

Original Article

Genetic variation in the association of air pollutants with a biomarker of vascular injury in children and adolescents in Isfahan, Iran

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Abstract

BACKGROUND: Some experimental studies revealed that exposure to air pollution increases the expression of tissue factor (TF) in atherosclerotic lesions. We aimed to investigate the role of TF +5466A>G (rs3917643) polymorphism in the association of air pollution on serum levels of TF as a biomarker of vascular injury in children.

METHODS: This cross-sectional study was conducted among 110 children, consisting of 58 (52.8%) girls and 52 (47.2%) boys with a mean age of 12.7 ± 2.3 years, living in Isfahan, Iran. Enzyme-linked immunosorbent assay were used for measurement of serum TF. Genotype of +5466A>G (rs3917643) polymorphism was determined by the polymerase chain reaction–restriction length fragment polymorphism (PCR–RFLP) method.

RESULTS: We identified 2 individuals with +5466AG genotype and 108 homozygous for the +5466A allele (no +5466GG homozygotes). The mean pollution standards index (PSI) value was at moderate level, the mean particular matter measuring up to $10 \mu\text{m}$ (PM₁₀) was more than twice the normal level. Multiple linear regression analysis showed that after adjustment for confounding factors (weight status, dietary and physical activity pattern), serum TF level had significant relationship with PSI (beta: 0.55, SE: 0.07, $p < 0.000$) and PM₁₀ (beta: 0.51, SE: 0.03, $p = 0.001$).

CONCLUSIONS: In spite of similar genetic polymorphism of TF, air pollutants might have an independent association with systemic inflammatory and coagulation responses. The harmful effects of air pollutants on the first stages of atherosclerosis in the pediatric age group should be underscored in primordial and primary prevention of chronic diseases.

KEYWORDS: Atherosclerosis; Prevention; Air pollution; Genetics; Children.

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A growing body of evidence supports the adverse effects of air pollution on the cardiovascular system and the progress of atherosclerosis¹⁻³ even from early life.⁴ The underlying mechanisms remain to be

determined. The blood vessel endothelium is a sensitive target for air pollutants.⁵ Some experimental studies showed that

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pulmonary exposure to the particulate matter (PM) enhances atherogenesis.⁶⁻⁸ Seaton has proposed that inhaled particles might increase pulmonary inflammation, possibly penetrate into the bloodstream, interact with platelets, and increase the level of blood coagulability.⁹

Various experimental studies showed that exposure to ultrafine PM increases expression of tissue factor (TF) in atherosclerotic lesions. During acute inflammatory states, the procoagulant response is characterized by increased cellular expression of TF, the physiological starter of coagulation. In chronic inflammatory diseases, including atherosclerosis, increased expression of TF and reduced anticoagulant activity, which may in turn stimulate thrombogenicity, have been documented.¹⁰⁻¹⁴

TF is well-known as a key initiator of coagulation, and is considered to have a pivotal role in atherothrombosis. In the normal vessel, TF is constitutively expressed in adventitial fibroblasts and at low levels in scattered cells in the tunica media. TF expression can be induced in both vascular and non-vascular cells by numerous pro-atherogenic stimuli. In atherosclerotic vessels, enhanced TF expression is definitely observed in vascular smooth muscle cells, monocytes/macrophages, foam cells and endothelial cells. Moreover, important non-hemostatic functions of TF in inflammation, cell migration and proliferation have been documented. Thus, TF has a crucial role not only in the late stage of thrombotic events, but also in early stages of atherosclerosis. The involvement of TF in these processes is modulated by genetic aspects influencing TF expression and activity.^{1,2,10-14}

For the first time, we documented significant association of air pollutants with serum TF level in children and adolescents. This association was independent of anthropometric measures and lifestyle habits,¹⁵ but considering the main interactions of environmental and genetic determinants in the expression of the systemic risk factors, the documented association of air pollutants with TF level might have been affected by the aforementioned genetic factors influencing TF expression and activity.

