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1 **Fibrin clot structure is affected by levels of particulate air**
2 **pollution exposure in patients with venous thrombosis**

3
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29 **ABSTRACT**

30 **Background:** Particulate air pollution is a risk factor for cardiovascular diseases and
31 thrombosis. Long-term exposure to particulate matter with a diameter $<10\ \mu\text{m}$ (PM₁₀)
32 has been associated with an increased risk of venous thrombosis.

33 **Objectives:** The aim of this study was to investigate whether or not particulate air
34 pollution alters fibrin clot structure and thus modulates thrombosis risk.

35 **Methods:** We investigated fibrin polymerization by turbidity (maximum absorbance
36 mOD), clot structure by confocal microscopy (fibre number per μm) and fibrin pore size
37 by permeability ($K_s \times 10^{-10}\ \text{cm}^2$) in 103 patients with deep vein thrombosis and 121
38 healthy controls, for whom levels of air pollution exposure had been recorded.
39 Exposure groups were defined by mean PM₁₀ concentrations over the 730 days before
40 the event.

41 **Results:** We found a higher average number of fibres per clot area in patients than
42 controls, but no difference in K_s or fibre thickness. When the two groups were divided
43 into high or low exposure to PM₁₀, a significantly denser fibrin clot network structure
44 with thicker fibres (higher maximum absorbance, $p < 0.05$), decreased permeability
45 (lower K_s value, $p < 0.05$) and higher average fibre numbers per clot area ($p < 0.05$) was
46 observed in patients in the high exposure group compared to those with low exposure.
47 There were no significant differences in fibrin clot structure between the two exposure
48 levels in healthy subjects.

49 **Conclusions:** PM₁₀ levels are associated with altered fibrin clot structure in patients
50 with deep vein thrombosis but not in controls, suggesting that air pollution may trigger
51 differences in fibrin clot structure only in patients predisposed to thrombotic disease.

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53

54 **HIGHLIGHTS**

- 55 • PM₁₀ levels are associated with denser fibrin clot structure in patients with DVT.
- 56 • In the control group, high PM₁₀ level did not contribute to denser fibrin clot
57 structure formation.
- 58 • Air pollution may trigger differences in clot structure in patients predisposed to
59 thrombosis.

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62 **KEY WORDS**

63 Air Pollution, Blood Clotting, Fibrin, Particulate Matter, Venous Thrombosis

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69 **1. INTRODUCTION**

70 Exposure to air pollution is associated with adverse effects on the pulmonary and
71 cardiovascular systems (Franchini and Mannucci, 2012; Peters, 2005). Urban air

72 pollution is composed of both gaseous pollutants (e.g. ozone, nitrogen dioxide, and
73 sulphur dioxide) and particulate matter (PM). The PM in air pollution is a mixture of
74 particles of different sizes, shapes, surface area, chemical composition, solubility and
75 origins that are suspended in the air (Pope 3rd, 2009). PM is categorized by
76 aerodynamic diameter, with PM₁₀ representing particles with a diameter of less than
77 10 µm (Brook, 2008; Polichetti et al., 2009). The smaller particles in PM₁₀, which have
78 a diameter of less than 2.5 µm (PM_{2.5}), are associated with combustion of fossil fuels
79 and high temperature industrial processes (Brook et al., 2010; Newby et al., 2014;
80 Pope 3rd, 2009). Ultrafine particulate matters with a diameter less than 100 nm (PM_{0.1})
81 are from fresh combustion and traffic-related pollution which can travel large distances
82 (Brook et al., 2010; Newby et al., 2014). The PM in urban air pollution has been
83 associated with cardiovascular mortality and morbidity in a number of studies (Morris,
84 2001; Polichetti et al., 2009; Shah et al., 2013).

85 A range of possible mechanisms by which PM may damage the cardiovascular system
86 have been proposed, including atherogenesis, thrombosis and endothelial dysfunction
87 (Mills et al., 2009). Observations that particulate air pollution exposure is associated
88 with increased levels of circulating coagulation proteins (eg. factor VIII [FVIII], von
89 Willebrand factor and fibrinogen) suggest that hypercoagulability due to PM exposure
90 could be an important risk factor for thrombosis (Baccarelli et al., 2007b; Nemmar et
91 al., 2006). A 70% increase in risk of deep vein thrombosis (DVT) for each 10 µg/m³
92 elevation of PM₁₀ was observed in a large cohort study in the Lombardy region
93 (Baccarelli et al., 2008). However, the mechanisms underpinning the increased risk of
94 thrombosis after exposure to ambient air pollution are still poorly understood.

