both baseline and follow-up, well trained public health nurses carried out the standardized personal interview based on a structured questionnaire to acquire information regarding demographic and socioeconomic characteristics, artesian well water usage, residential history, lifestyle variables, personal and family disease history of hypertension, diabetes, and cardiovascular diseases. Cumulative arsenic exposure (in ppm-years) was derived from the median arsenic concentration in artesian well water (ppm) in the village where the subject lived and the duration of consuming the artesian well water (years)while residing in the village. The human Ethical Committee of the National Health Research Institutes in Taiwan approved the study protocol which based on the ethical standards formulated from the Helsinki Declarations of 1964 and revised in 2000 [66]. Informed consent was provided to each subject before participation.

2.4 Biochemical measurements

Fasting plasma was used for quantitative determination of blood glucose, cholesterol, triglycerides, high- and low-density lipoproteins, and urine acid were analyzed using the same instrument.

Urinary samples were collected from each subject for arsenic species analyses. Subjects were asked not to consume seafood three days before urine collection. Arsenic species in urine including arsenite (AsIII), arsenate (AsV), monomethyl arsenical (MMA) and dimethyl arsenical (DMA) were quantified using high-performance liquid chromatography (HPLC) coupled with flow injection atomic absorption spectrometry. The HPLC system consisted of a solvent delivery pump (PU-1580, Jasco, Tokyo, Japan) and a silica-based anion-exchange column (Nucleosil 10 SB, 250 mm×4.6 mm; Phenomenex, CA, USA) with a guard column packed with the same material. A flow injection analysis system (FIAS-400, PerkinElmer, CT, USA) was designed as the on-line interface to the continuous hydride generation system (Analyst 100, PerkinElmer, CT, USA) used in this study. With this method, the within-say and between-day precision (coefficient of variance, CV%) for AsIII, AsV, MMA, and DMA determinations ranged from 1.0 to 3.7% were observed. Furthermore, the recoveries for AsIII, AsV, MMA, and DMA were 99.0, 98.9, 99.0, and 99.0% while the detection limits were 0.75, 1.47, 1.19, and 0.76 μg/L, respectively. The primary methylation index (PMI) was defined as the ratio between MMA and iAs levels, and the secondary methylation index (SMI) was defined as the ratio between DMA and MMA.

2.5 Genotyping

Eight functional polymorphisms: C-108T (promoter), L55M (exon 3) and Q192R (exon 6) of PON1, A148G (exon 5) and C311S (exon 9) of PON2, M287T (exon 9) of AS3MT, A140D (exon 4) of GSTO1, and N142D (exon 5) of GSTO2. SNPs were selected from NCBI's SNP database based on prior implication in disease and minor allele frequency. Genomic DNA was extracted from whole blood using standard techniques. The AS3MT M287T polymorphism was

determined using a commercially designed TaqMan SNP Genotyping Assay (Applied Biosystems, USA). All other genotypes will be conducted by PCR amplification followed by polymorphism-specific restriction enzyme digestion and gel analysis.

2.6 Physical examination

Resting twelve-lead conventional ECG recording was performed at the Beimen Branch, Shinyin Hospital. Minnesota standardized code classification [1] was evaluated for both baseline and follow-up ECG readings at Epidemiological Cardiology Research Center (EPICARE), Department of Public Health Sciences, Wake Forest University School of Medicine, Winston-Salem, North Carolina, USA (blinded to all other study data). ECG readings were classified into normal and abnormal (including minor and major abnormality) according to the definition of cardiac function by myocardial infarction or ischemia (Q wave and STT change) (MC_1, MC_4, MC_5, MC_92), conduction defect (MC_7), arrhythmias (MC_6, MC_81~MC_88), atrial enlargement or ventricular hypertrophy (LVH_MC3/LVH_CV), and prolonged ventricular repolarization. Fasting plasma was analyzed for blood glucose, cholesterol, triglycerides, high- and low-density lipoproteins, and urine acid by Beckmen SYNCHRON LX20 System (Beckman Coulter, Fullerton, CA).

