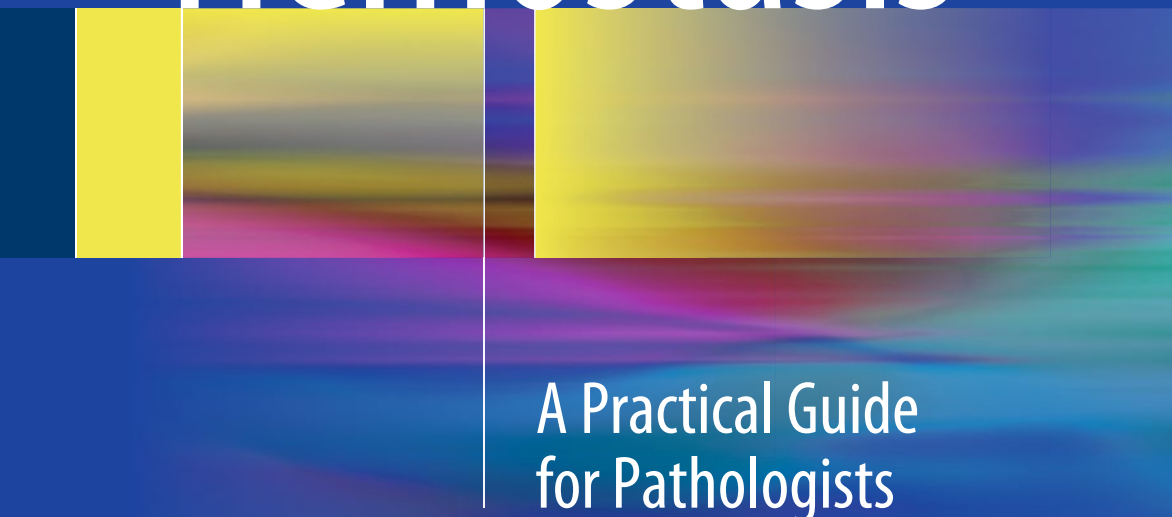
Laboratory Hemostasis



A Practical Guide for Pathologists

Second Edition

Sterling T. Bennett
Christopher M. Lehman
George M. Rodgers

Springer

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Preface

In the course of our practice as coagulation laboratory directors, we have acquired personal libraries of hemostasis and thrombosis texts that serve as excellent references for the basic science and clinical aspects of hemostasis. What was missing, though, was a single reference text that provided practical information and guidance on the topics relevant to directing a coagulation laboratory. After talking about it for several years, we attempted to fill the void with the first edition of a handbook containing the information we provide to the residents and fellows that we are helping train to become laboratory directors. We have now updated the information in our handbook to account for changes in the coagulation laboratory and transfusion medicine practice that have occurred since the initial edition was published.

In revising this book, we have tried to cite the consensus recommendations of authoritative bodies whenever possible and, otherwise, have cited reviews, chapters, and research articles of experts in their respective fields. These references should act as a starting point for a more detailed study of the various aspects of coagulation testing.

It is our intent that pathologists, clinical laboratory scientists, and other physicians serving as laboratory directors find this new edition of the book helpful in understanding and carrying out their responsibilities. We also hope that coagulation laboratory supervisors, technologists, and technicians find this to be a helpful reference for the day-to-day operation of the laboratory. Finally, we hope that residents and fellows find this book to be a useful tool for learning the basics of coagulation testing and for studying for board examinations.

Salt Lake City, UT, USA

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Role and Responsibilities of the Laboratory Director

1

Sterling T. Bennett

Clinical laboratories provide essential services and information for the practice of medicine. A substantial portion of electronic medical data consists of laboratory results [1], and laboratory results are vital information for many clinical decisions. Accordingly, laboratories potentially contribute to improved patient safety and medical outcomes when their services are timely and results are accurate.

Serving as a laboratory director is an interesting, challenging, and rewarding job, one that carries great responsibility. The director is accountable for all aspects of the laboratory service and is the key bridge between laboratory operations and clinical practice. As such, the laboratory director must be well-versed in clinical medicine, basic medical sciences, pathology disciplines, clinical laboratory sciences, laboratory operations, and quality management systems. Skills in informatics, data analysis, and business management are also important assets. The breadth of knowledge and skills required of a laboratory director is one aspect that makes the job so attractive.