95 Fibrin clot structure, mechanical properties and resistance to lysis, are emerging risk
96 factors in cardiovascular disease and venous thrombosis (Undas and Ariens, 2011;
97 Wolberg, 2007). Altered fibrin clot structure with compact, highly branched fibre
98 networks, reduced permeability and prolonged lysis time has been associated with
99 ischemic stroke and venous thrombosis (Undas and Ariens, 2011; Wolberg, 2007). In
100 view of these associations between thrombosis and fibrin structure, we previously
101 investigated the effects of particulate matter on fibrin clot structure. We found that
102 particulate matter caused changes in fibrin clot structure and function in purified
103 systems and human plasma (Metassan et al., 2010a). In contrast, we found no
104 changes in fibrin clot structure in healthy individuals after 2 hours exposure to
105 particulate matter while performing moderate exercise (Metassan et al., 2010b).
106 However, the exposure in the latter study was transient and of short duration. Whether
107 or not fibrin clot structure is affected by long-term exposure to high levels of air
108 pollution, and particularly in patients with thrombosis exposed to air pollution is
109 currently unknown.

110 In view of the association between exposure to particulate matter and thrombosis, and
111 of the association between thrombosis and abnormal fibrin clot structure, we
112 investigated the possible association between fibrin clot structure and PM₁₀ levels in
113 a well-characterized group of patients with DVT and healthy controls from the
114 Lombardy region in Northern Italy.

115

116 **2. METHODS**

117 **2.1 Study Population**

118 The study population of patients and controls has been previously described in detail
119 (Baccarelli et al., 2007; Baccarelli et al., 2007; Baccarelli et al., 2008; Baccarelli et al.,
120 2009). Briefly, patients from the Lombardy region, Northern Italy were referred to the
121 Angelo Bianchi Bonomi Thrombosis Center in Milan from January 1995 to September
122 2005 for a thrombophilia screening after a first episode of objectively confirmed lower-
123 limb DVT with or without pulmonary embolism. Controls were healthy individuals,
124 friends or partners of the patients, who were residents in the Lombardy region and
125 volunteered to undergo thrombophilia screening. Thrombophilia was classified as
126 being positive for at least one of the following: factor V Leiden, prothrombin G20210
127 mutation, antithrombin-, protein C-, protein S-deficiency, antiphospholipids antibodies
128 and hyperhomocysteinemia. All patients and controls provided written consent and the
129 study was approved by the local ethics committee. Methods for exposure assignment
130 were previously described in detail (Baccarelli et al., 2007; Baccarelli et al., 2007;
131 Baccarelli et al., 2008; Baccarelli et al., 2009). Hourly concentrations of PM₁₀ were
132 obtained from the Regional Environmental Protection Agency (ARPA Lombardia)
133 which recorded the hourly air pollution data from January 1994 to September 2005
134 using monitors located at 53 different sites throughout the Lombardy region (Baccarelli
135 et al., 2007). All patients and controls provided informal written consent and the study
136 was approved by the local ethics committee.

137

138 **2.2 Fibrin Polymerisation by Turbidity Analysis**

139 For turbidimetric analysis of fibrin polymerisation, plasma was diluted 1:3 in 0.05 M
140 Tris-HCl, 0.1 M NaCl, pH 7.5 in a 96-well plate, and 0.5 U/mL human thrombin (Sigma-
141 Aldrich, St. Louis, Mo) and 10 mM Calcium Chloride (final concentrations) were added.

142 Immediately after the addition of thrombin and calcium, absorbency was read every
143 12 seconds at 340 nm for 60 minutes with a Kinetic Plate Reader (Spectramax Plus
144 384, Molecular Devices, UK). Lag time (defined as the time required for the OD to
145 increase >0.01) and maximum OD (OD_{max}) were measured. The lag phase of the
146 turbidity curve reflects the time required for lateral aggregation of fibrin fibres to start
147 from the addition of the activation mixture. Maximum absorbance at the plateau phase
148 reflects the fibre diameter and fibrinogen concentration. Polymerisation rate was
149 analysed by measuring the slope of the turbidity curve at its steepest or inflexion point
150 (Mills et al., 2002).