2.7 Statistical analysis

Differences in demographic characteristics and cardiovascular risk factors between ECG normal and abnormal subjects were assessed. Continuous variables were expressed as mean ± standard deviation (SD) and evaluated by student's t or Wilcoxon rank-sum test. Categorical variables were expressed as proportions and compared using chi-square test or Fisher's exact test. Allele frequencies, genotype frequencies, and Hardy-Weinberg equilibrium were assessed separately in ECG abnormal and normal groups using SAS-genetics package. Relative distribution of polymorphisms in the ECG abnormal and ECG normal groups was assessed by chi-square analyses. Linkage disequilibrium (LD) as measured by D′ was assessed using Haploview 4.0 (http://www.broad.mit.edu/mpg/haploview/). Haplotypes and tag SNPs were inferred using SAS. Logistic regression analysis was used to assess the effect of cardiovascular risk factors and genetic polymorphisms in relation to ECG abnormality. Arsenic exposure and ECG abnormality in between study subjects in Putai and Chiali areas were also compared. Arsenic exposure in Putai area were stratified into two categories by median levels and subjects in Chiali area were used as reference group, and a trend test was conducted to evaluate the dose-relationship. ANOVA was conducted to evaluate urinary arsenic species between subjects with normal and abnormal ECG reading. A p value <0.05 was considered statistically significant. Permutation test, a significance test used to obtain the unknown reference distribution by calculating all possible values of the test statistic under random rearrangements of the disease status on the observed study subjects , was used to control for type 1 error for multiple testing due to the limited sample size, and the empirical p-values were reported [67]. Statistical analyses were conducted using SAS version 9.1 (SAS, Inc., Cary, NC).

3. Results

Baseline characteristics of arsenic exposure and cardiovascular risk factors among study subjects are summarized in Table 1. A total of 42 incident cases among the 121 baseline-

normal study subjects showed ECG deterioration at follow-up. Compared to ECG normal subjects, those with an ECG abnormality had significantly higher arsenic exposure as shown by both years of drinking artesian water and cumulative arsenic exposure index. Age and proportion of cigarette smoking in the ECG abnormal group tended to be higher but did not reach statistical significance. No differences were observed in other cardiovascular risk factors including gender, alcohol consumption, BMI, serum lipids, blood pressure, and plasma glucose.

Variable	ECG normal (n=79)	ECG abnormal (n=42)
Age (years)	62.0 ± 7.3	64.9 ± 8.7
Male (%)	29 (36.7)	18 (42.9)
Cigarette Smoking (%)	14 (17.7)	13 (31.0)
Alcohol consumption (%)	10 (12.7)	6 (14.3)
Residency (years)	41.5 ± 12.4	43.3 ± 14.3
Drinking Artesian Water (years)	19.2 ± 9.3	25.1 ± 9.7
Cumulative As exposure (ppm-years)	13.6 ± 8.4	18.0 ± 8.4
BMI (kg/m^2)	24.9 ± 3.13	24.5 ± 3.4
Triglycerides (mg/dl)	113.1 ± 64.7	132.3 ± 106.9
Total Cholesterol (mg/dl)	220.2 ± 45.0	211.7 ± 42.5
HDL (mg/dl)	60.3 ± 18.1	59.2 ± 14.3
LDL (mg/dl)	121.9 ± 48.1	124.6 ± 76.3
Cholesterol /HDL ratio	4.0 ± 1.6	3.8 ± 1.2
Uric acid (mg/dl)	6.1 ± 1.9	5.7 ± 1.8
SBP (mmHg)	124.8 ± 19.3	128.2 ± 17.3
DBP (mmHg)	82.2 ± 11.1	84.2 ± 8.6
AC glucose (mg/dl)	98.6 ± 23.1	104.4 ± 37.3
PC glucose (mg/dl)	127.3 ± 52.2	129.1 ± 78.1

Data are reported as mean ± SD or counts (%)
HDL: high density lipoprotein; LDL: low density lipoprotein; CHOL: total cholesterol levels; SBP: systolic blood pressure; DBP: diastolic blood pressure, AC: ante cibum, PC: post cibum

Table 1. Baseline characteristics of arsenic and CVD risk factors among baseline-normal study participants classified by ECG status at follow-up

3.1 Univariate SNPs association analysis

Eight functional polymorphisms: C-108T, L55M and Q192R of PON1, A148G and C311S of PON2, M287T of AS3MT, A140D of GSTO1, and N142D of GSTO2 were screened for association with ECG abnormality and Hardy-Weinberg equilibrium (HWE). None reached statistical significance, suggesting no univariate SNP association in the analysis. Genotypic frequencies of M287T showed a significant departure from HWE but because of the limited number of participants carrying the T alleles in this study population, they were excluded from subsequent analysis.

