

# Synergistic effects of genetic polymorphism and air pollution on markers of endothelial dysfunction in children

Parinaz Poursafa, Roya Kelishadi<sup>1,2</sup>, Shaghayegh Haghjooy-Javanmard<sup>3</sup>, Laleh Rafiei<sup>3</sup>, Kasra Keramatian<sup>2</sup>

Department of Environment Protection, Environment Research Center, Isfahan University of Medical Sciences (IUMS), Isfahan, Iran

<sup>1</sup>Department of Pediatrics, School of Medicine, <sup>2</sup>Child Growth and Development Research Center, IUMS, Isfahan, Iran, <sup>3</sup>Department of Physiology, Applied Physiology Research Center, IUMS, Isfahan, Iran

**Background:** This study aims to determine the association of some genetic polymorphisms in the relationship of air pollutants on the serum levels of thrombomodulin (TM) and tissue factor (TF) in a population-based sample of children and adolescents. **Materials and Methods:** This cross-sectional study was conducted among 110 participants (52.8% girls) with a mean age of 12.7 + 2.3 years, in Isfahan, Iran. Genotypes of TM G33-A and + 5466A > G polymorphisms were determined by the polymerase chain reaction – restriction length fragment polymorphism method (PCR-RFLP). The enzyme-linked immunosorbent assay (ELISA) was used for measurement of serum TM and TF. **Results:** The following genotypes were identified for TM: GG in 69.2%, GA in 27.2%, and AA in 3.6% of the participants. Considering TF, 108 participants were homozygous for the + 5466A allele, and two subjects had + 5466AG genotype. The mean pollution standards index (PSI) value was at a moderate level; the mean particulate matter measured up to 10 µm (PM<sub>10</sub>); and ozone (O<sub>3</sub>), nitrogen dioxide, and sulfur dioxide were considerably high. The mean serum TF and TM levels were not significantly different among the participants with the aforementioned genotypes. Among participants exposed to high quartiles of O<sub>3</sub>, PM<sub>10</sub>, and PSI, the TM-33G / A polymorphism (GA + AA genotype) increased the Odds ratio (OR) of the low serum TM level. There was no statistically significant association in the areas of low pollution. **Conclusion:** The findings of our study support the synergistic effect of the TM-33G / A polymorphism and air pollutants on factors associated with the onset of the atherosclerosis. This might be confirmatory evidence for gene-environment interaction, and related effects on atherogenesis from early life.

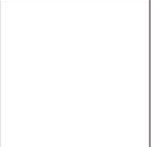
**Key words:** Air pollution, atherosclerosis, children, genetics, prevention

## INTRODUCTION

The harmful effects of air pollution on atherosclerotic cardiovascular diseases (ACVD) are well-documented;<sup>[1]</sup> even short-term exposure to air pollutants is associated with cardiovascular disorder.<sup>[2,3]</sup> The underlying mechanisms have yet to be determined; pulmonary and systemic oxidative stress and inflammatory responses provoking endothelial dysfunction, disturbance of the platelet function, blood coagulability, and thrombosis are suggested to be the effects of air pollutants.<sup>[4,5]</sup> Some experimental studies have confirmed the possible effect of air pollutants on the blood vessel endothelium and in turn on increasing the risk of atherosclerosis.<sup>[6-8]</sup>

Furthermore, the contribution of genetic factors in ACVD should be taken into account. Most of these genes participate in producing proteins that affect the progression of atherosclerosis or thrombosis. One of these proteins is thrombomodulin (TM), an endothelial cell membrane-bound glycoprotein, with its main physiological function being to bind thrombin.<sup>[9]</sup> On the other hand, increase in the level of blood coagulability is proposed as another underlying mechanism for air pollution-induced atherogenesis.<sup>[10]</sup> Exposure to ultrafine PM increases expression of the tissue factor (TF) in atherosclerotic lesions.<sup>[11]</sup> For the first time, we also found an independent association of air pollutants with the serum TF level in children and adolescents.<sup>[12]</sup>

Considerable interest has been focused over the years on examining the importance of gene-environment interaction on ACVD.<sup>[1]</sup> There is a growing body of evidence about the start of the aforementioned effects of air pollutants on the process of atherosclerosis from early life,<sup>[13-15]</sup> and studying the effects of genetic-environment factors on early stages of atherosclerosis in early life can help identify the underlying mechanisms.

