Persisting Endothelial Cell Activation and Hypercoagulability after COVID-19 Recovery—The Prospective Observational ROADMAP-PostCOVID-19 Study

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Abstract: Background. Hypercoagulable state and endothelial cell activation are common alterations in patients with COVID-19. Nevertheless, the hypothesis of persistent hypercoagulability and endothelial cell activation following recovery from COVID-19 remains an unresolved issue. **Objectives**. To investigate the persistence of endothelial cell activation and hypercoagulability after recovery from COVID-19. **Patients/Methods.** COVID-19 survivors (n = 208) and 30 healthy individuals were enrolled in this study. The following biomarkers were measured: procoagulant phospholipid-dependent clotting time (PPL-ct), D-Dimer, fibrin monomers (FM), free Tissue factor pathway inhibitor (free-TFP)I, heparinase, and soluble thrombomodulin (sTM). Antibodies against SARS-CoV-2 (IgG and IgA) were also measured. **Results**. The median interval between symptom onset and screening for SARS-CoV-2 antibodies was 62 days (IQR = 22 days). Survivors showed significantly higher levels of D-Dimers, FM, TFPI, and heparanase as compared to that of the control group. Survivors had significantly shorter PPL-ct. Elevated D-dimer was associated with older age. Elevated FM was associated with female gender. Elevated heparanase was independently associated with male gender. Decreased Procoag-PPL clotting time was associated with female gender. One out of four of COVID-19 survivors showed increase at least one biomarker of endothelial cell activation or hypercoagulability. **Conclusions**. Two months after onset of COVID-19, a significant activation of endothelial cells and in vivo thrombin generation persists in at least one out of four survivors of COVID-19. The clinical relevance of these biomarkers in the diagnosis and follow-up of patients with long COVID-19 merits to be evaluated in a prospective clinical study.

Keywords: COVID-19; SARS-CoV-2; endothelial activation; hypercoagulability; thrombin generation

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes the coronavirus disease 2019 (COVID-19), which led to death more than 5.6 million patients worldwide. In patients with severe COVID-19, activation of blood coagulation and endothelial cells orchestrated with complement activation and cytokine storm leads to disease worsening and death [1].

Hypercoagulable state and endothelial cell activation and fibrinolytic unbalance are common alterations in patients with COVID-19 hospitalized either at the conventional medical ward or at the intensive care unit (ICU) [2,3]. Two systematic reviews highlight that D-dimer values are higher in nonsurvivors as well as in patients with severe COVID-19 than in those with mild disease, showing the association between D-dimer levels and disease severity or death [4,5]. Nevertheless, there are limited data in small series of patient, on the persistence of hypercoagulability and endothelial cell activation following recovery from COVID-19. The answer to this question is required to proceed to the research of biomarkers mandatory for the risk of either vascular complications after recovery from COVID-19 or long COVID-19. This need is urgent since long COVID-19 is a new, frequent, and potentially disabling consequence of SARS-CoV-2 infection, with important impact on healthcare systems [6–10].

The prospective observational study ROADMAP-postCOVID-19 (pROspective Risk Assessment anD bioMArkers of hyPercoagulability) for the identification of patients with COVID-19 at risk for disease worsening) aimed to investigate whether biological signs of hypercoagulability and endothelial cell activation persist in survivors of COVID-19. The study was conducted on COVID-19 survivors that fitted into the selection criteria for convalescence plasma donation and were enrolled in a phase II clinical trial.

2. Materials and Methods

The ROADMAP-postCOVID-19 study included COVID-19 survivors who participated in the phase II study (NCT04408209) for the assessment of efficacy and safety of convalescent plasma treatment of COVID-19 infection started in Greece on 28 April 2020. Survivors enrolled in the study gave informed consent, as previously described, and the study design and screening results for these survivors postsymptom onset or -PCR positivity were previously reported [11]. Survivors were tested for the presence of anti-SARS-CoV-2 in the period 28 April 2020 to 30 July 2020 in four centers in Greece and, after their initial screening, in the same centers.

All inclusion criteria for the survivors of COVID-19 were previously described [12]. In summary, the main inclusion criteria included: (i) signed informed consent; (ii) confirmed SARS-CoV-2 infection by positive rtPCR of the nasal and/or pharyngeal swab; (iii) interval of at least 14 days after complete recovery from a SARS-CoV-2 infection (no symptoms, complete resolution of organ dysfunction which was caused by SARS-CoV-2); (iv) anti-SARS-CoV-2 immune response with detectable anti-SARS-CoV-2 antibodies; (v) two negative SARS-CoV-2 rtPCR results (nasal and/or pharyngeal swab); the time interval between the two negative tests should be at least 7 days; (vi) no transfusion within 3 months prior to enrollment in the study; (vii) absence of pregnancy; (viii) normal complete blood count, prothrombin time, activated partial thromboplastin time, and fibrinogen levels on the day of inclusion; (ix) negative serological tests for hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), venereal disease research laboratory (VDRL), human T cell lymphotropic virus(HTLV)-1 and negative for HIV, HBV, and HCV with nucleic acid amplification testing; and (x) absence of any antithrombotic treatment. Survivors with known cardiovascular disease, long-term medications, cancer, or a known autoimmune disease were excluded from the study. All survivors enrolled in the study had to fulfil all the general criteria for blood donation in terms of age, general condition, hemoglobin levels, and vital signs.