Malarstig et al. demonstrated that the TF +5466A>G (rs3917643) polymorphism may predict cardiovascular mortality in patients with acute coronary syndrome. While +5466G is found to associate with lower TF mRNA and basal TF activity in unstimulated monocytes from healthy donors, TF activity under lipopolisaccharide stimulation is shown to be two-fold higher in the +5466G allele carriers; this might provide a potential mechanism for the clinical association.¹⁶ The +5466G allele has a low frequency, and the minor allele of TF +5466A>G polymorphism is predominantly present in the heterozygous form; minor homozygous (+5466GG) genotype occurs infrequently. According to the Hardy Weinberg equilibrium, an assumed minor allele (+5466G) frequency of 7% would give an expected prevalence of the minor homozygote (+5466GG). Therefore, the TF +5466A>G polymorphism can only be analyzed using a dominant genetic model, even if thousands of subjects are being studied.¹⁷⁻¹⁸

Studying the effects of environmental factors on early life, before the process of aging and its related factors would affect the process of atherosclerosis, would reduce the confounders, and may help identify the underlying mechanisms, thus we conducted this study among a sample of children and adolescents. The current study aimed to investigate the role of TF +5466A>G (rs3917643) polymorphism in the association of air pollution on serum levels of TF as a biomarker of vascular injury in children and adolescents.

Methods

We have explained the full methodology of this study elsewhere,¹⁵ and here we report it in brief with focusing on its genetic aspects, not reported previously.

Participants

This cross-sectional study was conducted from November 2009 to February 2010 among 125 children and adolescents living in Isfahan, which is the second large and air-polluted city in Iran. The study was approved in the Re-

search Council & Ethics Committee of the School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran. It was conducted after obtaining written informed consent from the parents and oral assent from participants. The eligibility criteria were being aged 10 to 18 years, living for at least 6 months in areas of the city which had air pollution measurement stations, and location of homes and schools in the same area and less than 1 kilometer far from these stations. Those individuals who had a history of active or passive smoking, chronic disease, long-term medication use, or a history of acute infectious diseases in the past two weeks were not included in the study.

Study area

Isfahan is an industrial city with a population of 1894382, located in the center of Iranian plateau, with an average altitude of 1500 m from the sea level bounded by NW-SE mountain range of 3000 m. The air of this city is predominantly affected by industrial emissions and motor traffic.¹⁹⁻²⁰

Laboratory methods

While one of the parents accompanied his or her child, blood samples were taken from the ante-cubital vein, and were assayed and analyzed in the laboratory of the Applied Physiology Research Center affiliated to Isfahan University of Medical Sciences.

a. Genotyping methods

Genomic DNA was extracted using blood mini kit (DNP kit, CinnaGen, Iran) from whole peripheral blood leukocytes collected into ethylene diamine tetra acetic acid (EDTA) tubes and stored at -20°C. Genotype of +5466A>G (rs3917643) polymorphism was determined by polymerase chain reaction–restriction length fragment polymorphism (PCR–RFLP) method. The TF gene region surrounding +5466A>G polymorphism was amplified using ATG CAG TCA CTG TGC TGA GGA/GGC AAA TTA CAG AGC CAT CC primer pair. PCR was run under standard conditions using Taq DNA po-

lymerase (Cinna Gen, Iran), at annealing temperature of 58°C. The underlined nucleotide introduced digestion site for *Hinf*I (Fermentas UAB, Vilnius Lithuania) restriction endonuclease. Restriction fragments were separated by 2.5% agarose gel electrophoresis. Because minor allele (+5466G) was expected to be found mostly in a heterozygous form, the amplicon was designed to contain an additional, constitutive *Hinf*I restriction site as a digestion reaction positive control. Therefore, allele-specific *Hinf*I restriction product lengths were either 170/39 (+5466A allele) or 149/39/21 bps (+5466G allele).

b. Serum TF measurement

The enzyme-linked immunosorbent assay (ELISA) kits (R & D systems, USA) were used for measurement of serum TF according to the manufacturer instruction.

Air Pollution data

Data from 5 air pollution measurement stations in Isfahan city were recorded daily for the 7 days prior to blood sampling from participants. Daily data pertaining to main air pollutants, i.e., sulfur dioxide (SO₂), ozone (O₃), PM₁₀, nitrogen dioxide (NO₂) and carbon monoxide (CO) as well as the Pollutant Standards Index (PSI) were recorded. The mean values of seven 24-hour means of air pollutants and PSI were considered for statistical analysis.