151

152 **2.3 Laser Scanning Confocal Microscopy**

153 Laser scanning confocal microscopy provides detailed analysis of the fully hydrated
154 fibrin clot structure, allowing visualization of the 3D structure of the clot and direct
155 quantification of fibrin clot structure using image analysis tools. Human fibrinogen
156 labeled with fluorescein isothiocyanate (FITC) (final concentration 50 µg/ml) and 0.5
157 M CaCl₂ (final concentration 10 mM) were mixed with plasma samples (diluted 1:3 in
158 0.05 M Tris-HCl, 0.1M NaCl, pH 7.5). Next, human thrombin (final concentration 0.5
159 U/ml) was added to the mixture. Fibrin clots were prepared in a total volume of 60 µl,
160 and immediately upon the addition of thrombin, samples were briefly mixed and 30 µl
161 was transferred to a 6 channel µ-slide VI 0.4 (Ibidi, Martinsried, Germany), which was
162 placed in a humidity chamber for 30 minutes to prevent dehydration of the clot. The
163 3D structure of the clot was visualized by laser scanning confocal microscopy using a
164 LSM 700 T-PMT ZEISS microscope (ZEISS, Jena, Germany). Clot structure was
165 analysed using a 63x oil immersion lens with a 5W argon laser and 488 nm laser filter.

166 The images were collected in the format of 512x512 pixels. Fibre density was
167 calculated as the number of fibres crossing a straight line of fixed length across the
168 scan-field (Bhasin et al., 2008). All measurements were performed with Image J
169 version 1.25s software (Wayne Rasband, National Institutes of Health).

170

171 **2.4 Analysis of Pore-structure by Permeation**

172 Permeability analysis was performed to study the average pore-structure of the fibrin
173 network. In this method, the flow rate of a liquid passing through the fibrin clot is
174 measured under a constant pressure drop. A total of 10 mM calcium chloride and 1
175 U/ml human thrombin (Sigma) (final concentrations) were added to 30 μ L plasma
176 samples. After incubation in a wet chamber for 120 minutes at room temperature,
177 plastic tubes containing the clots were connected to a reservoir with buffer (0.05 M
178 Tris-HCl, 0.1 M NaCl, pH 7.5) and the buffer was allowed to flow through the fibrin
179 gels with a pressure drop of 4 cm. After washing for 120 minutes, the volume of the
180 buffer that flowed through the clot was measured in 4 sequential 30 minutes collections
181 over 120 minutes. The flow rate represents the pore size of fibrin clot structure,
182 expressed as Darcy constant or Ks. The Ks was calculated as previously described
183 (Mills et al., 2002).

184

185 **2.5 Statistical Analysis**

186 All experiments were performed in triplicate. Fits of the Ks, Δ Abs_{340nm}, and fibre
187 number distributions to the normal distribution were tested using the Kolmogorov-
188 Smirnov test. Pearson's correlation test was used to analyze the association between

189 clot parameters and patients' characteristics. The ANOVA and Chi Square tests were
190 used for investigating the intergroup differences for continuous variables and
191 categorical variables respectively. P-values less than 0.05 were considered to indicate
192 statistical significance. Analysis was performed with SPSS version 20 (IBM,
193 Portsmouth, UK).

194 To analyze the data, we categorized several parameters from continued data to
195 categorical data. As the concentrations of PM₁₀ were variable between days, we
196 decided to average all PM₁₀ individual exposure data from the day of event (DVT) up
197 to 730 days before the event. We chose 45.6 µg/m³ as the cut-off point to divide
198 patients and controls into two exposure groups because it represented the mean value
199 of PM₁₀ concentrations over the total period of 730 days. The participants with mean
200 exposure PM₁₀ levels below 45.6 µg/m³ were classed as low exposure and those with
201 PM₁₀ exposure levels above 45.6 µg/m³ were classed as high exposure. The number
202 of subjects with positive thrombophilia in each group was represented by percentages.

203

204 **3. RESULTS**

205 There were significant correlations between maximum absorbance and the number of
206 fibres ($r = 0.4$, $p < 0.001$), maximum absorbance and Ks ($r = -0.5$, $p < 0.001$), and
207 number of fibres and Ks ($r = -0.5$, $p < 0.001$). Maximum absorbance and fibre number
208 were both positively correlated with age, body mass index (BMI), fibrinogen
209 concentration and plasma level of FVIII, whereas Ks was negatively correlated,
210 indicating that with increasing age, BMI, fibrinogen concentrations and FVIII levels, the
211 fibrin fibres grew thicker, and were more compactly woven in the three-dimensional

212 clot network, and that the clot was less permeable. Except for Ks, both fibre thickness
213 ($r = 0.1$, $p = 0.042$) and fibre number ($r = 0.2$, $p = 0.001$) were associated with PM10
214 concentrations. Representative fibrin clot structures formed from plasma as imaged
215 using laser scanning confocal microscopy are shown in Fig 1.