The control group consisted of 30 healthy individuals who provided informed written consent before inclusion in the study. Individuals included in the control group were free

of any known hereditary or acquired thrombophilia or personal history of thrombotic or bleeding disorders, absent of pregnancy, had a negative serological test for cardiovascular diseases, and were not infected by the SARS-CoV-2.

Blood samples were obtained by atraumatic antecubital venipuncture from a peripheral vein on the day of inclusion in the study, using a 19-gauge needle. Blood was collected at Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ, USA; 3.8% sodium citrate 109 mMol/L). Platelet poor plasma (PPP) was prepared within 1 h of blood collection by double centrifugation (2×20 min at $2000 \times g$) at room temperature and stored in aliquots at -80 °C, and expended to the core laboratory at the Research Group "Cancer-Haemostasis-Angiogenesis" (INSERM UMRS 938, Sorbonne University, Paris, France) in a partnership with the Department of Clinical Research of Diagnostica Stago (Genevilliers, France). All preanalytical procedures were in conformity with the recommendations proposed by Laxroix et al. [13].

Biomarkers of hypercoagulability and endothelial cell activation: The procoagulant phospholipid-dependent clotting time (PPL-ct) was measured using a factor Xa-based clotting assay STA®-Procoag-PPL. The assay is performed using phospholipid depleted substrate plasma to eliminate the influence of any coagulation factors upstream [14,15]. Free-TFPI levels were measured with the ELISA Free TFPI, Asserachrom®; D-dimer and fibrin monomers (FM) were measured by STA Liatest D-Di and STA Liatest FM; soluble thrombomodulin (sTM) was measured by Asserachrom® TM. All assays were from Diagnostica Stago, Asnières, France. Heparanase-levels were measured with ELISA kits R&D Systems (Lille France). All samples were measured in duplicate. Samples were analyzed only after a single freeze-thaw cycle and thawing for 5 min in a water bath at 37 °C immediately before assay. All assays were performed according to the manufacturer's instructions. The inter- and intra-assay coefficients of variation of the assays ranged from 3–7%, and the detection limit of the assays was determined to be at zero.

Detection of anti-SARS-CoV2 antibodies: IgG and IgA anti-SARS-CoV-2 antibodies were detected in the sera of the survivors using a semiquantitative commercial ELISA methodology (Euroimmun Medizinische Labordiagnostika AG, Lubeck, Germany), according to the manufacturer. The method detects antibodies against the recombinant spike protein of the virus (S1 domain) and is fully described previously. The measurements were performed at the Hematology Laboratory Blood Bank, Aretaieion Hospital (Athens, Greece).

Ethics: All study procedures were carried out in accordance with the declaration of Helsinki (18th World Medical Association Assembly), its subsequent amendments, Greek regulations and guidelines, and the Good Clinical practice Guidelines (GCP), as defined by the International Conference of Harmonization. The study was approved by the local ethics committees of all participating hospitals. All participants in the study (survivors and controls) provided written informed consent.

3. Statistical Analysis

Levels of biomarkers measured in the cohort of survivors were compared against those measured in the control group. The normal values of the studied biomarkers were defined in the control group and were compared to the corresponding normal reference range used by the core laboratory. These normal ranges were established according to the requirements for the good quality of laboratory practice by performing the tests on healthy individuals representative of the general population regarding age, sex, ethnicity, and BMI. The Upper Normal Limit (UNL) and the Lower Normal Limit (LNL) for each studied biomarker were defined in the control group as follows: UNL = mean + 2 SD and LNL: = mean-2 SD. The UNL and LNL of the studied biomarkers were compared to the corresponding normal reference range used by the core laboratory.

Continuous variables were described as median (interquartile range, IQR) and categorical variables as frequencies (percentage). Alterations in hypercoagulability variables were treated as dependent variables in univariate logistic regression models, aiming to evaluate

independent associations with gender (male vs. female), age ($\geq$50 versus <50 years, with median age of the study cohort used as the cutoff), time from diagnosis ($\geq$62 versus <62 days, median time used as the cutoff), anti-SARS-CoV-2 IgG ($\geq$5.68 versus <5.68, median value used as the cutoff) and blood group (A/B/AB versus O, available in a subcohort of 112 patients). In case of two or more significant associations, multivariate logistic regression analysis followed. Odds ratios (OR) and 95% confidence intervals (95% CI) were estimated.

In this instance, relative risk ratios (RRRs) and 95% CIs were estimated, as appropriate. Level of significance was set at 0.05. Statistical analysis was performed using STATA/SE version 13 statistical software (Stata Corp., College Station, TX, USA).

