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2 Increased circulating endothelial cells in acute heart failure: Comparison 3 with von Willebrand factor and soluble E-selectin

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7

8 Abstract

9 *Background:* Circulating endothelial cells (CECs) in the peripheral blood, probably representing the most direct evidence of endothelial cell
10 damage, are increased in myocardial infarction, unstable angina and critical limb ischaemia. As chronic heart failure is also associated with
11 endothelial abnormalities, we hypothesised that CECs are raised in acute heart failure and that they would correlate with plasma indices of
12 endothelial perturbation, that is, von Willebrand factor (vWf) and soluble E-selectin.

13 *Methods:* We studied 30 patients with acute heart failure (venesection within 24 h of emergency hospital admission), 30 patients with chronic
14 stable heart failure (venesection as out-patients, all patients in sinus rhythm with ejection fraction $\leq 40\%$) and 20 healthy controls. CECs were
15 quantified using epifluorescence microscopy after CD146-immunomagnetic separation and phenotyped by streptavidin/biotin immunocy-
16 tochemistry. Citrated plasma was analysed for soluble E-selectin and vWf by ELISA.

17 *Results:* Levels of CECs, vWf and soluble E-selectin were significantly higher (all $p < 0.01$) in patients with heart failure compared to
18 controls, with no significant differences between acute and chronic heart failure. CECs correlated with plasma vWf ($p < 0.0001$) and soluble
19 E-selectin ($p = 0.022$) but not ejection fraction or NYHA class. In multiple regression analysis, heart failure was the only independent
20 predictor of raised CECs ($p < 0.0001$). Immunoperoxidase-defined surface expression of CD34, CD45 and CD36 by CECs was $< 2\%$, 0% and
21 8% , respectively.

22 *Conclusion:* CECs, a possibly heterologous population, may be used as a novel measure of endothelial damage in acute heart failure and
23 may have implications for the thrombotic risk associated with acute and chronic heart failure and prognosis in this condition.

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25

26 *Keywords:* Circulating endothelial cells; von Willebrand factor; Soluble E-selectin; Heart failure; Endothelium

27

28 1. Introduction

29 Good endothelial function and integrity are of undoubted
30 importance in cardiovascular disease, including chronic
31 heart failure, and can be assessed by physiological
32 techniques such as flow-mediated dilatation and changes
33 in specific plasma markers (e.g. von Willebrand factor, vWf)
34 [1,2]. Increased numbers of circulating endothelial cells
35 (CECs) in the peripheral blood are present in various
36 pathological conditions involving severe endothelial pertur-
37 bation, including inflammatory disease, acute myocardial

infarction, chronic stable heart failure, unstable angina and 38
critical limb ischaemia, but not in angina or intermittent 39
claudication compared to healthy controls [3–6]. Since 40
CECs are rare in the blood of healthy persons, increased 41
levels imply the most severe form of blood vessel injury, 42
(i.e. that removes adherent endothelial cells from the 43
internal elastic lamina). It follows that desquamated and 44
detached CECs imply that areas of the endothelium are 45
denuded, thus exposing the underlying prothrombotic sub- 46
endothelial layer to the circulating blood. As a consequence, 47
this may activate platelets and the coagulation cascade and 48
be partly responsible for the prothrombotic or hypercoagu- 49
lable state in these conditions. Indeed, this, in turn, may 50
contribute to the high morbidity and mortality [7,8]. 51

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52 However, CECs are not the only index of endothelial
53 perturbation. As indicated, plasma vWf (a marker of
54 endothelial damage/dysfunction) is increased in all major
55 risk factors for atherosclerosis and in clear atherosclerotic
56 disease (including heart failure) and increased levels also
57 predict poor long-term outcome [9,10]. An alternative
58 endothelial cell plasma marker, soluble E-selectin (possibly
59 reflecting inflammatory endothelial activation), is also
60 raised in numerous cardiovascular diseases although the
61 data on its ability to predict adverse cardiovascular events is
62 not as convincing [4,11,12]. Both vWf and soluble E-
63 selectin have been shown to be abnormal in chronic stable
64 heart failure [13,14].