Gene	SNP	ECG status		
		Normal	Abnormal	OR (95% CI)
PON1	Q192R			
	RR	18	14	1.00 (reference)
	QR	27	9	0.43 (0.15-1.20)
	QQ	11	3	0.35 (0.08-1.50)
	L55M			
	LL	48	21	1.00 (reference)
	LM	14	4	0.65 (0.19-2.22)
	MM	0	0	-
	C-108T			
	CC	17	7	1.00 (reference)
	CT	30	15	1.21 (0.41-3.56)
	TT	15	6	0.97 (0.27-3.54)
PON2	C311S			
	SS	31	18	1.00 (reference)
	CS	24	7	0.50 (0.18-1.40)
	CC	2	2	1.72 (0.22-13.30)
	A148G			
	AA	30	15	1.00 (reference)
	AG	28	10	0.71 (0.28-1.85)
	GG	3	2	1.33 (0.20-8.86)
AS3MT	M287T			
	MM	67	30	1.00 (reference)
	MT	2	1	1.12 (0.10-12.80)
	TT	0	0	-
GSTO1	A140D			
	AA	41	21	1.00 (reference)
	AD	22	9	0.80 (0.31-2.04)
	DD	5	2	0.78 (0.14-4.37)
GSTO2	N142D			
	NN	35	16	1.00 (reference)
	ND	30	14	1.02 (0.43-2.43)
	DD	4	2	1.09 (0.18-6.60)

Hardy-Weinberg Equilibrium (HWE) test was conducted among all study subjects

Table 2. Association of SNPs and Hardy-Weinberg equilibrium test

Figure 1 shows the related position and linkage disequilibrium (LD) between SNPs in the PON and GSTO gene clusters. Two SNPs within PON2 (C311S and A148G) and GSTO1-A140D and GSTO2-N142 were in high LD but SNPs within PON1 or adjacent SNPs between PON1 and PON2 (C-108T and C311s) had low LD measurements, implying they were not in the same LD block. Q192R, C-108T, C311S and A140D were identified as tag-SNPs.