4. Results

In total, 208 COVID-19 survivors were included in the study; 125 males (60%) and 83 females (40%) with a median age of 50 years (IQR = 20, range 18 to 78 years). The median time between symptoms onset and rtPCR diagnosis was 3 days. The median interval between symptom onset and screening for IgG SARS-CoV-2 antibodies was 62 days (IQR = 22 days). Antibodies against SARS-CoV-2 were detected in all survivors enrolled in the study. The median level of IgG and IgA antibodies was 5.68 U (IQR = 6.19) and 3.62 U (IQR = 5.36), respectively. In 112 survivors with known blood group, 48 (43%) were group O, 44 (39%) were group A, 14 (13%) were group B, and 6 (5.4%) were group AB. Since survivors were plasma donors for the phase II clinical study, which assessed the efficacy and safety of convalescence plasma treatment of COVID-19, they did not suffer any chronic disease nor did they receive any long-term treatment (as defined at the inclusion criteria at the Materials and methods section). According to the inclusion criteria of the study, all survivors had normal blood coagulation tests and fibrinogen levels. Demographics and clinical data are summarized in Table 1.

Table 1. Demographics and clinical data of COVID-19 survivors and controls.

	COVID-19 Survivors (*n* = 208)	Control (*n* = 30)
Age (range)	50 years (18–78 years)	48 years (18–75)
Sex Males Females	 60% (125/208) 40% (83/208)	 67% (20/30) 33% (10/30)
Comorbidities	no	no
Interval between symptom onset and PCR diagnosis of COVID-19 (median/range)	3 (0–29)	-
Interval between symptom onset and screening for IgG SARS-CoV-2 antibodies (median/range)	62 days (14–178)	-
Blood group		
O	43% (48/112)	10 (33%)
A	39% (44/112)	12(40%)
B	13% (14/112)	5 (17%)
AB	5.4% (6/112)	3 (3%)

5. Biomarkers of Hypercoagulability in COVID-19 Survivors

Survivors showed significantly higher levels of D-dimer, FM, TFPI, and heparanase as compared to that of the control group. Survivors had significantly shorter PPL-ct.

As compared to the UNL for each biomarker, elevated D-dimer and FM levels were noted in 25.1% (52/208) and 9.1% (19/208) of survivors, respectively. Elevated heparanase

was evident in 27.4% (57/208) of the survivors, elevated TM in 4.8% (10/208), and elevated TFPI in 23.1% (48/208).

Procoag-PPL clotting time was shorter than the LNL in 8.2% (17/208) of the survivors. The levels of biomarkers of hypercoagulability in survivors and controls are reported in Table 2.

Table 2. Profile of hypercoagulability in the COVID-19 survivors and healthy controls. Values are mean $\pm$ standard deviation. ** $p < 0.001$, * $p < 0.01$.

	Normal Reference Range	Control Group (n = 30)	COVID-19 Survivors (n = 208)	% Frequency of Alterations
Cellular derived hypercoagulability				
Procoag-PPL (sec)	42–85	60.9 $\pm$ 2.63	78.93 $\pm$ 32.72 **	Decreased: 8.2% (17/207)
Endothelial cell activation				
Heparanase (ng/mL)	0.08–0.16	0.13 $\pm$ 0.03	0.32 $\pm$ 0.84 **	Elevated: 27.7% (57/206)
sTM (ng/mL)	70–120	90.1 $\pm$ 18.1	80.76 $\pm$ 64.86 *	Elevated: 4.8% (10/208)
TFPI (ng/mL)	8–12	11.2 $\pm$ 2.0	21.33 $\pm$ 9.08 **	Elevated: 23.1% (48/208)
In vivo fibrin formation/lysis				
D-dimer (µg/mL)	<0.50	0.31 $\pm$ 0.08	0.51 $\pm$ 0.62 **	Elevated: 25.1% (50/199)
FM (µg/mL)	0.5–5.5	2.5 $\pm$ 0.5	6.12 $\pm$ 10.37 **	Elevated: 9.1% (19/208)

Procoag-PPL: procoagulant phospholipid dependent clotting time; sTM: soluble thrombomodulin; TFPI: tissue factor pathway inhibitor; FM: fibrin monomer.

6. Alterations in Biomarkers of Hypercoagulability COVID-19: Associations with Sex and Age

Increase of D-dimer was associated with older age; D-dimer elevation was noted in 34.7% of patients aged 50 years or more vs. 15.8% of those aged less than 50 years, (OR = 2.82, 95% CI: 1.43–5.55). Elevated FM was associated with female gender, as FM elevation was documented in 14.5% of females vs. 5.6% of males (univariate OR = 0.35, 95% CI: 0.13–0.93).

Elevated heparanase was independently associated with male gender (OR = 3.57, 95% CI: 1.70–7.48), but inversely correlated with older age (adjusted OR = 0.35, 95% CI: 0.18–0.70). No significant associations were noted between elevated TM and age or sex, whereas increase of TFPI was correlated with older age. TFPI elevation was noted in 32.7% of subjects aged 50 or more versus 13.5% of subjects aged less than 50 years, (OR = 3.12, 95% CI: 1.56–6.27).

Decreased Procoag-PPL clotting time was associated with female gender. Decrease in Procoag-PPL was observed in 14.6% of females versus 4.0% of males, (OR = 0.24, 95% CI: 0.08–0.72 for the male versus female).

The interval between COVID-19 diagnosis and assessment was not associated with the changes of the studied biomarkers.

Detailed data are depicted in Table 3.

Table 3. Results of univariate and multivariate logistic regression analysis examining predictors of hypercoagulability in study cohort of COVID-19 survivors. Bold cells denote statistically significant associations (*p* < 0.05).