65 Thus, there is considerable evidence of abnormal
66 endothelial function in chronic stable heart failure
67 [6,8,13,14]. We therefore hypothesised that CECs are also
68 raised in acute heart failure and that they correlate with
69 established plasma indices of endothelial perturbation (i.e.
70 vWf and soluble E-selectin). To test this hypothesis, we
71 conducted a cross-sectional study of patients with acute
72 onset heart failure who were compared to patients with
73 chronic stable heart failure and to healthy age- and sex-
74 matched controls. We also performed immunocytochemistry
75 to phenotype these CECs.

76 2. Patients and methods

77 2.1. Subjects

78 We recruited 30 patients with acute heart failure, 30
79 patients with chronic heart failure and 20 healthy controls.
80 Clinical characteristics of patients and controls are summar-
81 ised in Table 1. Heart failure was defined according to the
82 European Society of Cardiology guidelines [15]. Acute
83 heart failure patients were recruited and venesected within
84 24 h of hospital admission and had radiographic evidence of
85 pulmonary oedema, as well as clinical evidence of heart
86 failure. Therefore in virtually all cases, pharmacotherapy
87 had already commenced. Chronic heart failure was defined
88 as patients being in a stable NYHA class for at least 3
89 months and were recruited from out-patient clinics. All heart
90 failure patients were in sinus rhythm and had documented
91 left ventricular ejection fraction of $\leq 40\%$ either by M-mode
92 echocardiography or Simpson's method in the presence of
93 significant regional wall motion abnormality. Patients were
94 also classified according to the New York Heart Association
95 (NYHA) criteria, with I–II being mild symptoms and III–
96 IV being moderate to severe symptoms. Patients with acute
97 heart failure were studied within 24 h of admission of
98 hospital, whereas the (entirely separate) cohort of patients
99 with chronic heart failure was studied in our out-patient
100 research clinic. The study protocol was approved by the
101 West Birmingham Research Ethics Committee and all
102 patients gave written informed consent to the study
103 according to the Declaration of Helsinki.

Table 1

Baseline characteristics of study population

	Acute HF	Chronic HF	Controls	<i>p</i>	
<i>N</i>	30	30	20	–	t1.1
Sex (M:F)	20:10	22:8	7:13	0.03	t1.2
Age (years)	65 (11)	64 (13)	63 (9)	0.891	t1.3
NYHA I–II versus III/IV	4/26	17/13	–	0.001	t1.4
LVEF (%)	30 (22–32)	30 (29–34)	–	0.341	t1.5
<i>Comorbidity</i>					t1.6
IHD (%)	21 (70)	24 (80)	–	0.375	t1.7
HT (%)	16 (64)	10 (40)	–	0.069	t1.8
DM (%)	8 (32)	13 (42)	–	0.190	t1.9
Cholesterol (mmol/dL)	4.3 (1.4)	4.5 (1.0)	5.7 (1.0)	0.001	t1.10
Smoking (%)	3	10	5	0.546	t1.11
<i>Treatment (n and percentage of patients taking)</i>					t1.12
ACEI/ARB	23 (77)	29 (97)	–	0.023	t1.13
Spiroinolactone	9 (30)	6 (20)	–	0.371	t1.14
Beta blocker	6 (20)	13 (43)	–	0.052	t1.15
Anti-platelet	19 (63)	23 (80)	–	0.260	t1.16
Warfarin	6 (20)	3 (10)	–	0.278	t1.17
Statin	10 (33)	20 (67)	–	0.010	t1.18

Data expressed as mean (SD) (age and cholesterol), median (IQR)(LVEF) or *n* (%) unless stated.–not estimated. HF=heart failure; NYHA I–IV=New York Heart Association classification; LVEF=left ventricular ejection fraction based on M-mode echocardiography or Simpson's; IHD=ischaemic heart disease; HT=hypertension; and DM=diabetes mellitus. *p* values based on chi-square except age and cholesterol by ANOVA and LVEF by Mann–Whitney.