Fundamental knowledge is not enough. For the laboratory to succeed, it is vital for the director to clearly understand his or her role and responsibilities. Definitions of the laboratory director's responsibilities have been published by the College of American Pathologists [2], American Association of Blood Banks [3], International Organization for Standards (ISO) [4], National Pathology Accreditation Advisory Council (Australia) [5], European Society of Pathology [6], and Canadian Association of Pathologists [7], to name a few. In addition, the United States' Code

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Table 1.1 Laboratory director's responsibilities

General responsibilities	Personnel	Facilities and safety	Test procedures	Quality management	Consultation and education	Communication	Operational management
Overall operation and administration	Needs assessment	Biosafety	Test menu selection	Quality program	Accessible to medical staff and laboratory staff	Clinicians	Strategic planning
Definition of others' duties	Job descriptions	Chemical hygiene	Method selection	Quality standards	Test ordering	Patients	Capital budgets
Delegation of authority	Hiring standards	Ergonomics	Method validation	Quality assessment	Result interpretation	Administration	Operational budgets
Retention of responsibility	Staff selection		Quality control program	Proficiency testing	Therapeutic recommendations	Lab staff	Research and development
Licensure and accreditation	Assignment of duties		Harmonization or standardization of methods	Inter-method comparisons	Troubleshooting	Risk management	Contracting
Medical staff privileges	Delegation		Requisitions/ordering processes	Audits	Utilization patterns	Regulators	Marketing
	Training standards		Specimen acceptance and rejection criteria	Surveys	Test menu changes	Accreditation or certification bodies	Billing
	Staff training		Specimen collection and handling	Inspections	Staff continuing education		Organizational goals
	Competency standards		Transportation	Problem reports	Clinician education		Metrics
			Result reporting	Remedial actions	Institutional education program support		Contingency plans
	Performance		Standard operating procedures	Quality improvement			
	Performance standards		Reference laboratory selection and monitoring				
	Performance reviews						
	Remedial actions						

of Federal Regulations defines the laboratory director's responsibilities under the Clinical Laboratory Improvement Amendments of 1988 (CLIA-88) [8]. Table 1.1 provides a summary listing of the laboratory director's responsibilities.

For any given laboratory, the laboratory director's responsibilities are defined in large measure by prevailing regulations and the standards of accreditation bodies. Responsibilities may be further defined by the organizational structure of the institution and contractual arrangements between the institution and laboratory director; however, organizational structure and contracts do not absolve the laboratory director of responsibilities defined by regulations and accreditation standards.

Each set of regulations or accreditation standards is unique, with its own emphasis on the director's responsibilities. Some define detailed responsibilities while others provide few specifics. Table 1.2 illustrates these differences by listing the duties specifically attributed to the laboratory director in three sets of regulations or standards. The one point on which all agree is that the laboratory director is responsible for assuring that the laboratory complies with all provisions of the regulations or standards. An abstraction from the various definitions suggests that the laboratory director's responsibilities may be broadly categorized into general responsibilities, personnel, facilities and safety, test procedures, quality management, consultation and education, communication, and operational management. This chapter will discuss specific responsibilities within each of these categories.

1.1 General Responsibilities

The laboratory director is responsible for the overall operation and administration of the laboratory. As the sign on the late USA President Harry Truman's desk read: "The buck stops here" [9]. Although it takes many people in a variety of roles to make a laboratory run well, the ultimate accountability for success or failure rests with the laboratory director. It is imperative that the laboratory director assures that he or she has adequate authority to discharge this accountability. When the laboratory director is a private contractor rather than an employee of the organization that owns the laboratory, the laboratory director's span of authority is often ambiguous. Even when the laboratory director is an employee of the organization, others in management may see the director as a figurehead with limited authority in day-to-day operations. Nevertheless, in the event of regulatory infractions or failure to meet accreditation standards, the laboratory director will be held accountable, so it is important for the director to establish, both contractually and functionally, the authority necessary to truly direct the overall operation and administration of the laboratory.

With the complexity of contemporary clinical laboratories, it is not practical (or even possible) for the laboratory director to personally perform every facet of his or her responsibilities. The laboratory director may not even have the expertise to fulfill some responsibilities. Fortunately, the laboratory director is allowed to delegate responsibilities to other qualified individuals. To maintain order and clarity—and,

Table 1.2 Examples of regulations and accreditation standards defining the laboratory director's role and responsibilities

Category	Laboratory director's responsibilities[a] as defined by:		
	US Clinical Laboratory Improvement Amendments of 1988 (CLIA)	College of American Pathologists (CAP)	International Organization for Standardization (ISO)
General responsibilities			
Overall operation and administration	Take responsibility for the overall operation and administration of the laboratory, including employment of personnel who are competent to perform test procedures, record and report test results promptly, accurately and proficiently	Assume professional, scientific, consultative, organizational, administrative, and educational responsibilities	Take responsibility for the professional, scientific, consultative or advisory, organizational, administrative and educational matters relevant to the services offered by the laboratory
		Be ultimately responsible for compliance with the Standards for Accreditation	
Duties and delegation	If desired, delegate duties of the technical supervisor, technical consultant, clinical consultant, general supervisor, and testing personnel to qualified personnel	If desired, delegate administrative, medical and technical responsibilities to qualified individuals. If not qualified to direct any laboratory sections, retain qualified individuals to direct those sections	If desired, delegate selected duties and/or responsibilities to qualified personnel
	Specify, in writing, the responsibilities and duties of each consultant, supervisor, and person engaged in any phase of testing		
Retention of responsibility	If responsibilities are reapportioned, remain responsible for ensuring that all duties are properly performed	If duties are delegated, remain responsible to ensure that quality patient services are provided	If responsibilities are delegated, maintain ultimate responsibility for the overall operation and administration of the laboratory
Licensure and accreditation	Assure compliance with applicable regulations	Maintaining CAP Standards, implementing requirements of Accreditation Checklists, and documenting compliance	
Medical staff privileges		As applicable, serve as an active member of the medical staff for facilities served	Serve as a contributing member of the medical staff for those facilities served