	Compared Categories	Frequencies of Alterations in the Compared Categories	Univariate OR (95% CI)	Multivariate OR (95% CI)
Cellular Derived Hypercoagulability				
Decreased Procoag-PPL Clotting Time				
Male gender	Male vs. female	4.0% vs. 14.6%	**0.24 (0.08–0.72)**	Not entered
Age (years)	≥50 vs. <50	8.7% vs. 7.7%	1.15 (0.43–3.10)	Not entered
Time from diagnosis (days)	≥62 vs. <62	11.7% vs. 4.3%	2.97 (0.92–9.55)	Not entered
Anti-SARS-CoV-2 IgG	≥5.68 vs. <5.68	9.7% vs. 6.8%	1.47 (0.54–4.04)	Not entered
Blood group [§]	Blood group A/B/AB vs. O	6.4% vs. 10.4%	0.58 (0.15–2.30)	Not entered
Elevated Heparanase				
Male gender	Male vs. female	35.0% vs. 16.9%	**2.65 (1.34–5.25)**	**3.57 (1.70–7.48)**
Age (years)	≥50 vs. <50	20.4% vs. 35.0%	**0.48 (0.25–0.89)**	**0.35 (0.18–0.70)**
Time from diagnosis (days)	≥62 vs. <62	22.3% vs. 35.5%	**0.52 (0.28–0.98)**	0.57 (0.29–1.11)
Anti-SARS-CoV-2 IgG	≥5.68 vs. <5.68	29.4% vs. 26.2%	1.17 (0.64–2.16)	Not entered
Blood group [§]	Blood group A/B/AB vs. O	30.2% vs. 38.3%	0.70 (0.31–1.54)	Not entered
Elevated TM				
Male gender	Male vs. female	5.6% vs. 3.6%	1.58 (0.40–6.30)	Not entered
Age (years)	≥50 vs. <50	7.7% vs. 1.9%	4.25 (0.88–20.52)	Not entered
Time from diagnosis (days)	≥62 vs. <62	2.9% vs. 7.5%	0.37 (0.09–1.47)	Not entered
Anti-SARS-CoV-2 IgG	≥5.68 vs. <5.68	5.8% vs. 3.9%	1.55 (0.42–5.65)	Not entered
Blood group [§]	Blood group A/B/AB vs. O	3.1% vs. 4.2%	0.74 (0.10–5.46)	Not entered
Elevated TFPI				
Male gender	Male vs. female	26.4% vs. 18.1%	1.63 (0.82–3.23)	Not entered
Age (years)	≥50 vs. <50	32.7% vs. 13.5%	**3.12 (1.56–6.27)**	Not entered
Time from diagnosis (days)	≥62 vs. <62	25.0% vs. 21.3%	1.23 (0.63–2.40)	Not entered
Anti-SARS-CoV-2 IgG	≥5.68 vs. <5.68	27.2% vs. 19.2%	1.57 (0.82–3.01)	Not entered
Blood group [§]	Blood group A/B/AB vs. O	18.8% vs. 18.8%	1.00 (0.38–2.61)	Not entered
In Vivo Fibrin Formation/Lysis				
Elevated D-dimer				
Male gender	Male vs. female	21.9% vs. 30.0%	0.65 (0.34–1.24)	Not entered
Age (years)	≥50 vs. <50	34.7% vs. 15.8%	**2.82 (1.43–5.55)**	Not entered
Time from diagnosis (days)	≥62 vs. <62	19.4% vs. 30.8%	0.54 (0.28–1.06)	Not entered
Anti-SARS-CoV-2 IgG	≥5.68 vs. <5.68	26.3% vs. 23.2%	1.18 (0.62–2.25)	Not entered
Blood group [§]	Blood group A/B/AB vs. O	25.4% vs. 22.2%	1.19 (0.48–2.98)	Not entered
Elevated FM				
Male gender	Male vs. female	5.6% vs. 14.5%	**0.35 (0.13–0.93)**	Not entered
Age (years)	≥50 vs. <50	10.6% vs. 7.7%	1.42 (0.55–3.69)	Not entered
Time from diagnosis (days)	≥62 vs. <62	10.6% vs. 7.5%	1.47 (0.55–3.96)	Not entered
Anti-SARS-CoV-2 IgG	≥5.68 vs. <5.68	8.7% vs. 9.6%	0.90 (0.35–2.32)	Not entered
Blood group [§]	Blood group A/B/AB vs. O	7.8% vs. 12.5%	0.59 (0.17–2.07)	Not entered

[§] available in a subcohort of 112 patients.

7. Discussion

SARS-CoV-2 has a direct effect on endothelial cells and amplifies their activation [16]. Endothelial cells are also targets of the inflammatory and immunological reaction triggered after SARS-CoV-2 infection. Activated endothelial cells play key role in orchestration of the inflammatory and hypercoagulable state in COVID-19 patients [17–21]. It is well established that in patients with COVID-19, endothelial cell activation and hypercoagulability are independently associated with the risk of disease worsening, admission in ICU and intubation, as well as with high mortality rate [22]. Endothelial cell activation is also implicated in the manifestation of long-COVID-19. The European Society of Cardiology suggests that the evaluation of endothelial function, in addition to myocardial injury and respiratory function markers in COVID-19 survivors, may represent possible means for early detection of vascular sequelae post-COVID-19 [23]. Endothelial activation and hypercoagulability might persist in COVID-19 survivors and could have some clinical implications in long-COVID-19.