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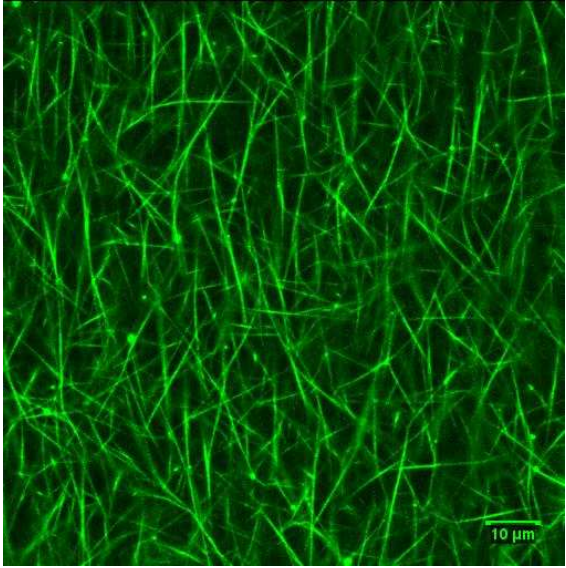
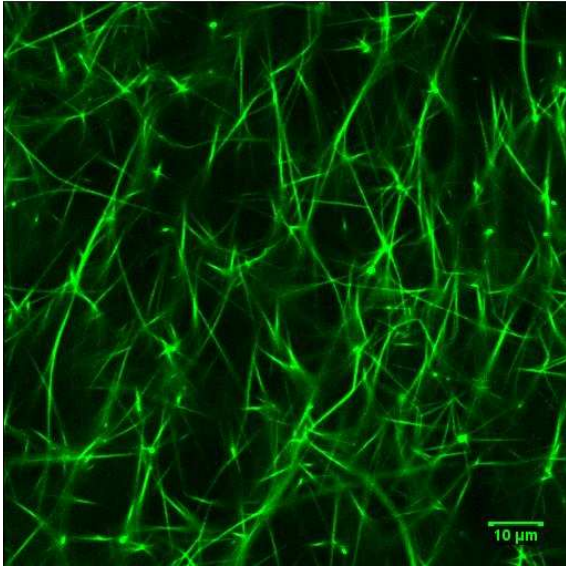
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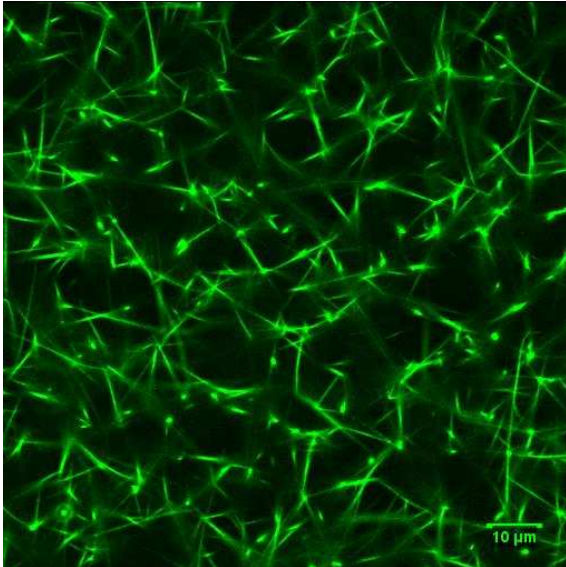
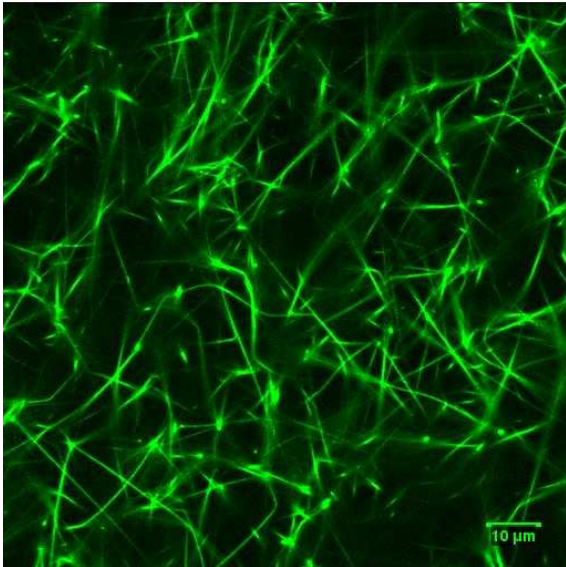
227 **Figure 1. Representative fibrin clot structures formed from plasma of subjects**
228 **exposed to high and low levels of PM₁₀**



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A. Patient A exposed to low PM10 levels

B. Patient B exposed to high PM10 levels



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233 C. Control A exposed to low PM10 levels

D. Control B exposed to high PM10 levels

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235 General characteristics, FVIII, fibrinogen, thrombophilia abnormalities and fibrin clot
236 structure parameters of patients and controls are shown in Table 1. Patients and
237 controls differed in sex distribution (more women among patients), BMI (higher in
238 patients), and thrombophilia abnormalities (more frequent in patients). FVIII and
239 fibrinogen were also higher in patients than controls. In terms of the clot structure, only
240 fibre number was significantly different between patients and controls ($p = 0.018$).