Personnel			
Supervision	Ensure that a general supervisor provides on-site supervision of high complexity testing		
	Identify whether supervision is required for specimen processing, test performance or result reporting		
	Identify whether supervisory or director review is required prior to reporting patient test results		
Adequate staffing	Employ a sufficient number of laboratory personnel with the education, training, and experience to provide appropriate consultation, properly supervise and accurately perform tests and report test results	Ensure that there are sufficient qualified personnel with adequate documented training and experience to meet the needs of the laboratory	Ensure that there are appropriate numbers of staff with the required education, training and competence to provide medical laboratory services that meet the needs and requirements of the users
Training and competence	Ensure that prior to testing patients' specimens, all personnel have appropriate education and experience, appropriate training for the type and complexity of the services offered, and demonstrated that they can perform testing operations reliably to provide and report accurate results		
Monitoring competence	Ensure that policies and procedures are established for monitoring individuals to assure personnel are competent and maintain their competency to process specimens, perform test procedures and report test results promptly and proficiently, and to assure identification of needs for remedial training or continuing education to improve skills		
Test authorization	Identify which examinations and procedures each individual is authorized to perform		
Personnel management	Identify which examinations and procedures each individual is authorized to perform		

(continued)

Table 1.2 (continued)

Category	Laboratory director's responsibilities[a] as defined by:		
	US Clinical Laboratory Improvement Amendments of 1988 (CLIA)	College of American Pathologists (CAP)	International Organization for Standardization (ISO)
Facilities and safety			
Facilities and safety	Ensure that the physical plant and environmental conditions of the laboratory are appropriate for the testing performed and provide a safe environment in which employees are protected from physical, chemical, and biological hazards	Implement a safe laboratory environment in compliance with good practice and applicable regulations	Implement a safe laboratory environment in compliance with good practice and applicable requirements
Test procedures			
Quality in preanalytic, analytic and postanalytic phases	Ensure that testing systems provide quality laboratory services for all aspects of test performance		
Test methods	Ensure that test methodologies can provide the quality of results required for patient care	Provide input into the selection of equipment, methods, and reagents appropriate to the needs of patients, the scope of testing, and the financial constraints on the laboratory or institution	
Verification of performance characteristics	Ensure that verification procedures are adequate to determine the accuracy, precision, and other pertinent performance characteristics of test methods		
Test performance	Ensure that personnel perform test methods as required for accurate and reliable results		
Quality control	Ensure that the quality control program is established and maintained to assure the quality of laboratory services and identify failures in quality as they occur	Define, implement, and monitor standards of performance in quality control	
Analytical performance levels	Ensure the establishment and maintenance of acceptable levels of analytical performance for each test system		

Remedial actions	Ensure that all necessary remedial actions are taken and documented whenever significant deviations from the laboratory's established performance characteristics are identified, and that patient test results are reported only when the system is functioning properly		
Reports	Ensure that reports of test results include pertinent information required for interpretation		
Data management		Monitor all work performed in the laboratory to determine that medically reliable data are being generated	Monitor all work performed in the laboratory to determine that clinically relevant information is being generated
		Correlate laboratory data for diagnosis and patient management	
Procedure manual	Ensure that an approved procedure manual is available to all personnel responsible for any aspect of the testing process		
Suppliers			Select and monitor laboratory suppliers
Anatomic pathology		Perform anatomic pathology procedures as appropriate	
Reference laboratories		Select and monitor all reference laboratories for quality of service	Select referral laboratories and monitor the quality of their service

(continued)

Table 1.2 (continued)

Category	Laboratory director's responsibilities[a] as defined by:		
	US Clinical Laboratory Improvement Amendments of 1988 (CLIA)	College of American Pathologists (CAP)	International Organization for Standardization (ISO)
Quality management			
Proficiency testing	Ensure enrollment in an approved proficiency testing program		
	Ensure proficiency testing samples are tested in the same manner as patients' specimens		
	Ensure results are returned within the timeframes established by the proficiency testing program		
	Ensure proficiency testing reports are reviewed by appropriate staff to evaluate the laboratory's performance and to identify any problems that require corrective action		
	Ensure an approved corrective action plan is followed when any proficiency testing result is found to be unacceptable or unsatisfactory		
Quality program	Ensure that the quality assurance program is established and maintained to assure the quality of laboratory services and identify failures in quality as they occur	Assume responsibility for implementation of the quality management plan	Ensure the implementation of the quality policy
Quality improvement		Participate as a member of the quality improvement committees of the institution	Define, implement and monitor standards of quality improvement of the medical laboratory service. This may be done within the context of the various quality improvement committees of the parent organization
Record-keeping		Establish and implement an adequate record-keeping system and monitor adherence to that system	