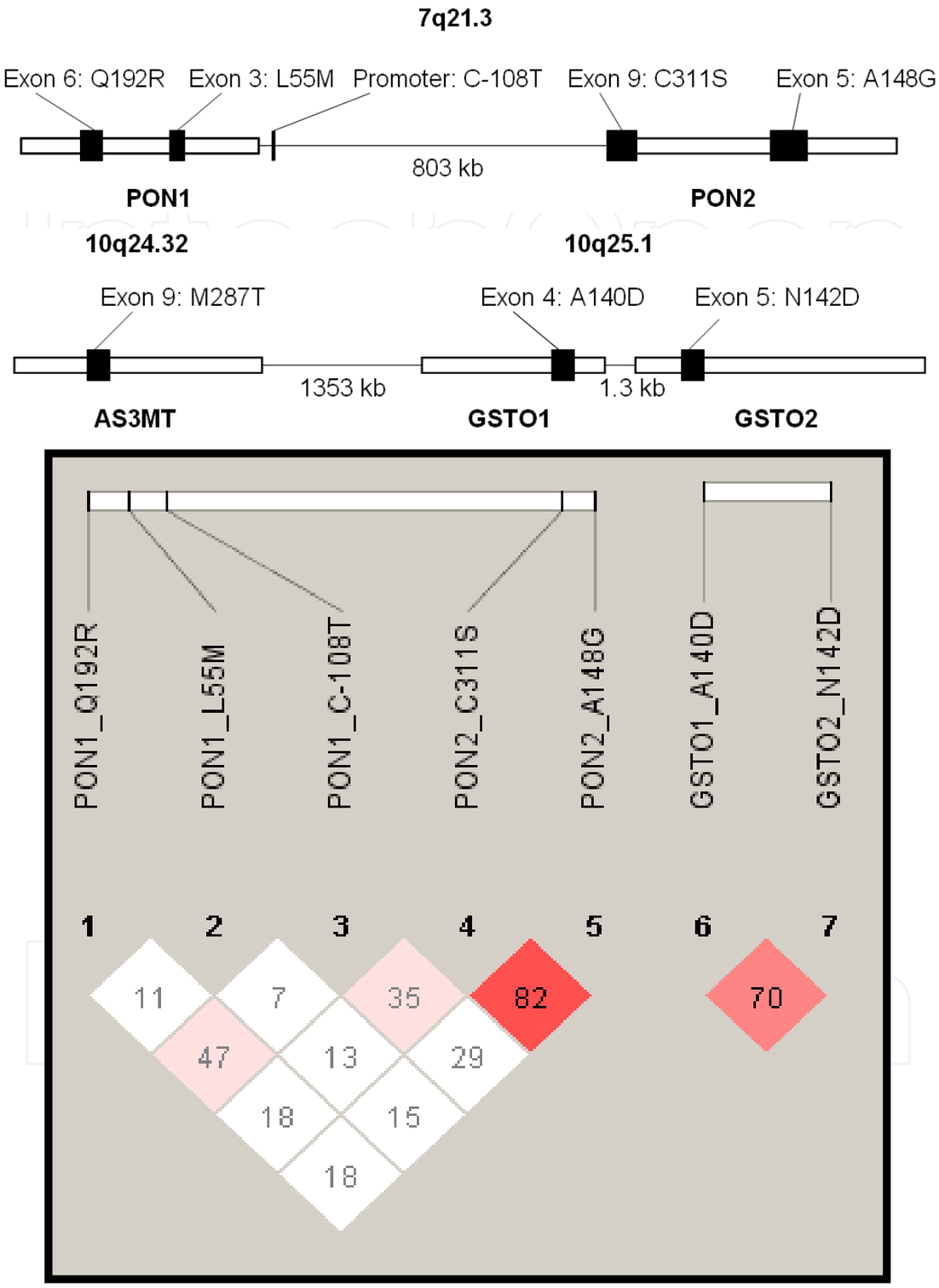

Fig. 1. Linkage disequilibrium (LD) plot of PON1 and GSTO gene clusters in 121 study subjects. The measure of LD (D′) among all possible pairs of SNPs is shown graphically. Dark red represents high D′ while white represents low D′

3.2 Haplotype analysis and association with ECG abnormality

Haplotypes of PON1, PON2, GSTO1, GSTO2 and tag-SNPs of the PON gene cluster were constructed, and those whose frequencies were <5% were excluded from association analysis (Table 3). Overall, the effects of these haplotypes on ECG abnormality were not statistically significant after 10,000 permutations; however, the haplotype R-C-S constructed by Q192R, C-108T and C311S had the highest odds, 1.92 (95% CI: 0.76-4.85) times increased risk toward ECG abnormality.

Haplotypes			ECG normal	ECG abnormal	OR
Q192R	L55M	C-108T			
R	L	T	46 (37.1%)	24 (42.9%)	1.00 (reference)
Q	L	C	38 (30.7%)	10 (17.9%)	0.50 (0.22-1.18)
R	L	C	18 (14.5%)	15 (26.8%)	1.60 (0.69-372)
Q	M	C	8 (6.5%)	4 (7.1%)	0.96 (0.26-3.51)
Q	L	T	8 (6.5%)	3 (5.4%)	0.72 (0.17-2.96)
Q192R	C-108T				
R	T		52 (41.9%)	24 (42.9%)	1.00 (reference)
Q	C		46 (37.1%)	14 (25.0%)	0.66 (0.31-1.42)
R	C		18 (14.5%)	15 (26.8%)	1.81 (0.78-4.18)
Q	T		8 (6.5%)	3 (5.4%)	0.81 (0.20-3.33)
C311S	A148G				
S	A		89 (71.8%)	40 (70.4%)	1.00 (reference)
C	G		32 (25.8%)	10 (17.9%)	0.70 (0.31-1.56)
A140D	N142D				
A	N		96 (69.5%)	43 (67.2%)	1.00 (reference)
D	D		28 (20.3%)	10 (15.6%)	0.80 (0.36-1.77)
A	D		10 (7.3%)	8 (12.5%)	1.79 (0.66-4.84)
Q192R	C-108T	C311S			
R	T	S	47 (37.9%)	21 (37.5%)	1.00 (reference)
Q	C	S	27 (21.7%)	9 (16.1%)	0.75 (0.30-1.86)
R	C	S	14 (11.3%)	12 (21.4)	1.92 (0.76-4.85)
Q	C	C	19 (15.3%)	5 (8.9%)	0.59 (0.19-1.79)
Q	T	S	8 (6.5%)	3 (5.4%)	0.84 (0.20-3.48)

Haplotypes with a frequency less than 5% were removed.
Empirical P-value: [a] Haplotype-specific test, [b] Haplotype-global test

Table 3. Estimated haplotype frequencies and haplotypes association analysis with ECG abnormality

The relative odds of lipid profiles for PON-haplotype R-C-S carrier compared with non-carriers are shown in Figure 2. The R-C-S haplotype was positively correlated with higher serum HDL-cholesterol, LDL-cholesterol, and triglyceride levels without statistical significance, but was significantly associated with increased total cholesterol levels (OR=2.91, 95% CI: 1.13-7.70).