241 However, there was a tendency for patients to show denser fibrin clot structure with
242 thicker fibres, more number of fibres per clot area and less permeable clots compared
243 to controls.

244 Patients exposed to high levels of air pollution showed higher concentrations of
245 fibrinogen compared to those exposed to low levels, whereas thrombophilia was
246 similarly distributed (Table 2). We also compared the fibrin clot structure parameters
247 by exposure levels in patients and controls. Patients in the high exposure group had
248 thicker fibres, more compactly arranged fibres and less permeable structures
249 compared to those in the low exposure levels. However, in controls only plasma levels
250 of coagulation FVIII were different between the two exposure groups ($p = 0.029$).

251 Table 3 shows logistic regression analysis of risk factors for DVT. The continuous data
252 age, BMI, fibrinogen concentration FVIII level were categorized into high and low
253 groups, the cut-off points were 51.9, 24.7, 299.7, and 125.3 respectively. The model
254 showed that increased age, BMI and fibrinogen concentrations did not contribute to
255 the development of DVT in this study. Male sex was a risk factor for DVT, as well as
256 FVIII, thrombophilia abnormalities and high level of PM₁₀.

257 Finally, we analyzed the relative contributions of age, sex, BMI, thrombophilia
258 abnormalities, PM₁₀ and interaction of thrombophilia abnormalities and PM₁₀ with the
259 variation in maximum absorbance, fibre number and Ks by linear regression in patients
260 and controls, respectively (Table 4). In the maximum absorbance model, BMI and
261 PM₁₀ exposure both significantly contributed to the formation of thicker fibres in
262 patients only, whereas age was significantly correlated with maximum absorbance in
263 controls. In the fibre number model, PM₁₀ and BMI were risk factors for more branched
264 fibre formation for both patients and controls. In the Ks model, exposure to PM₁₀ did

265 not contribute to the alterations of clot structure in patients or controls. Permeability of
266 the clot reduced with BMI increased in patients but not in controls. Neither
267 thrombophilia abnormalities nor the interaction of thrombophilia abnormalities and
268 PM₁₀ were contributing to the alteration of fibrin clot structure in this study. Due to the
269 relatively small sample size of patients who had thrombophilia, there was not enough
270 power to investigate the relationship between fibrin clot structures and thrombophilia.
271 Another reason for the absence of an effect of thrombophilia may be that there was a
272 large degree of heterogeneity of the causes of thrombophilia in this small group,
273 including defects of antithrombin, protein C, antiphospholipids, FV Leiden mutation or
274 prothrombin mutation. Each of these could have differential effects on fibrin clot
275 structure, and therefore overall, due to the small sample size, and heterogeneity of the
276 group, there was no effect on clot structure in our study (figure shown in appendix).

277

278 **Table 1. Characteristics of patients with deep vein thrombosis and controls**

Variables	Patients Mean ±SD or percentage%	Controls Mean ±SD or percentage%	P-Value
Number of subjects	103	121	
Age (years)	53.7 ±14.7	50.4 ±13.9	0.085
BMI	25.5 ±4.2	24.0 ±4.3	0.014
Men %	48.5%	25.6%	<0.0001
Primary education or below %	70.9%	77.7%	0.187
Non-Smoking %	33.0%	76.9%	0.584
Thrombophilia[^] %	40.8%	14%	<0.0001
Factor VIII (%)	141.6 ±43.1	108.1 ±27.4	<0.0001
Fibrinogen (mg/dl)	309.4 ±80.7	290.7 ±50.9	0.070
Maximum Absorbance (mOD)	719.2 ±175.2	679.3 ±156.9	0.073
Fibre Number (per µm)	22.5 ±3.5	21.3 ±3.8	0.018
Ks (x10⁻¹⁰ cm²)	28.8 ±8.8	31.4 ±12.2	0.248

279 [^] Thrombophilia was classified as being positive for at least one of the following: factor V Leiden, prothrombin G20210 mutation,
 280 antithrombin-, protein C-, protein S-deficiency, antiphospholipids antibodies and hyperhomocysteinemia.