The prospective observational ROADMAP-postCOVID-19 study was designed to identify biomarkers of hypercoagulability and endothelial cell activation mandatory of prolonged perturbation of the hemostatic equilibrium at the vascular level. Biomarkers of endothelial cell activation (TFPI, heparanase, soluble thrombomodulin) and in vivo hypercoagulability (D-dimer, FM) were measured. In addition, the PPL-ct, which is mandatory for the presence of procoagulant phospholipids in plasma, was also measured. Persistence of endothelial cell activation and hypercoagulability up to 62 days from symptom onset is a common alteration in COVID-19 survivors. Indeed, a pathological increase of at least one biomarker of endothelial cell activation was observed in almost 3 out of 10 survivors. Interestingly, among the studied biomarkers of endothelial cell activation, TFPI and heparanase most frequently increased. Almost 1 out of 4 survivors showed high levels of TFPI, whereas 25% of them showed a combined increase of TFPI and heparanase levels. Moreover, 5% of survivors had pathologically increased thrombomodulin levels. The TFPI, thrombomodulin, and heparanase are synthetized by endothelial cells and released upon their activation. Thus, although they have substantially different roles in coagulation mechanism, their increase in blood reflects systematic endothelial cell activation [24]. Release of TFPI and thrombomodulin by activated endothelial cells deprives vascular microenvironment of important inhibitors of TF and thrombin generation [25]. Heparanase, an endo-β-D-glucuronidase, stems from activated endothelial cells. It is the only enzyme in mammalians that cleaves heparan sulfate chains and is involved in a wide variety of pathological processes and diseases, including inflammatory and infectious processes [26]. Degradation of heparan sulfate by heparanase results in extracellular matrix remodeling and release of numerous sequestered components involved in several pathophysiological processes, including blood coagulation activation. In addition, heparanase directly interacts with TF, leading to enhancement of factor Xa formation and acceleration of thrombin generation [27]. Furthermore, the ROADMAP-postCOVID-19 study showed that 8% of the survivors had PPL-ct shorter than the LNL, and it documented that cell-derived hypercoagulability persists for at least two months after COVID-19, which is not a rare phenomenon.

Lastly, the ensemble of persistent thrombin generation, fibrin formation, and fibrinolysis is the third alteration of vascular/coagulation equilibrium identified by the ROADMAP-postCOVID-19 study. As much as 25% of survivors showed increased levels of D-dimer, and 9% had high levels of fibrin monomers. The frequency of increased D-dimers in COVID-19 survivors observed in our study was the same as that reported by Townsend et al. in COVID-19 survivors who were assessed after an interval of 80 days from COVID-19 diagnosis [28].

The COVID-19 survivors enrolled in the present study represent a highly selected population. They did not present any known chronic disease or cardiovascular risk factors that are related with chronic endothelial cell activation or the risk of COVID-19 worsening. Moreover, they had a mild disease trajectory (data not shown). Thus, the studied cohort

represents a fraction of patients with COVID-19 who do not present any evident cause of endothelial cell activation. In addition, the survivors had normal coagulation tests.

Nevertheless, the observed sustained hypercoagulability was of the same order as that observed in studies performed on unselected survivors of COVID-19 [29–31]. The survivors had normal blood coagulation tests, including fibrinogen levels. Thus, the increase of D-dimer in 25% of survivors cannot be attributed to the presence of hyperfibrinogenemia. The increase of FM in 9% of survivors reveals a persistence of in vivo thrombin generation. Enhanced fibrinolysis related to the presence of fibrin generated during COVID-19 and after disease recovery in some patients could be the origin of the persistence increase of D-dimer. Consequently, the increase of these biomarkers should be interpreted as an indication of long-lasting endothelial cell activation, with important reinforcement of procoagulant forces at the vascular microenvironment. The data reported by a study presented by Fogarty et al. in a small number of COVID-19 survivors further support our findings [29]. Prolonged overactive state of the immune system could be implicated with endothelial dysfunction in COVID-19 survivors [30]. von Maijenfeldt et al. showed that convalescent COVID-19 patients have sustained prothrombotic changes, as evidenced by enhanced thrombin-generating capacity and decreased plasma fibrinolytic potential at 4 months after hospital discharge. Cytokine profiling showed that cytokine production remained heightened postinfection [31]. On the other hand, persistent infection of endothelial cells by the SARS-CoV-2 could be a complementary pathway leading to sustained endothelial cell activation and hypercoagulability in COVID-19 survivors. The ROADMAP-postCOVID-19 study did not involve a control group of survivors of other viral infections (non-SARS-CoV-2) that provoke pneumonia or with other species of coronavirus. This is a common limitation of the studies that investigate immunological, vascular, or hematological alterations in patients with COVID-19, and it is imposed by emergency conditions of the SARS-CoV-2 outbreak. Consequently, we cannot evaluate the degree of endothelial cell activation induced by SARS-CoV-2 infection as compared to that of other viruses. Similarly, it is not possible to evaluate whether the endothelial cell activation is influenced by the type of the SARS-CoV-2 variant. Moreover, the present study was designed to assess the biomakrers of hypercoagulability and endothelial cell activation at a single point after recovery from COVID-19. Thus, it cannot provide any information on the kinetics of the hypercoagulable state upon infection by SRS-CoV-2 and manifestation of COVID-19. Furthermore, the absence of a baseline evaluation-prior or at the early phase of SARS-CoV-2 infection does not allow to evaluate the potential impact of pre-existing hypercoagulability or endothelial cell activation on the persistence and the intensity of the observed alterations of coagulation and endothelial cells in the survivors. It is well established that both male gender and increased age are independent risk factors for worsening of COVID-19 (reviewed in [1]). In line with this, the ROADMAP-postCOVID-19 study shows that male gender and age are also determinants of persistent activation of endothelial cells. The levels of heparanase were higher in males, and levels of TFPI were higher in survivors older than 50 years. Survivors older than 50 years showed increased levels of D-dimers but this was not the case for FM, indicating that additional mechanisms beyond age may lead to hypercoagulability. Once again, males were more prone to presenting increased FM. In contrast, blood group was not associated with the manifestation of persistent endothelial cell activation and hypercoagulability.

