Letter to the editors

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Modifications of protein Z and interleukin-6 during the acute phase of coronary artery disease

Francesca Cesari[a], Anna Maria Gori[a], Sandra Fedi[a], Rosanna Abbate[a], Gian Franco Gensini[a,b] and Francesco Sofi[a]

[a]Department of Medical and Surgical Critical Care, Thrombosis Centre, University of Florence, Italy; Center for the Study at Molecular and Clinical Level of Chronic, Degenerative and Neoplastic Diseases to Develop Novel Therapies, University of Florence and [b]Centro S. Maria agli Ulivi, Fondazione Don Carlo Gnocchi Onlus IRCCS, Impruneta, Florence, Italy

Correspondence and requests for reprints to Francesca Cesari, BS, Department of Medical and Surgical Critical Care, Thrombosis Centre, University of Florence, Azienda Ospedaliero-Universitaria Careggi, Viale Morgagni 85, 50134 Florence, Italy
Tel: +39 55 7949420; fax: +39 55 7949418;
e-mail: francesca.cesari@gmail.com

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Protein Z is a vitamin K-dependent plasma protein that acts as a cofactor for the inactivation of activated factor X by forming a complex with a specific plasma protein Z-dependent protease inhibitor [1]. Over the past years, protein Z has been investigated in different prothrombotic conditions, ranging from ischaemic cerebrovascular disease through pregnancy complications to coronary artery disease, and a role for altered levels of this protein has been reported [2–4]. However, some uncertainties regarding the real role of this protein on the occurrence of such diseases still remain. In particular, the relationship between this protein and the inflammatory pathway has been scarcely investigated. Only two previous studies have analysed the interaction between protein Z and proinflammatory cytokines: in 1999 Undar *et al.* showed an inverse correlation between protein Z and interleukin-6 in patients with haematological malignancies [5], while in 2002 Vasse *et al.*, in an 'in-vitro' study, demonstrated that protein Z biosynthesis by hepatic cells was only weakly affected by some inflammatory cytokines [6]. Actually, the very broad range of protein Z levels shown within the normal population suggests that protein Z may act as an acute phase protein, possibly being regulated by inflammatory cytokines. However, no clear data regarding the interplay between inflammatory reactants and protein Z in patients with atherosclerotic diseases are present.

The aim of this study was to evaluate the time-course of protein Z and interleukin-6 during and after an acute coronary event, in order to give an insight into the relationship between protein Z and the acute-phase state. We therefore enrolled and prospectively followed 10 patients (eight men, two women; median age, 64 years) with acute coronary syndrome who underwent primary percutaneous coronary intervention (PCI) at the Catheterization Laboratory of the Department of Heart and Vessels of the University of Florence. All the patients were followed up over several time-points [baseline (i.e. before PCI), 36 h, 72 h, 1 month and 3 months after PCI], remaining free from any adverse events throughout the follow-up period, and receiving standard therapy that included aspirin, clopidogrel, low-molecular-weight heparin, intravenous nitrates, statins, β-blockers and angiotensin-converting enzyme inhibitors where appropriate. Study approval was granted by the institutional ethics committee and all patients gave written informed consent. None of the patients were under anticoagulant treatment or reported a positivity to factor V Leiden mutation or antiphospholipid antibodies.

Fasting blood samples were withdrawn at each visit. Protein Z plasma levels were measured as previously described [4]. The intra-assay coefficient of variation was 4.9% and the interassay coefficient of variation was 8.4%. Interleukin-6 plasma levels were measured using a commercial enzyme-linked immunosorbent assay (high-sensitivity interleukin-6 human, Biotrak ELISA system; Amersham Biosciences, Little Chalfont, UK), according to the manufacturer's instructions.

Statistical analysis was performed using the SPSS software for Windows (version 12.0; SPSS Inc., Chicago, Illinois, USA). The Friedman test for related data was used to evaluate differences among several time-points for protein Z and interleukin-6 concentrations, whereas the Wilcoxon test for paired data was used for comparisons between two different time-points. The Spearman (R) test was used for correlation between the two parameters.

Time-course modifications of protein Z and interleukin-6 are shown in Fig. 1. Overall, protein Z and interleukin-6 did not change significantly from baseline to 3 months (P value for trend = 0.3 and 0.08, respectively). Protein Z, however, showed a significant increase of concentration up to a peak at 72 h after the acute event ($P = 0.04$, 72 h versus baseline), returning to similar values with respect to baseline and 3 months after the PCI, while interleukin-6 showed a parallel pattern with an earlier but not significant increase of values ($P = 0.8$, 36 h versus baseline). In addition, correlation analyses showed a strong positive correlation between protein Z and interleukin-6 at baseline and 3 months after the acute event ($R = 0.85$, $P = 0.002$ and $R = 0.89$, $P = 0.003$).