Communication			
Interaction with others		Relate and function effectively with applicable accrediting and regulatory agencies, appropriate administrative officials, physicians, the medical community, and patients	Relate and function effectively with applicable accrediting and regulatory agencies, appropriate administrative officials, the healthcare community, and the patient population served, and providers of formal agreements, when required
Response to lab staff and users			Address any complaint, request or suggestion from staff and/or users of laboratory services
Consultation and education			
Accessible to laboratory	Be accessible to the laboratory to provide onsite, telephone or electronic consultation as needed		
Medical significance, interpretation, and correlation of data	Ensure that consultation is available to the laboratory's clients on matters relating to the quality of the test results reported and their interpretation concerning specific patient conditions	Provide consultations about the medical significance of clinical laboratory data for purposes of further diagnosis and possible treatment	Ensure the provision of clinical advice with respect to the choice of examinations, use of the service and interpretation of examination results
		Interpret, correlate, and communicate laboratory data to clinicians, patients, and other appropriate requestors	
Educational responsibilities		Provide educational programs for the medical and laboratory staff, and participate in educational programs of the institution	Provide professional development programs for laboratory staff and opportunities to participate in scientific and other activities of professional laboratory organizations

(continued)

Table 1.2 (continued)

Category	Laboratory director's responsibilities[a] as defined by:		
	US Clinical Laboratory Improvement Amendments of 1988 (CLIA)	College of American Pathologists (CAP)	International Organization for Standardization (ISO)
Operational management			
Strategic planning		Perform planning for setting goals and developing and allocating resources appropriate to the medical environment	
Administrative and management responsibilities		Provide effective and efficient administration, including budget planning and control with responsible financial management, in accordance with regulations and institutional assignment of such responsibilities	Provide effective leadership of the medical laboratory service, including budget planning and financial management, in accordance with institutional assignment of such responsibilities
		Define, implement, and monitor standards of performance in cost-effectiveness of laboratory services	Define, implement and monitor standards of performance of the medical laboratory service
Research and development responsibilities		Plan and direct research and development appropriate to the facility	Plan and direct research and development, where appropriate
Contingency plan			Design and implement a contingency plan to ensure that essential services are available during emergency situations or other conditions when laboratory services are limited or unavailable

[a]This table is not a comprehensive cross-reference between various standards and regulations, but is intended to illustrate the commonalities and differences in the level of detail that different organizations give to the definition of specific responsibilities of the director. Many other regulations and standards exist beyond those listed. This table summarizes only those items that are specifically identified as responsibilities of the laboratory director

in some cases, to meet regulatory or accreditation requirements—delegation should be specific and in writing, and the laboratory director should be certain that designees understand the director's expectations. The director also needs to assure that the laboratory is organized in a manner that allows designees to fulfill their responsibilities.

The laboratory director retains accountability even for delegated responsibilities. In the event of an untoward event, the director cannot "pass the buck" to a designee, so the director needs to implement processes to ensure that designees are performing their delegated functions and to detect unsatisfactory performance.

The laboratory director is accountable for licensure and accreditation. He or she must be intimately familiar with applicable regulations and accreditation standards. The director's involvement is also required for licensure or accreditation applications, laboratory inspections, and correspondence with government agencies and accreditation bodies.

Time devoted to studying regulations, reading standards documents, consulting other laboratory directors, and conducting self-inspections or external inspections is time well spent. Time discussing clinical needs and laboratory issues with other members of the medical staff or physician clients is also time well spent. Understanding the role of the laboratory in clinical care and understanding the director's responsibilities in the laboratory will enable the director to help the laboratory succeed and will help the director to avoid the pitfalls that will be discussed later in this chapter.

1.2 Personnel

Personnel constitute the single most important asset of any laboratory. No matter how high the level of instrumentation, automation, computerization, or other technology, a laboratory cannot function without competent and dedicated people. From the highs of exceptional service to the lows of errors that jeopardize patients, the fruits of the laboratory service are attributable to its people. Accordingly, the laboratory director has many responsibilities related to human resources.

The director must assure that the laboratory has an appropriate number of trained and competent staff with adequate supervision to meet the demands of the laboratory service, regulations, and accreditation standards. The director needs to assure that job descriptions accurately reflect duties to be performed, that staff are selected in an equitable process from a pool of qualified applicants, and that each member of the laboratory staff is provided with a clear description of expectations and responsibilities.

The director is responsible for the implementation of a training program that includes training standards, acceptable methods of instruction, and a system of documentation. The program must be operated in a manner to assure that all staff members are adequately trained before working in the laboratory. The director is accountable for ongoing evaluation and improvement of the training program.

Closely related to training is competency, and the director is responsible for implementation of a competency assurance program, including competency standards, methods of assessment, and documentation systems. The program must assure that all personnel are competent to perform their duties and that any deficiencies in competency are detected and corrected through additional training, adjustments in supervision, reassignment of duties, or other means. The director is accountable for ongoing evaluation and improvement of the competency assurance program.