Long-COVID-19 emerges as a new aspect of the public health crisis triggered by the pandemic [32]. Delayed arterial thrombosis or venous thromboembolism post-COVID-19 is an emerging health problem [33–35]. The data presented herein allow to propose that evaluation of blood hypercoagulability (using D-dimer for instance) and endothelial cell activation could offer the possibility of prompt identification of COVID-19 survivors at risk of post-COVID-19 vascular complications. The findings of the present study underline the need for a thorough evaluation of a potential correlation between the sustained hyperco-agulability and endothelial cell activation with the symptomatology of long-COVID-19 to apply anticipated diagnostic and therapeutic strategies. To optimize the strategy for prompt

identification of the patients at risk of vascular complications related to long-COVID-19, a longitudinal evaluation of the clinically relevant biomarkers of hypercoagulability and endothelial cell activation identified in the present study could be performed. This strategy might be useful for a personalized risk assessment strategy and targeted treatment, and it must be evaluated in a prospective clinical study. The ROADMAP-postCOVID-19 study was designed, and survivors were enrolled during the early phase of the pandemic, which was before the recognition of the long COVID-19 syndrome. Consequently, clinical data relevant of the long COVID-19 are not yet available, and the clinical relevance of our findings in early recognition of patients at risk of long-COVID-19 cannot be evaluated in the present study. In addition, the study enrolled a highly selected population of COVID-19 survivors (i.e., absence of underlying pathological conditions, normal clotting times, and fibrinogen levels) and thus, excluded a subset of survivors with underlying conditions, which are associated with hypercoagulable state or hyperfibrinogenemia. Consequently, this represents a selection bias resulting in underestimation of the frequency of persistent hypercoagulability and endothelial cell activation in COVID-19 survivors.

In conclusion, the ROADMAP-postCOVID-19 study offers an analysis of a large panel of biomarkers of endothelial cell activation and hypercoagulability in survivors of COVID-19. The study documents that two months after symptoms' onset, activation of endothelial cells, in vivo thrombin generation, and fibrin lysis are frequent phenomena up to two months from COVID-19 symptom onset. The clinical relevance of the identified biomarkers of endothelial activation and hypercoagulability in the assessment of the risk of long COVID-19 must be investigated in a prospective study.

References

1. Gerotziafas, G.T.; Catalano, M.; Colgan, M.P.; Pecsvarady, Z.; Wautrecht, J.C.; Fazeli, B.; Olinic, D.M.; Farkas, K.; Elalamy, I.; Falanga, A.; et al. Guidance for the management of patients with vascular disease or cardiovascular risk factors and COVID-19: Position paper from VAS-European independent foundation in Angiology/Vascular medicine. *Thromb. Haemost.* **2020**, *120*, 1597–1628. [PubMed]
2. Norooznezhad, A.H.; Mansouri, K. Endothelial cell dysfunction, coagulation, and angiogenesis in coronavirus disease 2019 (COVID-19). *Microvasc. Res.* **2021**, *137*, 104188. [CrossRef] [PubMed]

3. Słomka, A.; Kowalewski, M.; Żekanowska, E. Hemostasis in coronavirus disease 2019—Lesson from viscoelastic methods: A systematic review. *Thromb. Haemost.* **2021**, *121*, 1181–1192. [CrossRef] [PubMed]

4. Short, S.A.P.; Gupta, S.; Brenner, S.K.; Hayek, S.S.; Srivastava, A.; Shaefi, S.; Singh, H.; Wu, B.; Bagchi, A.; Al-Samkari, H.; et al. d-dimer and death in critically Ill patients with coronavirus disease 2019. *Crit. Care Med.* **2021**, *49*, e500–e511. [CrossRef]

5. Gris, J.C.; Quéré, I.; Pérez-Martin, A.; Lefrant, J.Y.; Sotto, A. Uncertainties on the prognostic value of D-dimers in COVID-19 patients. *J. Thromb. Haemost.* **2020**, *18*, 2066–2067. [CrossRef]