104 Exclusion criteria were concomitant atrial fibrillation,
105 acute coronary syndromes (hospital admission for acute
106 myocardial infarction or unstable angina in the previous 3
107 months), infection or pyrexial illness, recent (<3 months)
108 myocardial infarction or stroke, chronic and systemic ill-
109 nesses (including renal failure, hepatic impairment, cancer,
110 inflammatory connective tissue disease and inflammatory
111 bowel disease), past history of thromboembolism and the use
112 of oral steroids and hormone replacement therapy. Some of
113 the patients were on warfarin because of left ventricular
114 thrombus, echocardiographic contrast or large regional wall
115 motion abnormality. Healthy control subjects were recruited
116 from amongst healthy hospital staff, spouses of patients and
117 from subjects attending hospital for hernia repair, varicose
118 vein procedures or other relatively minor operations. All
119 healthy control subjects had no clinical evidence of vascular,
120 metabolic, neoplastic, diabetic or inflammatory disease on
121 careful history, examination and routine laboratory tests.
122 None were taking prescription medicines.

2.2. Laboratory

123 Citrated plasma was obtained from venous blood by
124 centrifugation at 3000 rpm (1000 ×g) for 20 min at 4 °C.
125 Aliquots of citrated plasma were stored at –70 °C to allow
126 batch analysis. Soluble E-selectin was measured by ELISA
127 with R and D Systems reagents (Abingdon, UK), with a
128

129 minimum sensitivity of 1.6 ng/mL. vWf was measured by
130 an established ELISA (Dako, Ely, UK), with a minimum
131 sensitivity of 5 IU/dL. The intra-assay coefficient of
132 variation was <5% and inter-assay variation was <10%.

133 Blood for CECs was collected in a sodium fluoride tube,
134 prepared for immunomagnetic separation within 1 h and
135 counted by a single observer under epifluorescence micro-
136 scopy (Zeiss, Welwyn Garden City, UK). The detailed
137 methodology for capturing CECs and criteria for counting
138 CECs have been widely described [3–6]. Briefly, we
139 defined a CEC primarily as an autofluorescent event with
140 cell-like morphology binding a minimum of 4 magnetic
141 beads coated with a monoclonal antibody to CD146 and
142 having a diameter of at least 20 μ M (perhaps 4 beads in
143 size). However, other objects, whose morphology was not
144 classically cell-like but were clearly not artefacts, were also
145 identified. In order to be defined as a CEC, an object
146 without a clear cell-like morphology (i.e. possibly a cell
147 carcass) had to bind 10 or more beads. CECs are also
148 present in sheets or clumps binding numerous beads. In such
149 cases, the final number of CECs is determined by
150 approximating the number of 4-bead diameters. Intra-
151 ($n=40$ determinations) and inter-assay ($n=20$) coefficients
152 of variation were <5% and <10%, respectively; the inter-
153 and intra-observer variations of the method in our laboratory
154 were <5% [4]. All laboratory work was performed in
155 blinded fashion with respect to the identity of the samples.

156 CECs were stained by indirect immunocytochemistry by
157 standard techniques at room temperature. Briefly, CECs
158 were air dried to glass slides and re-hydrated in phosphate-
159 buffered saline (PBS) plus 10% normal swine serum (Dako,
160 Ely, UK) for 10 min. Following a PBS wash, a 1/50 dilution
161 of monoclonal antibodies (all Becton Dickinson, Oxford,
162 UK) to CD34 (marking bone marrow-derived progenitor
163 stem cells), CD36 (the thrombospondin receptor, marking
164 microvascular endothelial cells, platelets and monocytes/
165 macrophages) or CD45 (the leukocyte common antigen)
166 was applied to different slides for an hour, followed by a
167 PBS wash. Colour was developed by the Dako LSAB-2
168 System (Dako, Ely, UK), using first a 10-min biotin step,
169 washes, a 10-min streptavidin step, washes, and 5 min of
170 diaminobenzidine substrate. Slides were washed and taken
171 through alcohol to xylene and mounted under a coverslip.
172 Auto-fluorescence was retained: positive cells stained black.
173 Positive controls were normal Ficoll-prepared (Sigma
174 Aldrich, Poole, UK) peripheral blood mononuclear cells.