The director is also accountable for periodic reviews of staff to evaluate additional aspects of performance such as attendance, punctuality, interpersonal skills, and customer service. A minimum frequency for reviews may be set by the organization or by regulations, but reviews should be held as often as necessary to develop attributes of staff members in accordance with the laboratory's needs and objectives.

1.3 Facilities and Safety

The director is accountable for assuring that laboratory staff, visitors, and patients have a safe and healthy environment and that the facilities are appropriate for the services provided by the laboratory. The safety program needs to encompass biological safety, chemical hygiene, and ergonomics. The director assures that safety policies and procedures are consistent with regulations and good laboratory practices and personnel are well trained in safety.

The director assures that all necessary personal protective equipment is available to and used appropriately by everyone in the laboratory. The director assures that fire extinguishers, eyewash stations, emergency showers, chemical spill kits, biohazard spill kits, and other equipment are in good working order and personnel are adequately trained in their use.

The director is accountable for the proper decontamination of equipment and work areas, the proper handling, storage, and disposal of hazardous chemicals, and the proper handling and disposal of contaminated waste.

The director assures that accidents and violations of safety policies are promptly evaluated and appropriate corrective measures are instituted, including reporting to government agencies when required. The director assures the ongoing evaluation and improvement of the laboratory's safety program.

1.4 Test Procedures

The laboratory director is accountable for all aspects of testing, including preanalytical, analytical, and post-analytical phases. Selection of the test menu is one of the key functions of the laboratory director. In this regard, important guidance may be derived from discussions with the medical staff or physician clients, consideration of the needs of other services in the institution, proximity of other laboratory services, requisite expertise, and evaluation of cost impact (on the laboratory and other services).

The laboratory director is responsible for test methods used in the laboratory. The director assures the selection of suitable analyzers, reagents, supplies, calibrators, and control materials, so that test methods' performance characteristics meet the needs of laboratory users [10]. The director assures that test methods are validated against established acceptance criteria before being placed into service for testing patients' specimens. The director assures that when two or more methods are used for the same analysis that results are equivalent.

The director is responsible for the quality control program, including types of controls, frequency of quality control testing, control limits, interpretation of control results, and procedures for out-of-range control values. The director assures that all assays are in control when patients' results are reported.

The director assures that only suitable specimens are analyzed. The director approves the types of specimens and anticoagulants that are acceptable for the methods employed and defines rejection criteria for unacceptable specimens. Rejection criteria include not only improper specimen types but also ordering, labeling, and handling issues that render a specimen unsuitable for testing.

The director is responsible for the reporting of test results. The director assures that reference intervals are appropriate for the laboratory's patient population and that any other information needed for proper interpretation of results is provided to clinicians. The director is accountable for the methods of reporting, whether by electronic means, printed reports, fax, telephone, etc., to assure that test results are accurately transmitted and available when needed by clinicians caring for the patients.

The director is responsible for standard operating procedures for all aspects of testing. The director assures that standard operating procedures are valid, updated as necessary, and accessible to laboratory personnel.

The director is accountable for the selection of reference laboratories for tests that are not performed in the laboratory. The director assures that reference laboratories are licensed and accredited, if required, that reference laboratories provide timely services, and that the laboratory receives the reference laboratories' results and reports them properly to its users.

1.5 Quality Management

The laboratory director is accountable for the quality of all services of the laboratory, including the implementation of a quality management system that includes an overarching quality plan, quality standards, and a quality manual with policies and procedures. The quality system addresses all aspects of the laboratory service including organization, personnel, equipment, purchasing and inventory, process control, documents and records, information management, error and incident management, assessments, process improvement, customer service and satisfaction, facilities, safety, and so forth.

The director is responsible for quality assessment methods including proficiency testing, inter-method comparisons, audits, surveys, inspections, and incident or

problem reports. The director assures that laboratory personnel evaluate the results of assessments, identify opportunities for improvement, and institute corrective or remedial actions. The director is responsible for the identification, prioritization, and implementation of quality improvement activities.

1.6 Consultation and Education

A visible role of the laboratory director is in the area of consultation and education. As both a laboratory and clinical expert, the laboratory director is in a unique position to help maximize the clinical value of laboratory testing. To do this, the director must be accessible to the medical and laboratory staff.

The director consults with clinicians about test selection and ordering, interpretation of test results, and therapeutic decisions. The director educates clinicians about changes in test methods or test menu, the utility of new tests, and opportunities to improve usage of existing tests. The director fields complaints or concerns from clinicians and assesses clinicians' needs. The wise director pays careful attention to astute clinicians' observations about laboratory results or services. The director participates in the institution's education program, as applicable.

The director consults with laboratory staff on issues related to test methods, quality control, client satisfaction, vendor relations, and so forth. The director evaluates the clinical and regulatory ramifications of laboratory problems or incidents. The director assures that laboratory staff receive education necessary to perform their duties and enhance the laboratory's services.