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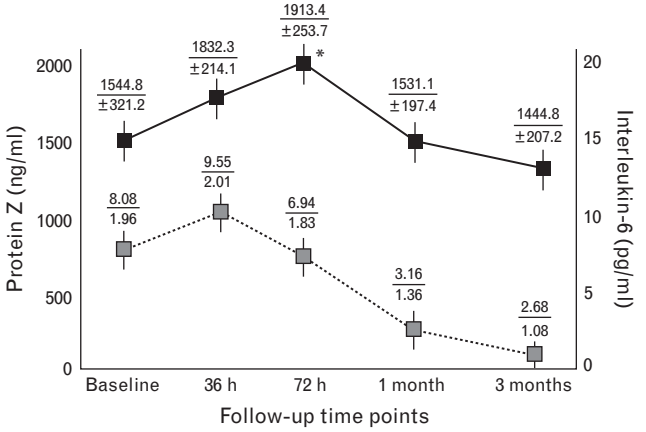
Fig. 1



Modifications of protein Z and interleukin-6 over the follow-up time points. All data reported as the mean ± SD. *P value versus baseline. Bold line, protein Z levels; dashed line, interleukin-6 levels.

This is the first study that prospectively investigated, in a limited number of patients with acute coronary syndrome, the time-course of protein Z during and after an acute event, in relation to a highly sensitive inflammatory cytokine such as interleukin-6. Protein Z showed a significant increase of values up to a peak at 72 h after the acute coronary event. At 3 months of follow-up, however, protein Z returned to similar values with respect to baseline. This pattern was similar and parallel to that reported by interleukin-6, which instead showed an earlier peak of levels (i.e. at 36 h after PCI). Furthermore, correlation analyses demonstrated a strong positive relationship between protein Z and interleukin-6 at baseline, possibly suggesting a regulatory role of this cytokine on protein Z hepatic biosynthesis. This datum seems to be in accordance with that of Vasse *et al.*, which showed a significant, albeit modest, influence of inflammatory cytokines on protein Z levels [6].

Several studies have to date demonstrated PCI to represent a procedure that induces a prothrombotic state and enhances the release of inflammatory markers, such as C-reactive protein, tumour necrosis factor-α and interleukin-6 [7–9]. It is therefore possible that protein Z levels have been somehow influenced by this kind of procedure. No 'ad-hoc' studies investigating the role of PCI on influencing protein Z levels, however, have been conducted. Besides, interleukin-6 has been recognized as one of the principal regulator of most acute phase proteins and a highly sensitive biomarker for detecting early inflammatory response after coronary stent implantation. The similar changes reported by protein Z over follow-up points with regard to interleukin-6 seem to indicate that protein Z is a weak acute-phase marker with a longer latency time. Nonetheless, the reason why the acute phase is able to determine a delayed increase of protein Z values with respect to inflammatory cytokines remains to be solved. This ability could, however, partly explain the conflicting findings reported for this protein in relation to the occurrence of thrombotic diseases.

In conclusion, we reported in patients prospectively followed up over several time points a characteristic pattern of modifications for protein Z after an acute event. In particular, a significant increase of values at 72 h after the acute event has been reported, with a further decrease up to similar values with respect to baseline after 3 months. This characteristic pattern, if confirmed in future and larger studies, seems to be of importance in clinical studies evaluating protein Z in relation to atherosclerotic diseases.

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Preanalytical variables in coagulation testing

Emmanuel J. Favaloro

Department of Haematology, Institute of Clinical Pathology and Medical Research (ICPMR), Westmead Hospital, NSW, Australia

Correspondence and requests for reprints to Dr E.J. Favaloro, Department of Haematology, Institute of Clinical Pathology and Medical Research (ICPMR), SWAHS, Westmead, NSW 2145, Australia
Tel: +61 2 9845 6618; fax: +61 2 9689 2331;
e-mail: emmanuel@icpmr.wsahs.nsw.gov.au

It was with considerable interest that I read the recent letter by Sheppard and colleagues [1]. These workers observed a prolongation of routine coagulation test times [prothrombin time and activated partial thromboplastin time (APTT)] following filtration of plasma samples intended for lupus anticoagulant testing, and thus identified this prolongation event as a novel preanalytical

variable. I particularly enjoyed reading how they solved the mystery of the prolonged test times, tracking the events to a particular batch lot of plasma filters, and to consequent reduction in coagulation factor V (FV) and factor VIII (FVIII). In the study by Sheppard and colleagues [1], the filtration of samples occurred 'in-house', and thus the identification of the pre-analytical event was achievable. Such 'detective work' is often employed by many of us involved in haemostasis, when attempting to solve a variety of inconsistencies in test results. Sheppard and colleagues [1] have resolved this particular issue in their laboratory by validating new filters prior to use.