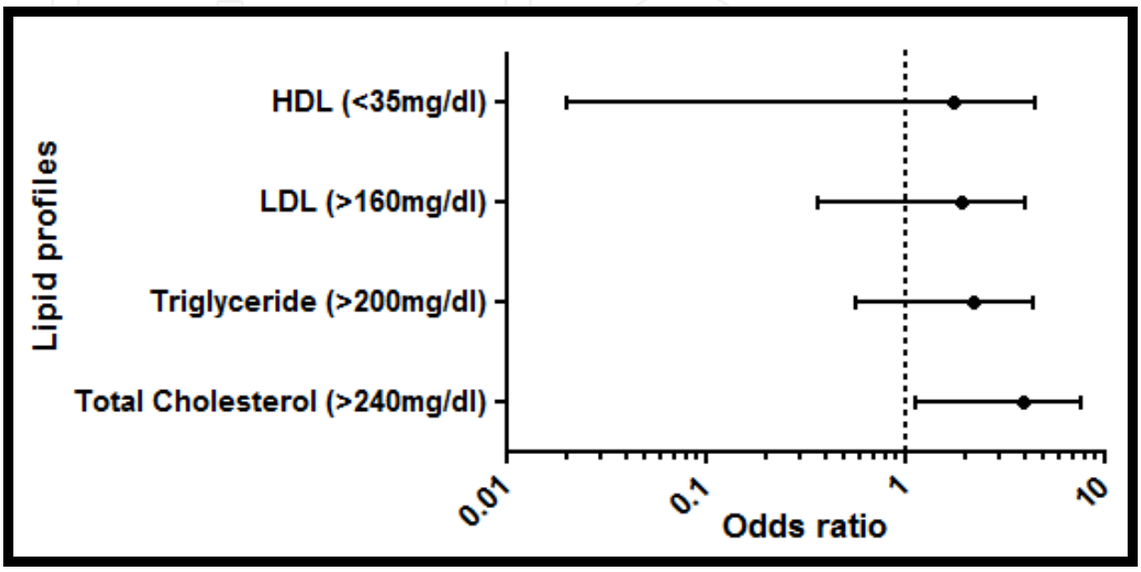

Fig. 2. Odds ratios of lipid profiles for Q192R, C-108T and C311S R-C-S haplotype carrier among study subjects (N=121)

3.3 Synergistic association of PON haplotype and arsenic on ECG abnormality

The synergistic associations between PON haplotype and arsenic exposure are summarized in Table 4. The PON R-C-S haplotype carrier with higher cumulative arsenic exposure (greater than the median value of 14.7 ppm-years) showed a >14.66 (95% CI: 1.83-117.64) increased risk for ECG abnormality compared to non-RCS haplotype carriers with low cumulative arsenic exposure (<14.7 ppm-years) (Table 4a). The PON R-C-S haplotype carrier with more years of drinking artesian water (greater than the median of 21 years) had a 10.83-fold (95% CI: 1.83-64.03) increased risk (Table 4b). These associations were even stronger after adjusting for age, gender, and cigarette smoking, when the odds increased to 19.19 (95% CI: 1.86-197.76) and 21.09 (95% CI: 2.77-160.35) for cumulative exposure index and drinking years, respectively.

Table 5 showed the correlation between cumulative arsenic exposure and urinary arsenic species from arsenic endemic and non-endemic areas in southwestern Taiwan. Subjects with higher cumulative arsenic exposure had significantly higher levels of As (III), iAs, MMA, Summation of iAs and MMA. However, DMA levels and SMI were significantly lower among subjects with high arsenic exposure. Similar pattern was observed when urinary arsenic was analyzed in percentage. Subjects with higher cumulative arsenic exposure had higher percentages of As(III), iAs and MMA in urinary and lower DMA percentage.