175 2.3. Power calculations

176 We have previously reported increased CECs in the
177 plasma of 26 subjects with acute myocardial infarction
178 (AMI) and 33 with unstable angina compared to 13 with
179 stable angina and 14 healthy controls with an overall F
180 statistic of 16 giving a p value of <0.001[3]. More recently
181 we found raised levels in 30 chronic heart failure patients
182 compared to 20 controls [6]. Consequently, we hypothesised

similar levels and distribution in acute heart failure 183
compared to chronic heart failure and versus healthy 184
controls. Thus, with three groups, our power calculation 185
required a minimum of 20 subjects per group to generate a 186
similar F statistic at $p<0.001$. This target number of 187
subjects ($n=60$) provides the power to detect a correlation 188
coefficient (r) of 0.35 at $p<0.05$ and $1-\beta=0.85$. 189

190 2.4. Statistical analysis

Data were analysed by the Shapiro–Wilks test to 191
determine distribution. Normally distributed data are 192
expressed as mean and standard deviation. As the data for 193
left ventricular ejection fraction (LVEF), sE-selectin and 194
CECs were not normally distributed, values were expressed 195
as median (inter-quartile range, IQR). Baseline cross- 196
sectional data among acute heart failure, chronic heart 197
failure and healthy controls were analysed by ANOVA, 198
Mann–Whitney or Kruskal–Wallis test as appropriate, with 199
between-group comparisons by Tukey’s post-hoc test and, if 200
appropriate, after log transformation. Categorical data were 201
compared using chi-squared test. Correlations were per- 202
formed using Spearman’s rank correlation method. Multi- 203
variate analysis was performed by stepwise multiple 204
regression analysis using CEC as the dependent variable 205
and clinical variables (e.g. age, gender, hypertension, 206
coronary artery disease, etc.) and the presence/absence of 207
heart failure as predictors. 208

209 3. Results

210 3.1. Cross-sectional analysis

Median number of CECs were significantly increased 211
(approximately three-fold) in both acute and chronic heart 212
failure compared to healthy controls, with no significant 213
difference between patient groups [Table 2]. Similarly (as 214
expected), mean vWf and median soluble E-selectin levels 215
were also both raised in both patients groups, but 216
(unexpectedly) there was no difference between the two 217
heart failure patient groups. Taking the mean plus two 218
standard deviations to be the top of the normal range, then 219

Table 2
Circulating endothelial cells, von Willebrand factor and soluble E-selectin
in heart failure patients and controls

Variable	Acute HF	Chronic HF	Controls	p	
CECs (cells/ml)	13 (7.0–18)	15 (8.4–20)	4.5 (1.6–7.2)	<0.0001	t2.4
vWf (IU/dl)	184 (62)	214 (82)	100 (51)	<0.0001	t2.5
sE-sel (ng/mL)	67 (43–142)	54 (40–100)	28 (20–56)	0.003	t2.6

sE-sel (soluble E-selectin) and CECs (circulating endothelial cells)
expressed as median (IQR) and vWf (von Willebrand factor) expressed
as mean (SD). p values from Kruskal–Wallis for sE-sel and CEC and
ANOVA for vWf. All indices were higher in both patient groups compared
to controls with no difference between patient groups ($p<0.05$ by Tukey’s
post-hoc test and sE-sel and CECs after log transformation). t2.7

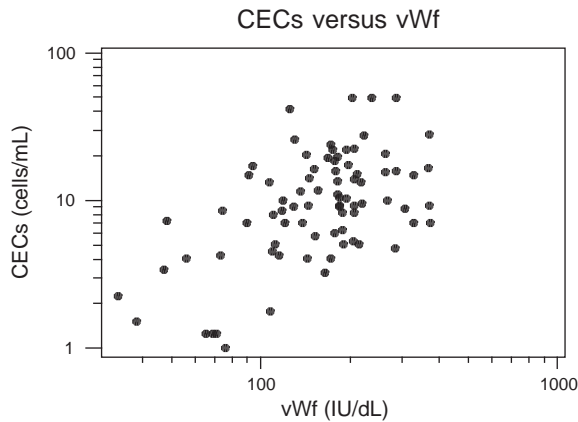


Fig. 1. Relationship of von Willebrand factor (IU/dl) and circulating endothelial cells (cells/ml). Spearman correlation, $r=0.463$; $p<0.0001$.

220 the top of the normal range for (normally distributed) vWf
 221 was exceeded by 21 of the 30 patients (70%) with chronic
 222 heart failure and by 15 of the 30 patients (50%) with acute
 223 heart failure. Similarly, taking the top of the normal range to
 224 be the 95th percentile for soluble E-selectin and CECs (as
 225 both are non-normally distributed), then 8 patients (27%)
 226 with chronic heart failure and 11 (37%) patients with acute
 227 heart failure had raised levels of soluble E-selectin, whilst
 228 22 patients (73%) in both heart failure groups had high
 229 levels of CECs.