These study findings deserve additional commentary. In part, the report from Sheppard and colleagues [1] may have under-emphasized the problem of preanalytical variables related to filtration of samples. In addition, there are a large number of other potential related preanalytical variable events that sometimes occur, which were not reported in that previous communication. In regards to the former, it should be recognized that filtration of plasma samples can lead not only to identification of falsely prolonged prothrombin time and APTT results, but also potentially to identification of false FV and FVIII deficiencies. The identification of a false FVIII deficiency (i.e. false diagnosis of haemophilia A) is particularly relevant here. Our laboratory previously identified the problem of sample filtration as a preanalytical problem some 10 years ago [2,3]. We also observed prolongation of prothrombin time and APTT results in filtered samples, as well as loss of FV and FVIII. Additionally, in filtered plasma samples, we also observed loss of factor IX and factor XII, and von Willebrand factor (VWF). We were further able to show differential loss, dependent on the point of sample filtration obtained (i.e. greater losses identified in early filtration samples compared with late filtration samples). This was presumably due to initial adhesion or entrapment of these factors by the filter, and eventual 'filter saturation' so that less protein material was removed from the late filtration sample. We also showed that loss of FVIII and VWF was particularly striking, and that a false identification of either haemophilia A or von Willebrand's disease (VWD) could ensue if the laboratory unknowingly tested filtered plasma. This is a real possibility, particularly if samples are derived from offsite sources.

The probable scenarios are as follows: (1) the routine coagulation laboratory reports a prolonged coagulation test time – either prior to filtration (e.g. normal range variation event or true prolongation) or post filtration event; (2) the clinician sees these results and requests further investigation, and may order lupus anticoagulant testing (a common reason for prolonged APTT results) and/or factor assays to exclude a coagulation defect (or haemophilia) and/or VWF studies to exclude VWD (a common bleeding disorder that can sometimes present with a prolonged APTT due to coincident FVIII deficiency; (3) the sample processing laboratory filters a plasma sample for lupus anticoagulant testing (as per current guidelines and recommendations); and (4) the specialized coagulation laboratory performs testing for lupus anticoagulant and/or factor assay and/or VWF testing. There are many possible failure points in this process. Often, the routine coagulation laboratory, the sample-processing laboratory, and the specialized coagulation laboratory comprises two or three distinct laboratories. The sample processing laboratory may not appropriately differentially label any filtered (for lupus anticoagulant) and nonfiltered (for factors and VWF) plasma samples, or may fail to provide a nonfiltered sample for coagulation factor/VWF testing. The specialized coagulation laboratory may unknowingly use a filtered plasma sample for factor assay and VWF testing, and thus report an abnormal finding. The clinician may request repeat tests for confirmation. Unless the possibility of the preanalytical event is recognized, however, the same scenarios may eventuate, and the laboratory may eventually report a similar (false) abnormal finding. The clinician may thus unwittingly 'diagnose' haemophilia or VWD in their patient.

This situation is not restricted to filtration of plasma samples. A similar finding of low FVIII and VWD-like test results will occur if the laboratory tests serum (instead of citrated plasma) [3], and low FVIII (and FV) may also be identified (as well as possible inhibitors to FV and FVIII) if ethylenediamine tetraacetic acid plasma is used instead of citrated plasma [4]. Although it may be expected that the likelihood of these events will be low (and certainly lower than the likelihood of testing filtered plasma), these events do occur in pathology practice; most often, following collection into an inappropriate collection tube by inexperienced clinicians or blood collectors, and/or provision of an inappropriate specimen to the specialized coagulation laboratory by the sample-processing laboratory [3,4]. The possibility of these events is highest when the specialized coagulation laboratory receives preprocessed samples from offsite routine coagulation or sample processing laboratories (i.e. the specialized coagulation laboratory does not see the original collection tube).