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282 **Table 2. Clotting parameters (mean ± SD) in patients and controls of high and low PM₁₀ exposure**

Variables	Patients			Control		
	Low Exp (n=23)	High Exp (n=80)	P-Value	Low Exp (n=72)	High Exp (n=49)	P-Value
PM₁₀ Levels (µg/m³)	39.3 ±8.5	48.9 ±2.6	<0.001	41.61 ±4.8	49.20 ±3.0	<0.001
Thrombophilia[≠]	47.8%	38.8%	0.435	12.5%	16.3%	0.552
Factor VIII (%)	132.2 ±38.0	144.2 ±44.3	0.260	114.2 ±30.2	101.7 ±22.9	0.029
Fibrinogen (mg/dl)	277.2 ±81.3	320.4 ±78.1	0.026	283.8 ±64.9	300.4 ±45.3	0.168
Maximum Absorbance (mOD)	626.4 ±155.5	745.8 ±172.2	0.003	675.9 ±171.2	684.2 ±134.7	0.776
Fibre Number (per µm)	20.4 ±3.9	23 ±3.1	0.001	21 ±4	21.7 ±3.4	0.307
Ks (x10⁻¹⁰ cm²)	33.7 ±11.2	26.3 ±6.1	0.006	30.1 ±11.4	35.2 ±14.5	0.236

283 [≠]The percentage of subjects with positive thrombophilia in each group

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Table 3. Logistic regression analysis of risk factors for deep vein thrombosis

Determinants or Variables	OR	95% CI	p-value
Age > 51.9 years	0.70	0.32-1.52	0.368
Men	3.02	1.36-6.74	0.007
BMI > 24.73	0.88	0.39-1.95	0.748
Thrombophilia	2.65	1.16-6.05	0.020
FVIII > 125.27%	5.52	2.52-12.10	<0.0001
Fibrinogen > 299.73 mg/dl	1.44	0.65-3.17	0.371
PM₁₀ Exposure Level > 45.6 µg/m³	3.85	1.79-8.28	0.001

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299 **Table 4. Linear regression analysis of risk factors for Maximum Absorbance, Fibre Number and Ks (cases/controls)**

Determinants	Maximum Absorbance		Fibre Number		Ks	
	Correlation Coefficient (95% CI)		Correlation Coefficient (95% CI)		Correlation Coefficient (95% CI)	
	Case	Control	Case	Control	Case	Control
Age (years)	0.47 (-1.75-2.70)	2.30* (0.10-4.49)	-0.02 (-0.07-0.03)	0.09 *** (0.04-0.14)	-0.15 (-0.33-0.02)	-0.26 (-0.52-0.01)
Men	9.01 (-56.58-74.60)	-47.2 (-112.65-18.24)	-0.18 (-1.58-1.22)	-0.42 (-1.84-1.00)	-1.16 (-6.64-4.33)	3.03 (-5.68-11.74)
BMI	18.12 *** (10.34-25.91)	5.93 (-1.26-13.12)	0.22 * (0.06-0.39)	0.22 ** (0.06-0.37)	-0.65 * (-1.30-(-0.01))	-0.53 (-1.30-0.24)
Thrombophilia	358.56 (-128.83-845.96)	-227.89 (-886.78-431.00)	8.03 (-2.67-18.74)	7.78 (-6.48-22.04)	-16.08 (-48.96-16.81)	-70.42 (-153.83-12.99)
PM₁₀ Exposure Level (µg/m³)	10.07 ** (3.58-16.56)	-1.44 (-6.90-4.03)	0.22 ** (0.08-0.35)	0.12* (0.01-0.24)	-0.32 (-0.75-0.12)	0.41 (-0.48-1.31)
Interaction (Thrombophilia and PM₁₀ exposure)	-7.62 (-17.95-2.71)	4.99 (-9.29-19.26)	-0.157 (-0.38-0.07)	-0.21 (-0.52-0.10)	0.34 (-0.39-1.08)	1.85 (-0.10-3.80)

300 The correlation coefficient is of statistical significance *p<0.05; **p<0.01; ***p<0.001.

301

302 4. DISCUSSION

303 We found that after long-term and high-level exposure to air pollution (PM₁₀
304 concentrations over 45.6 µg/m³), patients with DVT had significantly denser fibrin clot
305 structures compared to those living in areas with lower levels of exposure (PM₁₀ less
306 than 45.6 µg/m³). In the high exposure group, clots from patients contained thicker
307 fibres, more compact fibre arrangements and less permeable fibrin structures. There
308 were no significant differences in fibrin clot structure between the two exposure levels
309 in healthy subjects.