230 3.2. Correlations

231 In the whole study group, CECs correlated well with
 232 vWf (Spearman, $r=0.463$; $p<0.0001$, Fig. 1) and modestly
 233 with soluble E-selectin ($r=0.256$; $p=0.022$). We are under-
 234 powered to perform other un-hypothesised sub-group
 235 analyses, e.g. for the effects of different therapies, hyper-
 236 tension, smoking, diabetes or ischaemic heart disease. For
 237 the same reason, we have not performed a formal
 238 sensitivity/specificity analysis. However, in stepwise multi-
 239 ple regression analysis of the entire study group, only heart
 240 failure was an independent predictor of raised CECs
 241 ($p<0.0001$).

242 3.3. Characteristics of CECs

243 Fig. 2 shows a typical, single CEC binding to seven
 244 immunobeads and a small contaminant. Immunophenotyp-
 245 ing of 124 CECs from patients with heart failure found <2%
 246 to co-stain for progenitor cell marker CD34 and 8% to co-
 247 stain for microvascular marker CD36. None stained for the
 248 leukocyte-common antigen CD45.

249 4. Discussion

250 Heart failure is a leading cause of mortality and hospital
 251 admissions [16] and patients hospitalized for acute and
 252 decompensated heart failure are at particularly high risk of

253 death, with up to 30% inpatient mortality and nearly one- 253
 254 half of patients being re-admitted within 6 months [17,18]. 254
 255 The causes of death in acute heart failure are largely cardiac 255
 256 in origin, but pulmonary embolism and ischaemic strokes 256
 257 account for up to 20% of non-cardiac deaths [19]. While 257
 258 cardiac deaths usually result from lethal arrhythmias, both 258
 259 epidemiological and autopsy results suggest that acute intra- 259
 260 coronary thrombotic occlusion may be the probable 260
 261 triggering event [20–22]. Acute heart failure patients can 261
 262 therefore be regarded to be at high risk of thrombosis-related 262
 263 complications. Indeed, the components of Virchow's triad 263
 264 are fulfilled in heart failure, with abnormalities of flow (poor 264
 265 cardiac function), vessel wall (endothelial damage/dysfunc- 265
 266 tion) and blood constituents and with significant abnormal- 266
 267 ities of haemostatic factors and platelet function [5,7]. 267

268 The present study extends the demonstration of raised 268
 269 CECs in acute myocardial infarction, chronic stable heart 269
 270 failure and critical limb ischaemia [3,4,6] to include acute 270
 271 heart failure and also shows that increased CECs correlate 271
 272 with two other markers of endothelial perturbation, i.e. 272
 273 plasma vWf and soluble E-selectin [8–12]. Our previous 273
 274 work in peripheral atherosclerosis and chronic heart failure 274
 275 demonstrated a correlation between CECs and vWf [4,6], 275
 276 findings that may have implications for the thrombotic risk 276
 277 associated with heart failure and poor prognosis in this 277
 278 condition. Indeed, as CECs represent the most direct 278
 279 evidence of endothelial damage, it is not surprising to find 279
 280 increased numbers in heart failure, in view of the extensive 280
 281 evidence of endothelial perturbation in this condition [6– 281
 282 8,13,14]. However, we expected all three endothelial 282
 283 markers to be higher in acute heart failure (requiring 283
 284 hospital admission) compared to (out-patient) chronic stable 284
 285 heart failure. That this did not occur is puzzling and may 285
 286 represent a limitation of our approach or relatively small 286
 287 numbers of subjects with wide distribution and/or the effects 287
 288 of different treatments and co-morbidities. Alternatively, it 288
 289 may represent the upper limit of CECs that are compatible 289
 290 with life. Thus we conclude that the degree of endothelial 290
 291 perturbation is similar between heart failure groups, regard- 291
 292 less of 'acute/chronic' status. 292