6. Sheng, W.-H.; Liu, W.-D.; Wang, J.-T.; Chang, S.-Y.; Chang, S.-C. Dysosmia and dysgeusia in patients with COVID-19 in northern Taiwan. *J. Formos. Med. Assoc.* **2021**, *120*, 311–317. [CrossRef]

7. Belli, S.; Balbi, B.; Prince, I.; Cattaneo, D.; Masocco, F.; Zaccaria, S.; Bertalli, L.; Cattini, F.; Lomazzo, A.; Negro, F.D.; et al. Low physical functioning and impaired performance of activities of daily life in COVID-19 patients who survived hospitalisation. *Eur. Respir. J.* **2020**, *56*, 2002096. [CrossRef]

8. Zhao, Y.-M.; Shang, Y.-M.; Song, W.-B.; Li, Q.-Q.; Xie, H.; Xu, Q.-F.; Jia, J.-L.; Li, L.-M.; Mao, H.-L.; Zhou, X.-M.; et al. Follow-up study of the pulmonary function and related physiological characteristics of COVID-19 survivors three months after recovery. *EClinicalMedicine* **2020**, *25*, 100463. [CrossRef]

9. Sudre, C.H.; Murray, B.; Varsavsky, T.; Graham, M.S.; Penfold, R.S.; Bowyer, R.C.; Pujol, J.C.; Klaser, K.; Antonelli, M.; Canas, L.S.; et al. Attributes and predictorsof long COVID. *Nat. Med.* **2021**, *27*, 626–631. [CrossRef]

10. Parums, D.V. Editorial: Long COVID, or Post-COVID Syndrome, and the Global Impact on Health Care. *Med. Sci. Monit.* **2021**, *27*, e933446-1. [CrossRef]

11. Terpos, E.; Politou, M.; Sergentanis, T.N.; Mentis, A.; Rosati, M.; Stellas, D.; Bear, J.; Hu, X.; Felber, B.K.; Pappa, V.; et al. Anti-SARS-CoV-2 antibody responses in convalescent plasma donors are increased in hospitalized patients; Subanalyses of a phase 2 clinical study. *Microorganisms* **2020**, *8*, 1885. [CrossRef]

12. Kostopoulos, I.V.; Orologas-Stavrou, N.; Rousakis, P.; Panteli, C.; Ntanasis-Stathopoulos, I.; Charitaki, I.; Korompoki, E.; Gavriatopoulou, M.; Kastritis, E.; Trougakos, I.P.; et al. Recovery of innate immune cells and persisting alterations in adaptive immunity in the peripheral blood of convalescent plasma donors at eight months post SARS-CoV-2 infection. *Microorganisms* **2021**, *9*, 546. [CrossRef]

13. Lacroix, R.; Judicone, C.; Poncelet, P.; Robert, S.; Arnaud, L.; Sampol, J.; Dignat-George, F. Impact of pre-analytical parameters on the measurement of circulating microparticles: Towards standardization of protocol. *J. Thromb. Haemost.* **2012**, *10*, 437–446. [CrossRef]

14. van Dreden, P.; Rousseau, A.; Fontaine, S.; Woodhams, B.J.; Exner, T. Clinical evaluation of a new functional test for detection of plasma procoagulant phospholipids. *Blood Coagul. Fibrinolysis* **2009**, *20*, 494–502. [CrossRef]

15. Schneider, P.; Van Dreden, P.; Rousseau, A.; Kassim, Y.; Legrand, E.; Vannier, J.-P.; Vasse, M. Increased levels of tissue factor activity and procoagulant phospholipids during treatment of children with acute lymphoblastic leukaemia. *Br. J. Haematol.* **2010**, *148*, 582–592. [CrossRef]

16. Varga, Z.; Flammer, A.J.; Steiger, P.; Haberecker, M.; Andermatt, R.; Zinkernagel, A.S.; Mehra, M.R.; Schuepbach, R.A.; Ruschitzka, F.; Moch, H. Endothelial cell infection and endotheliitis in COVID-19. *Lancet* **2020**, *395*, 1417–1418. [CrossRef]

17. Castro, P.; Palomo, M.; Moreno-Castaño, A.B.; Fernández, S.; Torramadé-Moix, S.; Pascual, G.; Martinez-Sanchez, J.; Richardson, E.; Téllez, A.; Nicolas, J.M.; et al. Is the endothelium the missing link in the pathophysiology and treatment of COVID-19 complications? *Cardiovasc. Drugs Ther.* **2021**. [CrossRef]

18. Levi, M. Pathophysiology of coagulopathy in hematological malignancies and in COVID-19. *HemaSphere* **2021**, *5*, e571. [CrossRef]

19. Syed, F.; Li, W.; Relich, R.F.; Russell, P.M.; Zhang, S.; Zimmerman, M.K.; Yu, Q. Excessive matrix metalloproteinase-1 and hyperactivation of endothelial cells occurred in COVID-19 patients and were associated with the severity of COVID-19. *J. Infect. Dis.* **2021**, *224*, 60–69. [CrossRef]

20. Chauhan, A.J.; Wiffen, L.J.; Brown, T.P. COVID-19: A collision of complement, coagulation and inflammatory pathways. *J. Thromb. Haemost.* **2020**, *18*, 2110–2117. [CrossRef]