R-C-S	CAE	ECG Normal	ECG Abnormal	OR (95% CI)	OR (95% CI)[a]	Empirical P-value [a]
-	-	22	3	1.00 (reference)	1.00 (reference)	-
+	-	8	2	1.83 (0.26-13.06)	1.57 (0.19-13.00)	0.319
-	+	17	11	4.74 (1.14-19.72)	4.27 (0.83-22.08)	0.632
+	+	2	4	14.66 (1.83-117.64)	19.19 (1.86-197.76)	0.014

[a] Permutation analysis adjusted by age, gender and cigarette smoking
CAE: Cumulative arsenic exposure (ppm-years)
R-C-S: Q192R, C-108T and C311S R-C-S haplotype

Table 4a. Synergistic effects of Q192R, C-108T, and C311S R-C-S haplotypes carrier and high cumulative arsenic exposure (CAE) (> median of 14.7 ppm-years) on ECG abnormality

R-C-S	DAW	ECG Normal	ECG Abnormal	OR (95% CI)	OR (95% CI)[a]	Empirical P-value [a]
-	-	26	3	1.00 (reference)	1.00 (reference)	-
+	-	8	2	2.17 (0.31-15.33)	1.90 (0.24-14.94)	0.190
-	+	19	15	6.84 (1.73-27.02)	10.66 (2.12-53.55)	0.055
+	+	4	5	10.83 (1.83-64.03)	21.09 (2.77-160.35)	0.010

[a] Permutation analysis adjusted by age, gender and cigarette smoking
DAW: Drinking artesian water (years)
RCS: Q192R, C-108T & C311S R-C-S haplotype

Table 4b. Synergistic effects of Q192R, C-108T, and C311S R-C-S haplotypes carrier and more years of drinking artesian water (DAW) (> mean of 21 years) on ECG abnormality

Variable	Non-exposed (Chiali) (N=302)	≤ 14.7 (Putai) (N=191)	> 14.7 (Putai) (N=103)
Urinary arsenic level			
As (III) (μg/g creatinine)**	2.07 (2.98)	4.61 (9.46)	4.73 (6.24)
As (V) (μg/g creatinine)	2.78 (3.16)	3.13 (3.66)	2.21 (1.92)
iAs (μg/g creatinine)**	4.85 (4.66)	7.73 (11.54)	6.95 (6.74)
MMA (μg/g creatinine)**	3.13 (3.79)	4.48 (6.30)	4.21 (3.25)
Σ(iAs +MMA) (μg/g creatinine)**	7.98 (6.84)	12.21 (14.66)	11.15 (7.84)
DMA (μg/g creatinine)*	42.87 (34.48)	33.64 (27.34)	37.37 (24.32)
Σ(iAs + MMA + DMA) (μg/g creatinine)	50.85 (37.86)	45.87 (33.60)	48.58 (28.53)
PMI (MMA/iAs)	0.87 (1.02)	0.84 (0.75)	0.85 (0.60)
SMI (DMA/MMA)**	23.68 (22.80)	15.44 (28.38)	15.94 (20.86)
Urinary arsenic percentage			
As (III) %**	4.59 (5.18)	10.08 (13.22)	9.18 (9.08)
As (V) %**	6.23 (5.62)	8.27 (7.76)	5.81 (7.58)
iAs %**	10.82 (8.37)	18.35 (16.50)	14.99 (10.73)
MMA %**	7.45 (8.59)	10.90 (9.05)	10.12 (7.57)
DMA %**	81.73 (13.84)	70.75 (20.23)	74.89 (13.99)

[a] P-value for ANOVA
*P-value < 0.05, ** P-value < 0.01

Table 5. Correlation of cumulative arsenic exposure and urinary metabolism capacity

Distribution between levels of cumulative arsenic exposure and ECG abnormality was summarized in Table 6. Significant dose-response relationships were observed between higher levels of cumulative arsenic exposure and ECG reading regarding abnormalities, myocardial infarction or ischemia disease, and also atrial enlargement and ventricular hypertrophy. Increased cumulative arsenic exposure was also correlated with a higher proportion of abnormality in prolonged ventricular repolarization however not reach statistical significance.

Variable	Non-exposed (Chiali) (N=302)	≤ 14.7 (Putai) (N=191)	> 14.7 (Putai) (N=103)
ECG reading (ECG group)**			
0: Normal	155 (51.3)	96 (50.3)	40 (38.8)
1: Minor abnormal	122 (40.3)	70 (36.7)	40 (38.8)
2: Major abnormal	25 (8.3)	25 (13.0)	23 (22.4)
Myocardial Infarction or Ischemia (MC_MI)*			
0: Normal	216 (71.5)	136 (71.2)	68 (66.0)
1: Minor abnormal	72 (23.8)	37 (19.4)	20 (19.4)
2: Major abnormal	14 (4.6)	18 (9.4)	15 (14.6)
Conduction defect (BBB; Bundle Branch Block)			
0: Normal	261 (86.4)	174 (91.1)	86 (83.5)
1: Abnormal	41 (13.6)	17 (8.9)	17 (16.5)
Arrhythmia (Arrhythmia)			
0: Normal	265 (87.8)	170 (89.0)	97 (94.2)
1: Abnormal	37 (12.3)	21 (11.0)	6 (5.8)
Atrial enlargement/Ventricular hypertrophy (Hypertrophy)**			
0: Normal	286 (94.7)	167 (87.4)	85 (82.5)
1: Abnormal	16 (5.3)	24 (12.6)	18 (17.5)
Prolonged ventricular repolarization (Long_QT)			
0: Normal	299 (99.0)	187 (98.0)	99 (96.1)
1: Abnormal	3 (1.0)	4 (2.0)	4 (3.9)