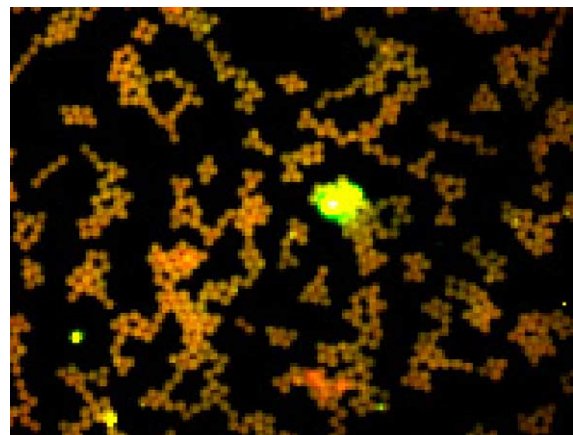


Fig. 2. A circulating endothelial cell rosetting with 7 immunobeads.

293 There is, at present, interest in both CECs and circulating
 294 endothelial progenitor cells (EPCs, originating from the
 295 bone marrow [23,24]). In our hands, CECs from patients
 296 with peripheral atherosclerosis are largely CD34 negative
 297 and are thus unlikely to be part of the EPC ‘family’ that
 298 bears this marker [4]. Furthermore, using exactly the same
 299 methodology, 93% of CECs from patients with acute
 300 coronary syndromes stained positive for endothelial nitric
 301 oxide synthase [25]. The present study of CECs from
 302 patients with heart failure found <2% to stain for CD34,
 303 again suggesting very few are EPCs. However, 8% stained
 304 positive for the microvascular cell marker CD36 [26,27],
 305 suggesting most CECs arise from large vessels. No cells
 306 stained for the leukocyte common antigen CD45 confirming
 307 the non-leukocyte nature of our CECs.

308 There is also some debate as to whether or not the CECs
 309 captured by this technique are apoptotic or non-apoptotic
 310 [3,28,29]. It is possible that the method may be capturing
 311 apoptotic cells in the early stages not detected by TUNEL
 312 staining [3]. However, the fact that these circulating cells (if
 313 still viable) are no longer attached to the basement
 314 membrane means that they will undergo apoptosis even-
 315 tually [28]. Certainly, *in vitro* studies show that apoptotic
 316 human umbilical vein endothelial cells become procoagu-
 317 lant by increased expression of phosphatidylserine and the
 318 loss of anticoagulant membrane components [30]. More-
 319 over, these ‘desquamated’ CECs indirectly suggest that the
 320 underlying prothrombotic subendothelial surface is exposed
 321 to the circulating blood resulting in activation of the
 322 coagulation cascade.

323 Whatever the origin or status of the CECs, the fact of
 324 increased numbers implies gross endothelial insult, although
 325 the immediate basic pathological event(s) responsible for
 326 this is/are unclear. Recent work supports the hypothesis that
 327 high shear stress in pulmonary hypertension could result in
 328 shedding of the involved endothelial cells, resulting in
 329 increased numbers of CECs [31]. The same study reported
 330 that 23% of CECs stained positive for E-selectin, implying a
 331 low degree of endothelial activation, compared to 7% of
 332 CECs from normal volunteers staining for this marker. This
 333 paper, like our present report, also showed an overlap
 334 between CECs in health and in disease, indicating the need
 335 for caution in interpreting causality or in additional assess-
 336 ment. Nevertheless, as raised vWf predicts poor outcome
 337 [9,10,14,25] and correlates with CECs (see Refs. [4,6] and,
 338 also, present data), it could be that the latter may also be
 339 useful in predicting those patients at risk of serious
 340 cardiovascular events and therefore worthy of more
 341 focussed care. Indeed, in acute coronary syndromes,
 342 increased CECs predict poor outcome at 30 days and 1-
 343 year follow up [25].

344 In conclusion, peripheral blood CECs are a relatively
 345 novel tool to assess endothelial damage in cardiovascular,
 346 autoimmune, cancer and other conditions, although they
 347 may be a collection of cells with different phenotypes [3–
 348 6,31–33]. However, CECs are arguably the most direct

evidence of endothelial damage *in vivo*. In heart failure, 349
 such endothelial damage (possibly induced by pathological 350
 processes such as hypoxia and/or increased oxidative stress) 351
 may play a role in the pathophysiology of the associated 352
 prothrombotic state. 353

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