1.7 Communication

Although most communication is made by managers, supervisors, and frontline staff, the laboratory director, by virtue of his or her position of accountability and authority, plays a critical role in communication with clinicians, patients, laboratory staff, administration, risk management, government regulators, inspectors, vendors, and others. The director's personal communications can often facilitate the resolution of troublesome situations.

1.8 Operational Management

Depending on prevailing regulations, accreditation standards, and contractual provisions, the laboratory director may or may not have direct accountability for operational management; however, the impact of operational management on the director's ability to discharge his or her other duties cannot be ignored and creates a virtual or implied accountability. By virtue of background, training, expertise, and responsibilities, the laboratory director is in a unique and critical position with respect to strategic planning, organizational goal setting, capital and operational

budgeting, research and development, marketing, and vendor contracting. The director must advocate for patients and clinicians in deliberations and decisions related to operational management because, in many cases, no one else will be in a position to do so.

1.9 Resources

It should be obvious by this point in this chapter that the responsibilities of the laboratory director are broad and deep. The knowledge and skills required are tremendous. Fortunately, laboratory directors are not left without resources.

Other laboratory directors are a great potential resource for practical ideas. Former mentors and respected laboratory directors in the community are generally more than willing to provide feedback or ideas. Professional societies at the local, regional, national, or even international level provide networking opportunities and educational forums for enhancing one's knowledge and skills. Private organizations offer training programs for laboratory directors. Some government agencies also offer training for laboratory directors.

Many publications address one or more aspects of the laboratory director's responsibilities. For example, the Clinical and Laboratory Standards Institute (CLSI) has published guidelines for quality system modeling, quality improvement, training and competency assessment, laboratory design, laboratory safety, technical procedure manuals, proficiency testing, reference laboratory selection and evaluation, cost accounting, assessment of test accuracy, method comparisons, and others [10–20]. The CLIA-88 regulations in the Code of Federal Regulations [8] and the standards of the College of American Pathologists, American Association of Blood Banks, International Organization for Standardization, and others contain much information about contemporary good laboratory practices [2–7].

1.10 Common Pitfalls

Before concluding this chapter, a final note about common pitfalls seems in order. Because the responsibilities of the laboratory director are so numerous, because most responsibilities can be delegated, and because the laboratory director often has other service responsibilities, it is easy for a lab director to fall into one or more traps. The nature and implications of the traps and the means of extrication are self-evident, so the traps are listed without elaboration.

- The laboratory director doesn't understand the scope of his or her responsibilities.
- Hospital administrators, lab managers, lab supervisors, and lab staff don't understand the role and responsibilities of the laboratory director.
- Regulators have a broader interpretation of the laboratory director's responsibilities than the employer or contracting facility.

- The laboratory director's delegation of responsibilities is informal or ambiguous.
- The laboratory director is unaware of problems or issues.
- The laboratory director's follow up is inadequate or non-existent.
- The laboratory director accepts multi-laboratory system directives that conflict with or fail to meet local needs.
- The laboratory director and subordinates are unaware of or don't understand regulations or accreditation standards.
- The laboratory director passively accepts the institution's failure to devote adequate resources to meet service needs and comply with regulations and accreditation standards.

1.11 Summary and Key Points

The position of laboratory director bestows many responsibilities requiring a broad set of knowledge and skills in clinical medicine, pathology, clinical laboratory sciences, basic sciences, operations, and quality management. Some key points for the director to keep in mind are:

- The laboratory director is accountable for all aspects of laboratory.
- Specific accountabilities may be further defined by prevailing regulations, accreditation standards, organizational model, and contracts.
- The laboratory director is the key bridge between laboratory operations and clinical practice.
- Delegation to others is required to fulfill the laboratory director's responsibilities.
- Common pitfalls can be avoided.

By understanding and fulfilling his or her responsibilities, the laboratory director not only assures that the laboratory makes a great contribution to clinical care and patient safety but also obtains the personal benefits of an interesting, challenging, and rewarding position.

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Collection of Coagulation Specimens

2

Sterling T. Bennett

2.1 Collection of Specimens for Hemostasis Testing

Specimen integrity is important for every laboratory test, but especially for coagulation testing, where even minor deviations from standard practices may lead to inaccurate results. Citrated plasma is the most common specimen type for routine and special coagulation testing, and the suitability of citrated plasma is particularly sensitive to anticoagulant concentration, container materials, collection technique, centrifugation, and storage. Accordingly, this chapter will emphasize citrated plasma specimens, but will also address other specimen types that are becoming increasingly important in hemostasis testing. The purpose of this chapter is to describe current recommendations for and points of ambiguity about specimens for hemostasis testing to assist the laboratory director in establishing standard operating procedures and evaluating the acceptability of non-ideal specimens.