21. Terpos, E.; Ntanasis-Stathopoulos, I.; Elalamy, I.; Kastritis, E.; Sergentanis, T.N.; Politou, M.; Psaltopoulou, T.; Gerotziafas, G.; Dimopoulos, M.A. Hematological findings and complications of COVID-19. *Am. J. Hematol.* **2020**, *95*, 834–847. [CrossRef] [PubMed]

22. de Bruin, S.; Bos, L.D.; van Roon, M.A.; Tuip-de Boer, A.M.; Schuurman, A.R.; Koel-Simmelinck, M.J.A.; Bogaard, H.J.; Tuinman, P.R.; van Agtmael, M.A.; Hamann, J.; et al. Amsterdam UMC COVID-19 Biobank investigators. Clinical features and prognostic factors in Covid-19: A prospective cohort study. *EBioMedicine* **2021**, *67*, 103378. [CrossRef] [PubMed]

23. Evans, P.C.; Rainger, G.E.; Mason, J.C.; Guzik, T.J.; Osto, E.; Stamataki, Z.; Neil, D.; Hoefer, I.E.; Fragiadaki, M.; Waltenberger, J.; et al. Endothelial dysfunction in COVID-19: A position paper of the ESC working group for atherosclerosis and vascular biology, and the ESC Council of Basic Cardiovascular Science. *Cardiovasc. Res.* **2020**, *116*, 2177–2184. [CrossRef] [PubMed]

24. Maruyama, I. Biology of endothelium. *Lupus* **1998**, *7* (Suppl. 2), 41–43. [CrossRef] [PubMed]

25. Yu, Y.; Böing, A.N.; Hau, C.M.; Hajji, N.; Ruf, W.; Sturk, A.; Nieuwland, R. Tissue factor coagulant activity is regulated by the plasma membrane microenvironment. *Thromb. Haemost.* **2018**, *118*, 990–1000. [CrossRef]

26. Jin, H.; Cui, M. New advances of heparanase and heparanase-2 in human diseases. *Arch. Med Res.* **2018**, *49*, 423–429. [CrossRef]

27. Nadir, Y.; Brenner, B. Heparanase procoagulant activity in cancer progression. *Thromb. Res.* **2016**, *140* (Suppl. 1), S44–S48. [CrossRef]

28. Townsend, L.; Fogarty, H.; Dyer, A.; Martin-Loeches, I.; Bannan, C.; Nadarajan, P.; Bergin, C.; Farrelly, C.O.; Conlon, N.; Bourke, N.M.; et al. Prolonged elevation of D-dimer levels in convalescent COVID-19 patients is independent of the acute phase response. *J. Thromb. Haemost.* **2021**, *19*, 1064–1070. [CrossRef]

29. Fogarty, H.; Townsend, L.; Morrin, H.; Ahmad, A.; Comerford, C.; Karampini, E.; Englert, H.; Byrne, M.; Bergin, C.; O'Sullivan, J.M.; et al. Persistent endotheliopathy in the pathogenesis of long COVID syndrome. *J. Thromb. Haemost.* **2021**, *19*, 2546–2553. [CrossRef]

30. Lim, J.; Puan, K.J.; Wang, L.W.; Teng, K.W.W.; Loh, C.Y.; Tan, K.P.; Carissimo, G.; Chan, Y.-H.; Poh, C.M.; Lee, C.Y.-P.; et al. Data-driven analysis of COVID-19 reveals persistent immune abnormalities in convalescent severe individuals. *Front. Immunol.* **2021**, *12*, 34867943. [CrossRef]

31. von Meijenfeldt, F.A.; Havervall, S.; Adelmeijer, J.; Lundström, A.; Magnusson, M.; Mackman, N.; Thalin, C.; Lisman, T. Sustained prothrombotic changes in COVID-19 patients 4 months after hospital discharge. *Blood Adv.* **2021**, *5*, 756–759. [CrossRef] [PubMed]

32. Fernández-Lázaro, D.; Sánchez-Serrano, N.; Mielgo-Ayuso, J.; García-Hernández, J.L.; González-Bernal, J.J.; Seco-Calvo, J. Long COVID a new derivative in the chaos of SARS-CoV-2 infection: The emergent pandemic? *J. Clin. Med.* **2021**, *10*, 5799. [CrossRef] [PubMed]

33. Fan, B.E.; Cheung, C. Post COVID-19 arterial thromboembolism: A clear and present danger. *Semin. Thromb. Hemost.* **2021**. [CrossRef] [PubMed]

34. Korompoki, E.; Gavriatopoulou, M.; Fotiou, D.; Ntanasis-Stathopoulos, I.; Dimopoulos, M.A.; Terpos, E. Late-onset hematological complications post COVID-19: An emerging medical problem for the hematologist. *Am. J. Hematol.* **2021**, *97*, 119–128. [CrossRef]

35. Engelen, M.M.; Vandenbriele, C.; Balthazar, T.; Claeys, E.; Gunst, J.; Guler, I.; Jacquemin, M.; Janssens, S.; Lorent, N.; Liesenborghs, L.; et al. Venous thromboembolism in patients discharged after COVID-19 hospitalization. *Semin. Thromb. Hemost.* **2021**, *47*, 362–371. [CrossRef]