Review

Giuseppe Lippi* and Emmanuel J. Favaloro

Laboratory hemostasis: milestones in Clinical Chemistry and Laboratory Medicine

Abstract

Hemostasis is a delicate, dynamic and intricate system, in which pro- and anti-coagulant forces cooperate for either maintaining blood fluidity under normal conditions, or else will prompt blood clot generation to limit the bleeding when the integrity of blood vessels is jeopardized. Excessive prevalence of anticoagulant forces leads to hemorrhage, whereas excessive activation of procoagulant forces triggers excessive coagulation and thrombosis. The hemostasis laboratory performs a variety of first, second and third line tests, and plays a pivotal role in diagnostic and monitoring of most hemostasis disturbances. Since the leading targets of Clinical Chemistry and Laboratory Medicine include promotion of progress in fundamental and applied research, along with publication of guidelines and recommendations in laboratory diagnostics, this journal is an ideal source of information on current developments in the laboratory technology of hemostasis, and this article is aimed to celebrate some of the most important and popular articles ever published by the journal in the filed of laboratory hemostasis.

Keywords: bleeding; coagulation; hemostasis; laboratory testing; thrombosis.

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Introduction on physiological and pathological hemostasis

Physiological hemostasis is a complex and multifaceted system, whereby pro- and anti-coagulant forces actively cooperate to either preserve blood fluidity under normal conditions or to trigger clot formation to prevent excessive bleeding when the integrity of the blood vessels is jeopardized. A fine balance between these opposite forces is essential (Figure 1). An excessive prevalence of anticoagulant forces following blood vessel injury would lead to various degrees of hemorrhage that can reach even life-threatening severity, whereas an excessive activity of procoagulant forces under physiological conditions (e.g., in the lack of blood vessel damage) would instead lead to extensive (excessive) coagulation and consequent thrombosis.

The definition of hemorrhagic disorders entails a heterogeneous range of inherited and acquired conditions characterized by abnormal and/or excessive bleeding. Basically, bleeding disorders are classified according to the phase of hemostasis that is principally affected, so that they can be typically divided into disorders of primary or secondary hemostasis. The former class comprehends most frequently thrombocytopenia as well as congenital or acquired disorders of platelet function, whereas the latter conditions are mostly due to inherited or acquired abnormalities of clotting factors, that can be also categorized as quantitative, qualitative or both. Von Willebrand disease (VWD) is characterized by qualitative or quantitative abnormalities of von Willebrand factor (VWF), and characteristically expresses disturbances of primary and secondary hemostasis (Figure 2) [1, 2]. In terms of frequency, the most recent survey of the World Federation of Hemophilia (WFH) reports that the total number of people with bleeding disorders identified is 242,517, whereas those carrying hemophilia A and B are 153,251, those with VWD are 62,158 and those carrying other bleeding disorders are 27,030 [3]. These figures, however, are likely to underestimate the true incidence of bleeding disorders, because of diagnostic challenges worldwide [2, 4].

Thrombosis is a highly complex and multifaceted phenomenon. Although blood clots can potentially develop in almost each and every human vessel containing platelets, coagulant factors and prothrombotic substances [5], lymphatic thrombosis is extremely rare [6] so that the leading clinical manifestations of thrombosis typically encompasses venous [7] and arterial occlusion [5] (Figure 3). Although the final event is virtually identical between
these two blood districts, i.e., the generation of a thrombus that may in part or completely obliterate the lumen of the vessel (i.e., cause vascular occlusion), the pathogenesis, the risk factors, the composition of the thrombus as well as the clinical signs and symptoms are different between veins and arteries, with very modest overlap [8]. Regardless of its origin, the consequences of both types of thrombosis, i.e., pulmonary embolism for venous thrombosis (VTE) and myocardial infarction or stroke for arterial thrombosis are the leading causes of death worldwide. The wide group of pathologies consequent to arterial thrombosis (i.e., cardiovascular disease which encompasses acute coronary syndrome, cerebral ischemia and peripheral artery occlusive disease) are in fact the first cause of death and morbidity in western countries, whereas VTE, which include both deep vein thrombosis (DVT) and pulmonary embolism (PE), is the leading cause of death and morbidity in hospitalized patients [9, 10].

Figure 1 The hemostatic balance: a delicate equilibrium between pro- and anti-thrombotic forces.