2.2 Citrate Anticoagulant Concentration

The recommended anticoagulant for coagulation specimens is 105–109 mmol/L (3.13–3.2 %) trisodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), commonly called 3.2 % citrate, with or without buffer [1]. A higher concentration of citrate (129 mmol/L or 3.8 %) is also commercially available and is considered acceptable by the Clinical and Laboratory Standards Institute (CLSI), but has some important disadvantages,

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including producing longer prothrombin time (PT) and partial thromboplastin time (PTT) results [2], lower activated protein C (APC) resistance results [3], and discrepant International Normalized Ratio (INR) values [4, 5]. Furthermore, thromboplastin International Sensitivity Index (ISI) values for calculating INRs are assigned using specimens collected in 3.2 % citrate, based on the World Health Organization protocol, and are not validated for specimens collected in 3.8 % citrate [5]. Laboratories should not use 3.2 and 3.8 % citrate interchangeably. Other citrate formulations are commercially available and CLSI considers them to be acceptable for some assays, but if used for routine coagulation testing, then anticoagulant-specific reference intervals should be developed.

The ratio of blood to anticoagulant is important because a relative excess of citrate, from under-filling specimen tubes, prolongs routine coagulation tests [6]. The ideal volume ratio of blood to citrate anticoagulant is 9:1 [1], but there is some degree of tolerance for deviations from this ratio, particularly for PT testing [7, 8]. A note of caution is in order. Studies demonstrating acceptable results with specimen volumes less than 90 % of the ideal volume have been conducted on only a very limited number of reagents, analyzers, and assays. It seems prudent, therefore, to require tubes to be filled in the range of 90–110 % of the ideal volume unless published or in-house studies with the laboratory's assay systems support different criteria.

Another circumstance that yields a relative citrate excess is high hematocrit, due to a relatively low plasma volume. CLSI has defined high hematocrit as being greater than 55 % (0.55 L/L) [1]. In this case, an alteration in the blood-to-anticoagulant ratio in the specimen is required to maintain a suitable citrate concentration in the plasma. CLSI's approach is to determine the adjusted volume of citrate to remain in the tube, remove sufficient citrate to achieve the adjusted volume, then add the volume of blood that would ordinarily be used (i.e., 90 % of tube capacity). Table 2.1 shows adjusted volumes for a variety of tube sizes and hematocrits. For example, if a 3.0 mL tube (usual fill volume of 0.3 mL citrate and 2.7 mL blood) is to be used and the patient has a hematocrit of 60 %, the adjusted volume of citrate is calculated as 0.22 mL. After removing 0.08 mL citrate, add 2.7 mL blood. The final citrate concentration in the specimen is 17 mmol/L of plasma plus citrate solution. Similarly, if the patient's hematocrit is 70 %, the required volume of citrate is 0.17 mL. After removing 0.13 mL citrate, add 2.7 mL blood. Again, the final citrate concentration in the specimen is 17 mmol/L of plasma plus citrate solution. In either example, the final specimen volume (blood and citrate) is less than the usual fill volume, so it is advisable to mark the new intended fill volume on the tube when the citrate volume is adjusted. Laboratory staff need to be instructed not to reject these specimens on the basis of a low volume.

Another convenient approach is to remove a specified volume of citrate from a coagulation tube, then fill the tube with blood to the usual fill volume. Table 2.2 shows the volume of citrate to remove, as a function of hematocrit and tube size. For example, if a 3.0 mL tube (0.3 mL citrate and 2.7 mL blood) is to be used and the patient has a hematocrit of 60 %, first remove 0.09 mL citrate, leaving 0.21 mL citrate in the tube. Add enough blood to bring the total volume of blood and citrate to 3.0 mL (i.e., add 2.79 mL blood). The final citrate concentration in the specimen is 17 mmol/L of plasma plus citrate solution. Similarly, if the patient has a

Table 2.1 Citrate volume required in specimens with high hematocrit

	Blood volume (excluding citrate), mL			
Hematocrit, %	*4.5*	*2.7*	*1.8*	*0.9*
56	0.41	0.24	0.16	0.08
57	0.40	0.24	0.16	0.08
58	0.39	0.23	0.16	0.08
59	0.38	0.23	0.15	0.08
60	0.37	0.22	0.15	0.07
61	0.36	0.22	0.14	0.07
62	0.35	0.21	0.14	0.07
63	0.34	0.21	0.14	0.07
64	0.33	0.20	0.13	0.07
65	0.32	0.19	0.13	0.06
66	0.31	0.19	0.13	0.06
67	0.31	0.18	0.12	0.06
68	0.30	0.18	0.12	0.06
69	0.29	0.17	0.11	0.06
70	0.28	0.17	0.11	0.06
71	0.27	0.16	0.11	0.05
72	0.26	0.16	0.10	0.05
73	0.25	0.15	0.10	0.05
74	0.24	0.14	0.10	0.05
75	0.23	0.14	0.09	0.05
76	0.22	0.13	0.09	0.04
77	0.21	0.13	0.09	0.04
78	0.20	0.12	0.08	0.04
79	0.19	0.12	0.08	0.04
80	0.19	0.11	0.07	0.04

Source: Clinical and Laboratory Standards Institute [1]