310 The mechanisms underpinning this difference between patients and healthy controls
311 are unknown but may be related to the differences in susceptibility of fibrin clot
312 structure to air pollution PM exposure. In a previous in vitro study we found that at
313 least part of the effect of particulate matter on fibrin structure could be attributed to
314 oxidative stress induced by the particles (Metassan et al., 2010). It is possible that
315 healthy subjects are more resistant to oxidative stress than patients with venous
316 thrombosis, since the latter may have an enhanced inflammatory state (Franchini and
317 Mannucci, 2011), that increases oxidative stress. Alternatively, due to increased levels
318 of inflammatory proteins and coagulation activation in patients with venous thrombosis,
319 any additional oxidative effects caused by air pollution on fibrin clot structure could be
320 more pronounced, perhaps due to a threshold effect, or a minimum level of oxidative
321 stress needed for effects on clot structure to become apparent. Finally, due to the
322 inflammatory state, pulmonary function may be impaired, leading to translocation of
323 ultrafine PM into the circulation. However, these considerations remain speculative as
324 there currently are no reliable methods to analyze PM in the blood, nor do we have
325 detailed information regarding the pulmonary function in our patients.

326 We previously investigated the effects of transient exposure (2 hours) to diesel particle
327 air pollution in a controlled environment in healthy, young individuals (Metassan et al.,
328 2010), finding that fibrin clot structure in plasma from subjects after short-term diesel
329 exhaust exposure was not significantly different compared to those who were exposed
330 to filtered air (Metassan et al., 2010). Our current findings extend these findings and
331 further indicate that even after chronic, long-term and high level exposure to particulate
332 matter, the fibrin clot properties in healthy controls remain similar to those who were
333 exposed to lower levels of PM exposure.

334 Consistent with a larger previous study on the association between air pollution and
335 venous thrombosis (Baccarelli et al., 2008), PM₁₀ exposure in the current study was a
336 strong risk factor for DVT and men had higher risk of DVT than women. Baccarelli et
337 al. (2008) showed that DVT risk was associated with the concentrations of PM₁₀
338 measured during the year before diagnosis (Baccarelli et al., 2008). In the current
339 study, sex, levels of factor VIII, thrombophilia abnormalities, and PM₁₀ exposure level
340 were all significantly associated with the risk of DVT. Increased levels of coagulation
341 factors, such as factor VIII, have previously been associated with increased risk of
342 thrombosis (A Undas et al., 2009). Thrombophilia abnormalities are also contributing
343 factors that modulate fibrin clot structure. The prothrombin G20210 mutation leads to
344 the increase plasma level of prothrombin which triggers the formation of denser clot
345 structure composed of more branched thinner fibres (Wolberg and Campbell, 2008).
346 However, age, BMI and fibrinogen concentrations were not significantly associated
347 with DVT.

348 We also observed some differences in clot structure between patients and controls.
349 Clots formed from plasma of patients had denser, less permeable fibrin clot structures

350 containing more, thicker fibres compared to controls, although the differences did not
351 reach statistical significance, possibly due to the relatively small number of subjects
352 studied. These data provide some support to previous studies by Undas et al.(2009),
353 in which plasma from patients with DVT and pulmonary embolism (PE) formed clots
354 with lower clot permeability and higher maximum absorbency than controls (A Undas
355 et al., 2009). But there were still some different findings that in our study in the patient
356 group, there were 93 patients with deep vein thrombosis (DVT) and 10 patients with
357 pulmonary embolism (PE). By comparing the fibrin clot structures, there were no
358 significant difference between patients with DVT and patients with PE (shown in the
359 table in the appendix). However, Undas et al. previously found differences in that
360 patients with DVT only (n=66) had denser fibrin clot structure and prolonged clot lysis
361 time compared to patients with DVT and PE (n=34) (Anetta Undas et al., 2009). The
362 finding that there were no differences in our study may due to the relatively small
363 sample size of patients with PE, since only 10 patients had DVT and PE compared
364 with 93 patients with DVT only.

365 One possible mechanism by which air pollution may contribute to the development of
366 thrombosis could involve local pulmonary inflammatory and oxidative responses with
367 the release of prothrombotic factors and inflammatory cytokines into the circulation
368 after the inhalation of particles (Emmrechts and Hoylaerts, 2012; Mills et al., 2009;
369 Newby et al., 2014). Previous animal studies showed that PM₁₀ caused lung
370 inflammation following intrapulmonary instillation of PM and inhalation of concentrated
371 ambient particles (Donaldson et al., 2005; Elder et al., 2004; Mills et al., 2009). In
372 clinical studies, pulmonary inflammation occurred after inhalation of both concentrated
373 ambient particulate matters and dilute diesel particles (Ghio et al., 2000; Mills et al.,
374 2009; Salvi et al., 1999). After exposure, plasma concentrations of pro-inflammatory

375 cytokines such as interleukin (IL) - 1 β , IL-6 and tumor necrosis factor- α increased
376 (Donaldson et al., 2005; Fujii et al., 2002; Mills et al., 2009). In both animal and clinical
377 studies, exposure of PM also led to the elevation of fibrinogen concentrations (Mills et
378 al., 2009; Schwartz, 2001). High concentrations of fibrinogen shorten the lag phase of
379 polymerization, increased branch point densities, fibre thickness and clot rigidity, with
380 concurrent increases in the resistance of the clot to fibrinolysis (Scott et al., 2004;
381 Weisel, 2007).