Statistical analysis

Analyses were initially stratified by gender, but as the differences were not significant, results are presented for girls and boys combined. We used log-transformed concentrations of variables to achieve normal distributions. The associations between air pollutants and serum TF were assessed by multiple linear regression after adjustment for age, gender, body mass index, as well as dietary and physical activity pattern, as described before^[15]. SPSS for Windows (version 15:0, SPSS Inc., Chicago, IL) was used for data analysis. The significance level was set at $p < 0.05$.

Results

Of the 125 participants, 118 serum specimens were available for measuring TF, and 110 whole blood samples for genotyping. Here, we report the findings of the latter group having the results of the genetic study.

The study participants consisted of 58 (52.8%) girls and 52 (47.2%) boys with a mean age of 12.7 ± 2.3 years. The mean (SD) of variables studied is presented in Table 1. It shows moderate levels of mean PSI, i.e., an inappropriate level for sensitive groups. Mean levels of ozone (O₃), nitrogen dioxide (NO₂) and sulfur dioxide (SO₂) were higher than acceptable values, which are presented in the table's footnote. The mean PM₁₀ level was remarkably high, reaching more than twice the normal level (120.48 vs. 50 $\mu\text{g}/\text{m}^3$).

Multiple linear regression analysis showed that after adjustment for confounding factors, i.e., weight status, dietary and physical activity pattern,¹⁵ serum TF level had significant relationship with PSI [β : 0.55, standard error (SE): 0.07, $p < 0.0001$] and PM₁₀ (β : 0.51, SE: 0.03, $p = 0.001$).

Genotyping identified 2 subjects with +5466AG genotype and 108 subjects, homozygous for the +5466A allele (no +5466GG homozygotes). Genotype distribution did not differ significantly from that predicted by the Hardy-Weinberg equilibrium law. Figure 1

presents the results of agarose gel electrophoresis of TF 5466A>G mutation amplification and restriction enzyme digests products. Because the polymorphism was found only in two participants, the plasma concentrations of TF were not comparable between +5466AG carriers and those with +5466AA genotype.

Discussion

In this study, which to the best of our knowledge is the first of its kind in the pediatric age group, we examined whether the TF +5466A>G polymorphism modified the association of air pollution with serum levels of TF, as a biomarker of vascular injury. While several prior studies have explored genetic determinants of systemic risk factors of atherosclerosis, relatively few have examined genes active in the blood cells and vessel wall.

The few studies that are available suggest that polymorphisms of genes specially expressed in the vasculature such as eNOS and matrix metalloproteinase may play an important role in the severity of vascular disease.²¹⁻²⁵ TF is one of the key procoagulatory mediators, which could be expressed by a dysfunctional endothelium associated with the atherosclerotic process. Plasma concentrations of TF are higher in patients with atherosclerotic cardiovascular diseases than in healthy controls.¹³ Some epidemiological studies have

Table 1. Mean (SD) of variables studied

Variables	Mean (SD)
Age (years)	12.8 (2.3)
Body mass index (Kg/m ²)	20.6 (3.3)
Tissue factor (pg/ml)	0.7 (0.1)
Pollutant standard index	74.6 (30.3)
Air pollutants	
PM ₁₀ ($\mu\text{g}/\text{m}^3$)	120.4 (62.8)
CO (ppm)	3.9 (2.5)
SO ₂ (ppb)	43.7 (30.5)
NO ₂ (ppb)	59.3 (35.5)
O ₃ (ppb)	33.6 (10.2)

SD: standard deviation; PM₁₀: particulate matter 10 (acceptable level: 50 $\mu\text{g}/\text{m}^3$); CO: carbon monoxide (acceptable level: 9ppm); SO₂: sulfur dioxide (acceptable level: 0.03 ppb); NO₂: nitrogen dioxide (acceptable level: 0.05 ppb); O₃: ozone (acceptable level: 0.08 ppb); PSI: pollution standards index (0-50: Good; 51-100: Moderate; 101-199: Unhealthy; 200-299: Very unhealthy \geq 300: Hazardous)