Laboratory hemostasis

Laboratory diagnostics plays an essential role in the diagnosis, follow-up and therapeutic monitoring of most – if not all – hemostasis disturbances. Laboratory hemostasis basically entails first, second and third line tests [11], which find their suitable collocation within well-established diagnostic algorithms. First line analyses, which are also conventionally called “screening” tests, include the activated partial thromboplastin time (APTT) [12–14], prothrombin time (PT)/International Normalized Ratio (INR) [15–17], fibrinogen [18, 19], D-dimer [20, 21], and platelet count [22]. Thrombin generation assays [23, 24] along with thromboelastography [25, 26] are additional useful “global” tests of hemostasis, which have not found, however, widespread application in clinical and laboratory practice. Second line assays are typically those designed to provide further insights into abnormalities of screening tests, or used to monitor more accurately some antithrombotic therapies, and thereby include clotting factors assays [27], ristocetin-induced platelet agglutination and VWF antigen tests [28], anticardiolipin (aCL) IgG and IgM, anti-β(2)glycoprotein I (anti-β(2)GPI) antibodies IgG and IgM and phospholipid-dependent coagulation assays [29, 30], platelet function tests such as Platelet Function Analyzer-100 (PFA-100) and aggregometry [31, 32], assays for heparin-induced thrombocytopenia [33, 34], additional tests for thrombophilia screening including resistance to activated protein C, antithrombin, proteins C and S, and genetic polymorphisms/mutations (e.g., prothrombin G20210A and factor V Leiden) [35, 36] along with ecarin clotting time, chromogenic anti-factor Xa and dilute Russell viper venom time (dRVVT) for monitoring novel anticoagulants [37, 38]. Both first and second line
tests might be available to most clinical laboratories, whereas third line tests – which are intended to troubleshoot the most challenging conditions and encompass analyses such as VWF collagen binding, VWF ristocetin cofactor assay, VWF-FVIII binding assay, multimer and molecular analysis for the precise classification of VWD [39, 40], coagulation factors inhibitors testing [41, 42], analyses of rare thrombophilic mutations [43], rare platelet functional disorders [44], pharmacogenetics testing [45, 46] – are occasionally used and typically available in specialized laboratories.

Quality in laboratory diagnostics is irrecusable, since spurious results obtained on unsuitable specimens may negatively bias the clinical decision-making and jeopardize patient safety. Laboratory errors may develop from any step throughout the testing process [47, 48], but are characteristically prevalent in the manually intensive activities of the preanalytical phase [49–51]. Although the classical model of the “brain-to-brain loop” applies to all fields of laboratory medicine, its significance is substantial for hemostasis testing [48]. The vast armamentarium of laboratory hemostasis share some basic requisites and quality characteristics of traditional clinical chemistry, immunochemistry and hematology diagnostics, but there are also some specific preanalytical, analytical and postanalytical requirements [52–54]. Basically, the sample matrix of clotting tests is peculiar since the clotting assays require the use of buffered sodium citrate plasma, with the anticoagulant provided preferably at a final concentration of 3.2% (i.e., 105–109 mM). Additional and specific preanalytical quality issues include order of collection and appropriate filling of primary collection tubes [55]. These two aspects are pivotal in order to prevent cross-contamination between sequential tubes and appropriate combination of blood and additive. Similar emphasis can be placed on sample processing in hemostasis [56]. Although there is large evidence to support implementation and monitoring of analytical quality specifications in clinical chemistry, less data has become available for coagulation testing and further efforts should be planned for improvement. The leading objectives of the journal include promotion of progress in fundamental and applied research, along with publication of guidelines and recommendations in laboratory diagnostics. As such, since the journal is an ideal source of information on current developments in the medical and laboratory technology arena, this article is aimed to celebrate some of the most important and popular articles ever published by the journal in the field of laboratory hemostasis.

**Laboratory hemostasis throughout Clinical Chemistry and Laboratory Medicine history**

The selection of the most representative hemostasis articles published in Clinical Chemistry and Laboratory Medicine...
Medicine since its founding in 1976 (formerly as Journal of Clinical Biochemistry and Clinical Biochemistry, and then European Journal of Clinical Biochemistry and Clinical Biochemistry) is obviously challenging and may be somehow arbitrary when objective criteria are not applied. As such, we chose to base our initial selection according to one of the most unbiased standards, that is the number of citations retrieved from the Thomson (former ISI) database (Table 1) (Thomson Reuters, Web of Science, available at: http://thomsonreuters.com), using the keywords “Clinical Chemistry and Laboratory Medicine” and/or “Journal of Clinical Biochemistry and Clinical Biochemistry” and/or “European Journal of Clinical Biochemistry and Clinical Biochemistry” and/or “hemostasis” and/or “coagulation”. The articles will be briefly described, in chronological order.