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**Address for correspondence:** Prof. Roya Kelishadi, Department of Pediatrics, Faculty of Medicine and Child Growth and Development Research Center, Isfahan University of Medical Sciences, Isfahan, Iran. E-mail: kelishadi@med.mui.ac.ir

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The aim of this study was to determine the association of some genetic polymorphisms in the relationship of air pollutants, on the serum levels of TM and TF, in a population-based sample of children and adolescents. We selected the pediatric age group in this regard to reduce the impact of the confounding factors related to the process of aging and the accumulation of the ACVD risk factors.

## MATERIALS AND METHODS

The study was approved in the Research Council and 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 the participants. We have published the full methodology of this study,<sup>[12,16]</sup> and here we report it in brief, while focusing on the genetic aspects, not reported previously.

### Study population

This cross-sectional study was conducted from November 2009 to February 2010, among 125 children and adolescents living in Isfahan, which is the second largest air-polluted city in Iran. The eligibility criteria were: Age between 10 and 18 years, living for at least six months in areas of the city that had air pollution measurement stations, and location of homes and schools in the same area, and less than 1 km 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 near 1,894,382, located in the center of the Iranian plateau, with an average altitude of 1500 m from the sea level, bounded by a NW-SE mountain range of 3000 m. The air of this city is predominantly affected by industrial emissions and motor traffic.<sup>[17]</sup>

### Clinical examination and assessment of lifestyle habits

Physical examination was done by the same trained team under the standard protocol by using calibrated instruments. Subcutaneous fat of the biceps and triceps muscles were measured with a skinfold caliper (Mojtahedi, Iran), the percent body fat was determined by bio-electrical impedance, using a Body Fat Monitor (Omron HBF-300, Japan). For assessment of dietary habits, the Healthy Eating Index (HEI) was computed as described before.<sup>[15]</sup> The physical activity level was assessed by the international Physical Activity Questionnaire for Children,<sup>[18]</sup> previously validated in Iranian children.<sup>[19]</sup>

### Laboratory methods

One of the parents accompanied his or her child and venous blood samples were taken. They were assayed

and analyzed in the laboratory of the Applied Physiology Research Center affiliated to the Isfahan University of Medical Sciences.

### Genotyping methods

Genomic DNA was extracted using a blood mini kit (DNP kit, CinnaGen, Iran) from the whole peripheral blood leukocytes collected into ethylene diamine tetra acetic acid (EDTA) tubes and stored at -20°C.

Genotyping for the TM G33-A polymorphism was carried out by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). A 259-bp DNA fragment, containing the G33-A polymorphism site for TM, was amplified by PCR using the following primers: forward, 5'-GGC CAG GGC TCG AGT TTA TAA AGG C-3'; and reverse, 5'-CGG GGA CAG TCG TCT GTT ACA G-3'. PCR was run under standard conditions, using Taq DNA polymerase (Cinna Gen, Iran), at an annealing temperature of 64°C. Digests (10 µl) containing one unit of restriction enzyme (StuI, from Biolabs) were incubated for 12 h at 37°C for the PCR products. The restriction site for StuI is 5'-AGG\*CCCT-3', which represents the mutant type A allele and produces two fragments for the AA genotype (24 and 235 bp). The wild-type G allele is not recognized by the StuI enzyme, and only shows one 259 bp band (for the GG genotype). The GA heterozygote has three bands, with sizes of 259, 235, and 24 bp. The digests are then separated on a 3% agarose gel and visualized by ethidium bromide staining.

Considering TF, a genotype of + 5466A > G (rs3917643) polymorphism was determined by the PCR-RFLP method. The TF gene region surrounding the + 5466A > G polymorphism was amplified using the 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 polymerase (Cinna Gen, Iran), at an annealing temperature of 58°C. The underlined nucleotide introduced the digestion site for a HinfI (Fermentas UAB, Vilnius Lithuania) restriction endonuclease. The restriction fragments were separated by 2.5% agarose gel electrophoresis. As a minor allele (+ 5466G) was expected to be found, mostly in a heterozygous form, the amplicon was designed to contain an additional, constitutive HinfI restriction site, as a digestion reaction positive control. Therefore, allele-specific HinfI restriction product lengths were either 170 / 39 (+ 5466A allele) or 149 / 39 / 21 bps (+ 5466G allele).

### Measurement of biomarkers

The enzyme-linked immunosorbent assay (ELISA) kits from Abcam Company (UK) with code Ab46508 were used for measurement of TM. The ELISA kits (R and D systems,

USA) were used for measurement of serum TF according to the manufacturer’s instruction.