Figure 1. Agarose gel electrophoresis of TF 5466A>G mutation amplification and restriction enzyme digests products. Lane 1, 2, 3, and 5: TF 5466A>G mutation product without digestion of restriction enzyme. Lane 4: AA Genotype. Lane 6: AG Genotype.

investigated the plasma levels of several coagulation factors as potential mediators of air pollution-related hypercoagulability. Coagulation factors such as FVII and fibrinogen, which are part of the acute-phase responses mediated by cytokines released during inflammatory reactions, increase after short-term exposure to particles.^{26,27}

Since TF +5466A>G polymorphism has comparatively low frequency, the minor allele of TF +5466A>G polymorphism is mainly exist in the heterozygous form, and minor homozygous (+5466GG) genotype occurs quite infrequently; according to the Hardy-Weinberg equilibrium, the TF +5466A>G polymorphism can only be analyzed using a dominant genetic model (for +5466G allele; AA vs. AG+GG),^{16,28} even if large number of individuals are to be studied.¹⁶ There is no explicit observations on homozygotes with the +5466GG genotype and its possible association with vascular diseases.

Some previous study investigated the role of TF +5466A>G polymorphism in vascular diseases or its complication. TF +5466A>G

(rs3917643) polymorphism may predict cardiovascular mortality in acute coronary syndrome patients. Despite the lower basal TF activity in unstimulated monocytes from healthy +5466G allele carriers, they have increased monocyte TF activity under lipopolysaccharide stimulation, which might provide a potential mechanistic link for the clinical observation.¹⁶ Moreover, it is found that this polymorphism modulates thrombin generation initiated by vascular injury in patients with ischemic heart disease.²⁸

Although the links between the role of TF +5466A>G polymorphism and increased risk of vascular disease are well-documented, the results of our study suggest that in spite of similar genetic background, exposure to air pollutants had an independent association with serum TF level.

Given the strong association of PM with atherosclerosis, the empirical pattern of PM mortality associations is more consistent with the inflammation/accelerated atherosclerosis hypothesis. The association of PM-induced low-grade inflammation with increased risk of

atherosclerotic events is supported by studies showing the relationship of PM exposure with elevated levels of C-reactive protein, inflammatory pulmonary injury, enhanced production of proinflammatory cytokines by human alveolar macrophages, bone marrow and blood cell reactions, blood viscosity, platelet aggregation, endothelial dysfunction and brachial artery vasoconstriction.²⁹⁻³⁷

Given that these associations are not limited to adults, and are also documented in adolescents^{38,39} and young adults,⁴⁰ the harmful effects of air pollution should be considered as a health priority for children and adolescents.

Study limitations & strengths

The findings of this study should be considered with its limitations. Similar to other ecological studies, this study is limited by the lack of exact exposure estimates. As the study was conducted with a cross-sectional design, cause-effect relations cannot be concluded. The existing equipment in monitoring stations was unable to measure PM_{2.5}; however, we found significant association of larger particle (PM₁₀) with biomarkers studied. In this study, system-

ic biomarkers were measured; assessment of broncho-alveolar lavage may reveal more specific results.

The strengths of this study are mainly its novelty in the pediatric age group, its population-based design and assessment of genetic polymorphism in addition to confounding factors for studying independent association of surrogate markers of endothelial dysfunction with air pollutants.

Conclusion

By studying genetic polymorphisms and adjustment for confounding factors as weight status and lifestyle behaviors, this study suggests an independent association of air pollutants with systemic inflammatory and coagulation responses. These changes in blood markers could represent additional risk factors, which in susceptible individuals, could increase the likelihood of serious arterial vascular thrombotic events on exposure to high levels of air pollutants. The effects of air pollution on the first stages of atherosclerosis in children and adolescents should be confirmed in longitudinal studies.

Conflict of Interests

Authors have no conflict of interests.

Authors' Contributions

PP, RK, and FM designed the study; PP, RK, FM, AL, MMA, AL, LF, and SHJ collected and analyzed the data; and PP, RK, and SHJ prepared and wrote the manuscript. All authors confirmed the content of the manuscript.

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