Finally, another 'generally unknown' but 'high-likelihood/significant' preanalytical problem is that of storage or transport of samples destined for coagulation factor and/or VWF testing. Current National Committee for Clinical Laboratory Standards guidelines [5] recommend that coagulation specimens be 'kept at 2 to 4°C or 18 to 24°C' until centrifuged and tested (within 4 h of collection). We and others [6–10], however, have now shown that handling these specimens according to these guidelines can yield loss of FVIII and VWF (and thus a false diagnosis of haemophilia or VWD) in a subset of

Table 1 Summary of preanalytical variables identified in the current report

Preanalytical variable (references)	Tests affected	Notes	Estimate of possible incidence	Solutions/checks
Filtration of plasma (e.g. for lupus anticoagulant testing) [1–3]	Prothrombin time, APTT, factor V, factor VIII, factor IX, factor XII, VWF (VWF:Ag, VWF:RCo, VWF:CB, multimers)	Greatest effect observed in early filtration samples (filter saturation effect?); may be some filter lot variability; VWF and factor VIII show greatest effects (VWF adhesion onto filter?); may therefore misidentify haemophilia or VWD	Around 20% of samples yielding a high APTT may feasibly yield simultaneous requests for lupus anticoagulant, and factors and/or VWF	Provide nonfiltered plasma for testing in all cases except lupus anticoagulant; use double centrifugation as an alternative to filtration for lupus anticoagulant testing; check filters for batch-based effects
Serum provided instead of citrate plasma [3,4]	Prothrombin time, APTT, most factors, VWF (primarily high-molecular-weight VWF; thus VWF:CB, VWF:RCo, multimers)	Uncommon event, but possible if inexperienced blood collector (or clinician) collecting blood, or inexperienced processor in sample-processing laboratory. More likely if sample-processing laboratory offsite or distinct from coagulation laboratory (primary tube not seen by coagulation laboratory). Possible identification of factor deficiency or VWD	Would be rare event, but nonetheless possible	Check whether prothrombin time/APTT give any clot value; 1:1 mixing tests with normal plasma will provide near-normal prothrombin time/APTT clot values; test fibrinogen levels
EDTA plasma provided instead of citrate plasma [4]	Prothrombin time, APTT, factor V, factor VIII; false factor V and factor VIII inhibitor	Uncommon event, but possible if inexperienced blood collector (or clinician) collecting blood, or inexperienced processor in sample processing laboratory. More likely if sample-processing laboratory offsite or distinct from coagulation laboratory (primary tube not seen by coagulation laboratory). May identify factor V or factor VIII deficiency or inhibitor	Would be rare event, but nonetheless possible. Provision of EDTA primary tube accompanied by 'coagulation test' requests might occur with some regularity but is usually detected by laboratory as an 'incorrect sample'. EDTA sample provision in non-EDTA primary tube has been identified on at least two separate occasions at two different institutions [4]	Check potassium level (biochemistry; high level expected); 1:1 mixing studies with normal plasma will show evidence of 'coagulation' inhibitors (false factor V and factor VIII inhibitor)
Low-temperature (~4°C) storage or transport of citrate whole blood [6–10]	APTT, factor VIII, VWF (particularly high-molecular-weight VWF; VWF:Ag, VWF:CB, VWF:RCo, multimers)	May falsely identify haemophilia A, or type 1 or type 2 VWD in normal individuals, or type 2 VWD in type 1 VWD individuals	Up to 30% of samples stored or transported in the cold will show a relative loss of factor VIII and VWF. The effect is clinically significant (i.e. abnormal results obtained) in about 20% of cases showing the effect	Do not store or transport citrate whole blood at low temperature; use ambient temperature (10–20°C)

APTT, activated partial thromboplastin time; EDTA, ethylenediamine tetraacetic acid; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:Ag, von Willebrand factor antigen; VWF:RCo, von Willebrand factor ristocetin cofactor; VWF:CB, von Willebrand factor collagen binding.

individuals when samples are stored or transported at low temperature. Samples stored at ambient temperature are resistant to such events [6–11].

There is no doubt that other significant preanalytical events may occur of which we are aware, or unaware [12,13]. I have presented a summary of the information in the current report in Table 1. In conclusion, it is important that collection staff, laboratory staff and clinical staff are all aware of the possibility of such events, and that mechanisms exist to avoid these situations. It is also important to follow established guidelines on sample processing [5], taking into account the caveats already noted. In the case of Sheppard and colleagues [1], some filter batch lots were found to be more problematic than others, and those problematic filters will be avoided. We did not study the possibility of filter variability in our previous studies [2,3]. Alternate solutions to filtration would be to double centrifuge samples, or to provide separate labelled samples for lupus anticoagulant (filtered or double centrifuged) and factor/VWF testing (standard processing). Since the object of sample processing for lupus anticoagulant testing is the provision of platelet-free material, double centrifugation is an acceptable alternative to filtration, and simply involves the initial standard centrifugation step, separating and recentrifugation of the separated plasma, and isolation and freezing of the double-centrifuged plasma from any residual cell pellet. We also reiterate that refrigerated transport of whole-blood coagulation samples should be avoided if possible.

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