**Air pollution data**

Data from five air pollution measurement stations in Isfahan city were recorded daily, for seven days, prior to blood sampling from the participants. Daily data pertaining to the main air pollutants, that is, sulfur dioxide (SO<sub>2</sub>), Ozone (O<sub>3</sub>), Nitrogen dioxide (NO<sub>2</sub>), carbon monoxide (CO), particulate matter with diameter less than 10 µg / m<sup>3</sup> in aerodynamic diameter (PM<sub>10</sub>), 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.<sup>[15]</sup>

**Statistical analysis**

Analyses were initially stratified by gender, but as the differences were not significant, results were presented for girls and boys combined. We used log-transformed concentrations of variables to achieve normal distributions.

The concentrations of biomarkers and air pollutants were categorized to quartiles, and the lower and upper quartiles were considered as low and high values. As there was no cutoff value to determine the high and low levels of TM and TF in the pediatric age group, we considered their lowest quartiles as low levels and their highest quartiles as high levels.<sup>[12]</sup> Then, we used logistic regression analysis to examine the association of the TM and TF genotypes, with low level of TM and high level of TF concentrations, in areas with low and high air pollution. This analysis was conducted after adjustment for the following potential confounders: age, gender, body mass index, waist circumference, healthy eating index, and physical activity level, as described earlier in the text.<sup>[12,15]</sup>

SPSS for Windows (version 19:0, 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 TM, and 110 whole blood samples (52.8% from girls) for genotyping, which were included in the statistical analysis.

The study participants had a mean (SD) age of 12.7 (2.3) years. The genotype distribution did not differ significantly from that predicted by the Hardy–Weinberg equilibrium law. The results of genotyping are depicted in Figure 1. The following genotypes were identified for TM: GG in 69.2% (*n* = 76), GA in 27.2% (*n* = 30), and AA in 3.6% (*n* = 4) of the participants. Considering TF, 108 participants

were homozygous for the + 5466A allele, and two subjects had a + 5466AG genotype (no + 5466GG homozygotes).

The mean (SD) of the variables studied is presented in Table 1; it shows that the mean pollutant standard index (PSI) was at a moderate level, that is, inappropriate for sensitive groups. The mean levels of ozone (O<sub>3</sub>), nitrogen dioxide (NO<sub>2</sub>), and sulfur dioxide (SO<sub>2</sub>) were higher than the acceptable levels. The mean PM<sub>10</sub> level was more than twice the normal level (120.48 vs. 50 mg / m<sup>3</sup>).

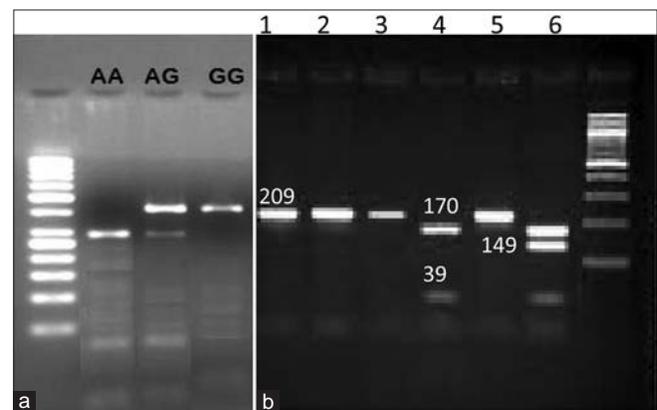
The mean serum TF and TM levels were not significantly different among participants with various genotypes.

Among the participants exposed to high quartiles of O<sub>3</sub>, PM<sub>10</sub>, and PSI, the GA and AA genotypes increased the OR of the low serum TM level [Table 2]. There was statistically no significant association in the areas of low pollution.

**Table 1: Mean (SD) of variables studied**

Variables	Mean (SD)
Age (years)	12.81 (2.30)
Body mass index (Kg / m <sup>2</sup> )	20.61 (3.34)
Waist circumference (cm)	72.62 (4.74)
Percent body fat (%)	22.85 (4.21)
Fat mass (kg)	12.46 (2.51)
Thrombomodulin (µmol / L)	5.64 (3.27)
Pollutant standard index	74.6 (30.3)
PM <sub>10</sub> (µg / m <sup>3</sup> )	120.4 (62.8)
CO (ppm)	3.9 (2.5)
SO <sub>2</sub> (ppb)	43.7 (30.5)
NO <sub>2</sub> (ppb)	59.3 (35.5)
O <sub>3</sub> (ppb)	33.6 (10.2)