Probably, the foremost article ever to appear in the journal and dealing with coagulation and hemostasis, was published by van Wersch et al. in 1990 [57]. The authors investigated 22 patients with exacerbation of inflammatory bowel disease and found that most of them exhibited abnormal platelet function (i.e., non-hyperaggregability by ADP 2 μmol/L), as well as enhanced fibrinogen and D-dimer concentrations. It was hence shown that there was a high prevalence of prothrombotic factors in patients with exacerbation of inflammatory bowel disease. One year later, the same team of authors published another seminal article about blood coagulation and fibrinolysis during normal pregnancy and provided valuable clues about activation of coagulation and fibrinolytic systems with ongoing pregnancy [58].

In 1998 Heil et al. carried out an influential study that is still the basis for several guidelines and recommendations about coagulation testing [59]. Basically, the authors determined the influence of storage time and temperature on PT, APTT, thrombin time (TT), fibrinogen, factors V and VIII, antithrombin, protein C and S in plasma from healthy subjects and patients receiving heparin therapy. When plasma stability was defined as the period during which a change <10% from the initial value could be recorded, significant biases were reported after 8 h for APTT, 24 h for PT, 48 h for factor V and 7 days for TT, fibrinogen, protein C and antithrombin in healthy subjects when plasma was held at 6°C. In volunteers untreated with heparin therapy, APTT was stable for 8 h, PT for 48 h, TT, fibrinogen and antithrombin for 7 days with plasma sample storage at room temperature. In patients under therapy with heparin, the stability in plasma stored at 6°C was 8 h for TT, 24 h for PT and APTT and 7 days for fibrinogen and antithrombin. Factors V and VIII displayed a reduction of 13% and 20% after 8 h. Nevertheless, when plasma was stored at room temperature, factor V was stable for 8 h, and PT for 24 h, whereas fibrinogen and antithrombin remained unchanged for up to 7 days. The APTT showed an increase of 13%, TT a fall of 16%, and factor VIII a decrease of 18% after 8 h. Another milestone published in the journal is the comprehensive overview by Rosén and Sturk, about the recently (at that time) discovered resistance to activated protein C [60]. Another important review article was that published by Korte in 2000 and dealing with changes of the coagulation and fibrinolysis system in malignancy and their possible impact on

### Articles

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### Table 1

Most popular articles published by Clinical Chemistry and Laboratory Medicine in the field of hemostasis and coagulation, according to the Thomson (former ISI) database, updated July 2012.
future diagnostic and therapeutic procedures [61]. Seven years later, in 2007, the journal published another highly cited article by Lippi et al., who provided a comprehensive overview on the physiopathology of hemostasis along with clear indications about the diagnostic approach to the inherited bleeding disorders [62]. In 2010, Favalaro et al. published an opinion paper that might be considered the ideal continuum of the previous article of Lippi et al., outlining the sequential process of platelet function investigations, and discussing each of the essential components in some detail [31]. In more recent times, although the number of citations is understandably lower, three articles will probably provide some predictable breakthrough into laboratory hemostasis. In 2011, Samama et al. published a seminal guide in the intricate field of laboratory assessment of new anticoagulants (i.e., fondaparinux, dabigatran, rivaroxaban or apixaban) [63], which has since been followed by a position paper issued by the Italian Society of Clinical Biochemistry (SIBioC), the Italian Society of Laboratory Medicine (SIMeL) and the Italian Federation of Centers for Surveillance of Anti-coagulated patients (FCSA), which analogously discusses guidelines and recommendations on the same topic [64]. Another influential article to have recently appeared in the journal is that of Parenmark and Landberg [65], who raised several doubts as to whether mixing of tubes with liquid-based citrate buffer for coagulation testing may be really necessary. Practically, avoidance of mixing the tubes seems to produce clinically negligible bias in results of screening coagulation tests, whereas instant and too vigorous mixing of blood samples after venipuncture may produce various degree of spurious hemolysis, and thereby jeopardize the reliability of several clotting [66] and platelet function tests [67], due to the presence of cell-free hemoglobin. This is truly important information, since it may pave the way to revising the current best practice guidelines for collection and handling of blood specimens for coagulation testing [68].

Conclusions

The abnormalities of primary and secondary hemostasis, either thrombotic or hemorrhagic, represent serious challenges for clinicians, while the contribution of laboratory diagnostics is indeed essential for clinical and therapeutic decision-making. Several preanalytical, analytical and postanalytical issues wait to be clarified in coagulation testing. All the previous articles noted above have produced breakthroughs in the field of laboratory hemostasis; some have also substantially influenced clinical and laboratory practice, many others will probably do so as well. With this expectation, we encourage all readers of Clinical Chemistry and Laboratory Medicine to submit further high quality articles about this topic to the journal.

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