382 A second possible mechanism may involve direct translocation of particulate matter
383 from the pulmonary alveoli into the blood circulation, crossing the pulmonary
384 epithelium and vascular endothelium barrier (Emmrechts and Hoylaerts, 2012; Mills
385 et al., 2009; Newby et al., 2014). Particles with diameters less than 10 μm can be
386 inhaled deeply into the lungs (Mills et al., 2009). A number of other factors may
387 influence the possible translocation of PM, including charge, chemical composition,
388 and propensity to form aggregates (Mills et al., 2009). The size and shape of the
389 particles could affect the region of deposition in the respiratory system, with smaller
390 sized particles penetrating deeper into the lung. Macrophages may not be able to
391 recognize particles with a diameter less than 500nm, and for this reason, ultrafine
392 particulate matters may enter the blood or lymphatic systems more easily and transfer
393 to different organs (Teow et al., 2011). Once in the circulation, the particles could
394 interact with vascular endothelial cells and have direct effects on the atherosclerotic
395 plaque (Mills et al., 2009), platelets (Lauer et al., 2009) and fibrin clot formation,
396 structure and stability.

397 As Mills et al. mentioned that diesel exhaust inhalation causes vascular dysfunction
398 and impaired endogenous fibrinolysis (Mills et al., 2005). Furthermore, previous

399 studies have shown that denser fibrin clot structures were associated with prolonged
400 lysis time (Ajjan and Grant, 2006; Ariens, 2013; Scott et al., 2004; Undas and Ariens,
401 2011). Therefore, as patients exposed to high level of air pollution had denser fibrin
402 clot structures, the lysis time compared to those patients only exposed to low level of
403 air pollution is likely longer. Future studies will be needed to further evaluate the effects
404 of air pollution exposure on fibrinolysis in patients with venous thrombosis.

405 Possible limitations of our study include the relatively small study sample size (due to
406 the time-consuming nature of fibrin structure analysis), and that we had no information
407 regarding personal levels of air pollution exposure for the participants. The
408 concentrations of PM₁₀ in this study were measured according to the area of residence
409 for the subjects, which were different for each subject and spanned several residential
410 areas in Lombardy. Therefore, although exposure to air pollution was not measured
411 with personal monitors, the data obtained did provide average daily, specific and long-
412 term individual exposure to air pollution.

413

414 **5. CONCLUSION**

415 In conclusion, this study showed patients with venous thrombosis exposed to high
416 level of air pollution had denser fibrin clot structures with thicker fibres (higher
417 maximum absorbance), decreased permeability (lower Ks value) and higher fibre
418 numbers compared to those in the low exposure group, indicative of a prothrombotic
419 clot structure. There were no differences in fibrin clot structure measurements between
420 the two exposure groups in controls, suggesting that air pollution may trigger
421 differences in fibrin clot structure only in patients predisposed to thrombotic disease.

422

423

424 **6. LIST OF ABBREVIATIONS**

425 PM -- Particulate Matter

426 FVIII -- Factor VIII

427 DVT -- Deep Vein Thrombosis

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430 **7. ACKNOWLEDGEMENT**

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570 **9. APPENDIX**

571 Table 5. Clotting parameters (mean \pm SD) in patients with DVT only and patients with DVT and PE

Patients with DVT Only Clotting Parameters	Mean (\pmSD)	Patients with DVT and PE Clotting Parameters	Mean (\pmSD)	P Value
Maximum Absorbance	68.799 (\pm 18.839)	Maximum Absorbance	59.806 (\pm 11.109)	0.167
Fibre Number	21.915 (\pm 3.844)	Fibre Number	20.677 (\pm 3.523)	0.206
Ks	2.868 (\pm 0.884)	Ks	2.913 (\pm 0.899)	0.890

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577 Table 6. Clotting parameters (mean \pm SD) in patients with and without thrombophilia abnormalities

Patients with Thrombophilia Abnormalities (n=61) Clotting Parameters	Mean (\pmSD)	Patients without Thrombophilia Abnormalities (n=42) Clotting Parameters	Mean (\pmSD)	P Value
Maximum Absorbance	71.815 (\pm 18.606)	Maximum Absorbance	71.989 (\pm 16.889)	0.568
Fibre Number	22.922 (\pm 3.043)	Fibre Number	22.182 (\pm 3.718)	0.365
Ks	2.840 (\pm 0.738)	Ks	2.901 (\pm 0.978)	0.205

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