SD = Standard deviation, PM<sub>10</sub> = Particulate matter 10 (acceptable level: 50 µg / m<sup>3</sup>), CO = Carbon monoxide (acceptable level: 9 ppm), SO<sub>2</sub> = Sulfur dioxide (acceptable level: 0.03 ppb), NO<sub>2</sub> = Nitrogen dioxide (acceptable level: 0.05 ppb), O<sub>3</sub> = Ozone (acceptable level: 0.08 ppb), PSI = Pollution standards index (0 – 50: Good; 51 – 100: Moderate; 101 – 199: Unhealthy; 200 – 299: Very unhealthy; > 300: Hazardous)



**Figure 1:** Genotypes of thrombomodulin and tissue factor in the study participants (a) Genotypes of TM polymorphism (G-33A) AA: Homozygote of the mutant type A allele; AG: Heterozygote; GG: Homozygote of the wild-type G allele (b) Genotypes of TF 5466A > G polymorphism Lane 1,2,3,5: TF 5466A > G mutation product without digestion of restriction enzyme, Lane 4: AA Genotype, Lane 6: AG Genotype

**Table 2: Association of genotypes with low thrombomodulin level in high and low exposure to air pollutants**

	Ozone				PM <sub>10</sub>				Pollutant Standard Index			
	Low		High		Low		High		Low		High	
	12.71 – 32.14		50.72-56.14		50.65 – 121.74		224.88 – 281.55		45.18 – 72.64		121.20 – 135.11	
	OR	95% CI	RR	95% CI	RR	95% CI	RR	95% CI	RR	95% CI	RR	95% CI
<b>GG</b>	1:00		1:00		1:00		1:00		1:00		1:00	
<b>GA or AA</b>	1.14	0.95,1.81	1.25	1.07,2.11	1.11	0.91,1.96	1.34	1.17,1.81	1.24	0.92,1.85	1.37	1.18,1.94
<b>P</b>	0.09		0.04		0.07		0.03		0.08		0.04	

\*: Values represent odds ratio (95% CI) adjusted for age, gender, anthropometric measures, dietary and physical activity habits, Low: quartile 1; high: quartile 4, Thrombomodulin (pmol / L); O<sub>3</sub>: ozone (ppb); PM<sub>10</sub>: particulate matter <sub>10</sub>(µg / m<sup>3</sup>); PSI: Pollution Standards Index

## DISCUSSION

In this study, which to the best of our knowledge is the first of its kind in the pediatric age group, although the levels of surrogate markers of endothelial dysfunction and vascular injury did not have a significant difference according to their various genotypes, among those participants exposed to high levels of some air pollutants, the minor alleles (GA + AA) of the TM G33-A polymorphism increased the risk of low TM levels. This finding suggests the synergistic effect of this polymorphism and air pollutants on early stages of atherosclerosis, and may be confirmatory evidence of a gene-environment interaction.

According to the World Health Organization (WHO), environment has a role in the development of more than 80% of the common diseases. Globally, environmental factors affect nearly one-quarter of all-cause mortality, as well as one-quarter of the total disease burden, and slightly more than one-third of the disease burden in children.<sup>[20]</sup> The findings of this study and other reports on the association of air pollutants with the surrogate markers of atherosclerosis and endothelial dysfunction from early life, suggest that such long-term effects should be considered in the primordial and primary prevention of ACVDs.

The underlying mechanisms of the association of air pollutants with the atherosclerosis process remains to be determined; inflammation and coagulation are the most important factors linking air pollution to ACVD.<sup>[1-8]</sup> TM plays a protective role in atherosclerosis; it acts as an endothelial anticoagulant cofactor and prevents dissemination of pro-coagulant and pro-inflammatory molecules.<sup>[9]</sup> The thrombin-thrombomodulin complex catalyzes the activation of protein C, which may proteolytically inactivate factors Va and VIIIa. Thrombin not only takes on the role of blood coagulation, but also has important roles to play in the aggregation of inflammatory cells, proliferation of mesenchymal cells, in platelet activation, by releasing the platelet-derived growth factor, as well as, vascular smooth muscle cell generation. Alterations in expression of TM and its partner proteins may increase the risk of inflammatory and thrombotic disorders.<sup>[9,21]</sup> All these cellular effects may end up with atherosclerosis and its sequelae. Furthermore, there are

unexplained associations of cardiometabolic risk factors and markers of endothelial dysfunction, with subclinical atherosclerosis. A recent population-based study reported that the association of a metabolic syndrome with subclinical atherosclerosis was independent of its association with the biomarkers of endothelial damage and oxidative stress. It suggested that the underlying mechanisms of the effects of metabolic abnormalities and oxidative endothelial damage on ACVDs were not similar.<sup>[22]</sup> Considering the associations of air pollutants with oxidative stress, metabolic disorders, and insulin resistance, as well as with surrogate markers of atherosclerosis and endothelial dysfunction from a young age,<sup>[1,12,13,15]</sup> the effects of environmental factors, notably air pollution, should be considered in the above-mentioned distinct mechanisms suggested for ACVD.