[a] P-value for trend test
*P-value < 0.05, ** P-value < 0.01

Table 6. Distribution of ECG reading by cumulative arsenic exposure

4. Discussion

Various ECG abnormalities have been observed among cases of acute arsenic poisoning and in acute promyelocytic leukemia patients treated with arsenic trioxide. Individuals exposed to excess arsenic through drinking water showed some of the ECG abnormalities[51]. Several epidemiologic studies showed that QT prolongation and increased CVD mortality among high levels of arsenic-exposed subjects. However, the results might not be applicable in subjects with low to moderate arsenic. Our data replicated this association in an arseniasis-endemic area and a well-matched control area which no previous history of water contamination. We highlighted the correlation between previous chronic arsenic exposure and ECG abnormalities after cessation of arsenic-contaminated water consumption for decades.

The major strength of this study was to apply a standardized Minnesota coding classification of ECG reading that ensures good quality assurance and control. Furthermore, detailed parameters regarding ECG abnormalities allowed us to evaluate the minor changes due to arsenic toxicity. In current analyses, higher duration of arsenic water consumption was associated with ECG abnormality, myocardial infarction or ischemia, atrial enlargement or ventricular hypertrophy in a dose-response relationship. Besides, it was also positively correlated to arrhythmia and prolonged ventricular repolarization without reached the statistical significance. Moreover, higher levels of cumulative arsenic exposure were also associated with ECG parameters including higher PR duration, QRS duration and QRS axis and smaller JT duration, JT index and RaVL (data not shown).

There were still some limitations for this study. First, results from this study were based on a population with history of arsenic exposure. Insignificant association between urinary methylation capabilities might due to attrition of high-level arsenic exposed subjects, competing risks for CVD mortality was not considered in current analysis. Another limitation of the present study was that the measurement of urinary metabolism species and physical evaluation were conducted at a cross-sectional design. The causal-relationship between urinary species of arsenic and ECG abnormality could not be inferred given current evidence. However, the previous exposure status was significantly correlated with current urinary arsenic species implied it was more efficient among subjects after cessation of long-term exposure to high levels of arsenic. These results may have implications for arsenic mediation strategies in areas currently exposed to potentially harmful levels of arsenic in drinking water. Furthermore, CVD usually took years for disease development. Correlation may be biased due to unmeasured factors during a relative short follow-up period. Longer duration of follow-up with serial changes for ECG abnormality would help better understand the underlying mechanism regarding arsenic-induced hazards.

The major significance by this study was the assessment of arsenic risk from subjects without exposure of inorganic arsenic to moderate and relatively high levels of cumulative arsenic exposure. Causal inference can be strengthened by the dose-response relationship by the stronger effect in a susceptible subgroup of the population. Besides, this study demonstrated significant gene-gene and gene-environment interactions by showing PON1 gene cluster including polymorphisms of PON1: Q192R, PON1: C-108T, and PON2: C311S and latent effect of arsenic exposure on incidence of ECG abnormality. Besides, PON2: C311S was independently associated with LDH elevation and further predicted future CVD mortality independent to other conventional risk factors including age, gender, cigarette smoking, hypertension and diabetes mellitus. After cessation of arsenic-contaminated water consumption for decades, biomarkers for CVD mortality and morbidity was still associated with reduced risks for arsenic and attributable to underlying genetic predisposition. Such data may also help risk assessment in the population and provide knowledge about the underlying mechanisms.

HDL has been shown to prevent atherogenesis in vivo and in vitro through anti-oxidative and anti-inflammatory activities[35]. The major part of anti-atherogenic properties associated with HDL is explained by the activity of Paraoxonase 1[68]. Both PON1 and PON2 belong to the protein family of Paraoxonase 1 that includes PON3 which has been suggested that involved in CVD[69]. PON1 directly form part of HDL particles whereas PON2 found in endothelial cells, smooth muscle cells and macrophages that possesses antioxidant properties similar to PON1 by delays cellular oxidative stress and prevents apoptosis in

vascular endothelial cells[70,71]. Regarding three common polymorphisms in coding region of the human PON1 gene, the frequencies of Q192R, L55M, and C-108T for Taiwanese population were similar to those reported in the literature for the Chinese population [72,73]. Besides, we confirmed that paraoxonase, diazoxonase and arylesterase activities were directly influenced by the Q192R and C-108T polymorphisms. Previous studies had also shown that 311 C allele in PON2 was associated with increased risks of coronary artery disease, MI and also diabetic nephropathy[74-76]. Our data confirmed the significance of PON2: C311S polymorphism during pathogenesis of CVD among chronic arsenic exposed subject. This finding could help to identify subjects at higher risk of cardiovascular damage for arsenic toxicity.

However, some other factors that might have influenced the arsenic methylation profiles were not considered. Nutritional status and dietary intake may also be uncontrollable factors. Besides, we could not obtain the accurate data on allele distribution three polymorphisms: PON1: C-108T, GSTO1:A140D, and AS3MT: L55M in our samples. We still could not rule out the intra- and inter-individual variability to arsenic methylation and also their impacts on pathogenesis of CVD morbidity and mortality. Besides, the arsenic levels in rice growing in the arsenic contaminated area or inorganic arsenic from fish intake may be elevated. This might potentially increase arsenic exposure in the endemic area[77-80]. Future study of exposure assessment is needed. In addition, arsenic exposure has also been shown to alter the methylation level of both global DNA and certain genes in studies that analyzed a limited number of epigenetic endpoints[81]. Therefore, it is necessary to enlarge sample size for the evaluation of genetic association and ECG abnormality and other confounders that may be directly related to arsenic risks.

Genome-wide association studies (GWAS) have been applied in the search for suscepitibility genes to coronary artery disease, myocardial infarction and heart failure[82-84]. However, none of the candidate regions and genes showed a powerful association with CVD at genome-wide significance and the molecular and biological mechanisms remains unclear. Atherosclerosis is a multifactorial disease that may lead to myocardial infarction or heart failure. A conservative estimate would be that at least 100 genes have the potential to affect the modifying factors including atherosclerosis, myocardial infarction and congestive heart failure with each having a genetic contribution of as much as 2% to the phenotype[84]. Since CVD usually took years to develop, correlation may be biased due to unmeasured factors during a relative short follow-up period or relatively underestimated by competing risk. Our studies with longer duration of follow-up with serial changes for ECG abnormality did help better understand the underlying mechanism and duration regarding arsenic-induced hazards. These findings emphasize the importance of long term arsenic effect, along with the necessity of intensive follow-up for preclinical or subclinical phenotypes such as ECG abnormality for preventing excessive CVD mortality.

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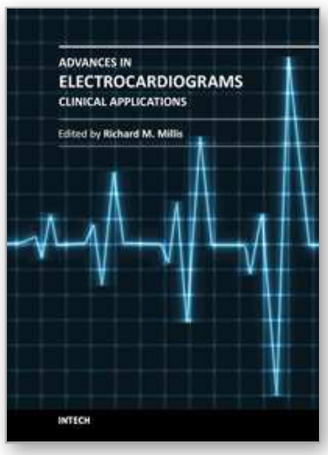

Advances in Electrocardiograms - Clinical Applications
Edited by PhD. Richard Millis

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Electrocardiograms have become one of the most important, and widely used medical tools for diagnosing diseases such as cardiac arrhythmias, conduction disorders, electrolyte imbalances, hypertension, coronary artery disease and myocardial infarction. This book reviews recent advancements in electrocardiography. The four sections of this volume, Cardiac Arrhythmias, Myocardial Infarction, Autonomic Dysregulation and Cardiotoxicology, provide comprehensive reviews of advancements in the clinical applications of electrocardiograms. This book is replete with diagrams, recordings, flow diagrams and algorithms which demonstrate the possible future direction for applying electrocardiography to evaluating the development and progression of cardiac diseases. The chapters in this book describe a number of unique features of electrocardiograms in adult and pediatric patient populations with predilections for cardiac arrhythmias and other electrical abnormalities associated with hypertension, coronary artery disease, myocardial infarction, sleep apnea syndromes, pericarditides, cardiomyopathies and cardiotoxicities, as well as innovative interpretations of electrocardiograms during exercise testing and electrical pacing.

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