The pivotal role of genetic factors, including the polymorphisms of TM and TF, on development of atherosclerosis is well-established.<sup>[9,11]</sup> Ireland *et al.* identified the 33G > A polymorphism in the 5¢-promoter region of the TM gene for the first time.<sup>[23]</sup> In Asians, the prevalence of the mutation A allele has been seen more frequently than in Caucasians (8 – 10% vs. 1%).<sup>[23-27]</sup> In both Taiwanese and Korean populations, the GA/AA genotypes have been asserted to be accompanied with decreased promoter activity and increased risk factor for ACVD. Likewise, it has been reported that the plasma TM level is significantly higher in patients with ACVD, except in those with a 33G > A mutation, as reduced TM promoter transcriptional activity and soluble TM levels are related to the increased risk of ACVD.<sup>[24-26]</sup> By showing a greater number of AA / GA genotypes of G-33A A in ACVD patients than in the controls, a study in Korea suggested that G-33A has a significant effect on the TM promoter activity as well as a substantial risk factor for ACVD.<sup>[26]</sup> Additionally, it is documented that reduction in soluble plasma TM levels is related to a 33G > A mutation, as well as to an increased risk of ACVD.<sup>[24-26]</sup>

The initial results of the current study showed that air pollutants, particularly PM<sub>10</sub> and O<sub>3</sub>, are associated with decreased levels of TM, and this association is independent of the anthropometric indices and lifestyle factors,<sup>[12]</sup> and independent of TF genotypes.<sup>[15]</sup> The current findings showed that in the presence of high levels of PM<sub>10</sub>, O<sub>3</sub>, and

PSI, the TM-33G / A polymorphism (GA + AA genotype) increased the risk for low serum TM.

Our findings are in line with a study in Taiwan, which examined the possible association between t-33G / A polymorphism and acute myocardial infarction. It found significantly higher frequency of the TM GA + AA genotype among patients with myocardial infarction than in the controls. This polymorphism and smoking were the only independent risk factors for young (i.e., age < 45 years) myocardial infarction. Although among non-smoker patients, this polymorphism was associated with a non-significant increase in the risk of young myocardial infarction, in the presence of smoking, the increase was significant, and those smoker patients who carried this polymorphism had an approximately 10-fold increased risk of young myocardial infarction compared to the non-smoking non-carriers. This study revealed that the clinical effect of this genetic factor was enhanced by smoking.<sup>[25]</sup> The findings of our study support the synergistic effect of the TM-33G / A polymorphism and air pollutants on factors associated with the onset of atherosclerosis from childhood. This might be a confirmatory evidence for gene-environment interaction, and the related effects on atherogenesis from early life.

The tissue factor, as a biomarker of vascular injury and a main pro-coagulatory mediator, may be expressed by endothelial dysfunction and ACVD.<sup>[28]</sup> It increases after short-term exposure to air particulates, and may be one of the potential mediators of air pollution-related hypercoagulability.<sup>[29]</sup> The TF + 5466A > G polymorphism increases the risk of vascular disease, and has a role in modulating thrombin generation, initiated by vascular injury.<sup>[30]</sup> In our study, TF had an independent association with low air quality, but the TF + 5466A > G polymorphism was rare, and did not modify this association. Our findings suggest that in spite of a similar genetic background, exposure to air pollutants had an independent association with the serum TF level from childhood.

#### Study limitations and strengths

One of the limitations of this study is its cross-sectional design, which does not allow for the establishment of causal relationships. Moreover, similar to other ecological studies, this study could not determine the exact exposure estimates. We could not measure PM<sub>2.5</sub> by the equipment available in the monitoring stations; however, the larger particles (PM<sub>10</sub>) had a significant association with the variables studied. Study of the bronchoalveolar lavage may reveal more specific results than the systemic biomarkers measured in the current study. The strengths of this study are the novelty in studying a population-based sample of very young and healthy individuals, as well as in determining genetic polymorphism and lifestyle factors.

## CONCLUSION

The findings of our study suggest a synergistic effect of the TM-33G / A polymorphism and air pollutants on a low serum TM level. Given the protective function of TM on the endothelial function, this synergistic effect might play an essential role in the onset of subclinical atherosclerosis from early life. The clinical and public health aspects of such proposed gene-environment interactions should be confirmed in future cohort studies.

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