This table shows the volume of citrate (mL) to remain in the tube before filling with a standard volume of blood. Note that the amount of blood to add is the same for all hematocrits, so the final fill volume of citrate plus blood is less than the ideal fill volume of the tube

The volume of citrate to remain in the tube is given by the formula

$C = (1.85 \times 10^{-3})(100 - H)V_{blood}$

where

C is the volume of citrate to remain in the tube, in mL

H is the hematocrit, in %, and

V_{blood} is the standard volume of blood to add, in mL

This procedure yields a final citrate concentration of 0.017 mmol per mL of plasma plus citrate solution

hematocrit of 70 %, first remove 0.14 mL citrate, leaving 0.16 mL citrate in the tube. Add enough blood to bring the total volume of blood and citrate to 3.0 mL (i.e., add 2.84 mL blood). Again, the final citrate concentration in the specimen is 17 mmol/L of plasma plus citrate solution. An advantage of this approach is that tubes are filled to their usual volume, circumventing the need to precisely measure the volume of blood to be added.

Table 2.2 Citrate reduction in specimens with high hematocrit, tubes filled to ideal volume

	Ideal fill volume of tube (blood + citrate), mL			
Hematocrit, %	*5.0*	*3.0*	*2.0*	*1.0*
56	0.12	0.07	0.05	0.02
57	0.13	0.08	0.05	0.03
58	0.14	0.08	0.06	0.03
59	0.15	0.09	0.06	0.03
60	0.16	0.09	0.06	0.03
61	0.16	0.10	0.07	0.03
62	0.17	0.10	0.07	0.03
63	0.18	0.11	0.07	0.04
64	0.19	0.11	0.08	0.04
65	0.20	0.12	0.08	0.04
66	0.20	0.12	0.08	0.04
67	0.21	0.13	0.09	0.04
68	0.22	0.13	0.09	0.04
69	0.23	0.14	0.09	0.05
70	0.24	0.14	0.09	0.05
71	0.25	0.15	0.10	0.05
72	0.25	0.15	0.10	0.05
73	0.26	0.16	0.10	0.05
74	0.27	0.16	0.11	0.05
75	0.28	0.17	0.11	0.06
76	0.29	0.17	0.12	0.06
77	0.30	0.18	0.12	0.06
78	0.30	0.18	0.12	0.06
79	0.31	0.19	0.13	0.06
80	0.32	0.19	0.13	0.06

Source: Derived from Clinical and Laboratory Standards Institute [1]

This table shows the volume of citrate (mL) to remove from common sizes of coagulation specimen tubes prior to specimen collection. After removal of the specified volume of citrate, enough blood is added to fill the tube to its ideal volume

The volume to remove is given by the formula

$$R = V\left(0.1 - \frac{100 - H}{641 - H}\right)$$

where

R is the volume of citrate to be removed, in mL

V is the ideal fill volume of the tube (blood + citrate), in mL, and

H is the hematocrit, in %

This formula was derived from the equation in Table 1.1. This procedure also yields a final citrate concentration of 0.017 mmol per mL of plasma plus citrate solution

It should be noted that the hematocrit threshold of 55 % appears conservative. Simple calculations show that if a tube containing 3.2 % sodium citrate is 90 % filled with blood with a hematocrit of 55 %, the final plasma specimen's citrate

concentration is 23.7 mmol/L. By inference, the maximum allowable citrate concentration in a plasma specimen under the CLSI guidelines is therefore 23.7 mmol/L. In a tube filled to its ideal volume (e.g., 3.0 mL blood plus citrate in a 3.0 mL tube), the citrate concentration is less than or equal to 23.7 mmol/L until the hematocrit is higher than 60 %, suggesting that a higher hematocrit threshold may be acceptable in completely filled tubes. Due to the difficulty, expense, and time delays involved in the preparation and use of citrate-reduced tubes, laboratories may wish to consider validating a hematocrit threshold higher than 55 % for citrate volume reduction.

2.3 Plastic vs. Glass Tubes

Over the past several years, the use of plastic specimen tubes throughout the laboratory has been on the rise for several reasons, including lower risk of breakage, which reduces biohazard exposure risk, and lighter weight, which reduces shipping and disposal expenses. Regulatory agencies have also strongly encouraged the use of plastic tubes and tube manufacturers have discontinued the production of many types of glass tubes.

Coagulation testing is known to be sensitive to the composition of specimen tubes [9], and historically, coagulation specimens have been collected in siliconized glass tubes to limit contact activation of clotting factors. Several studies comparing coagulation test results on plastic versus glass tubes found statistically significant differences in PT results between tube types, but in most cases the differences were deemed to be clinically insignificant [10, 11]; however, the differences were deemed potentially clinically significant in two studies [8, 12]. Rodgers and colleagues evaluated the effect of plastic tubes on esoteric coagulation tests, including factor assays, thrombin time, lupus anticoagulant, von Willebrand factor, protein C, protein S, APC resistance, and antithrombin, with normal donor plasma specimens. They found that the only test significantly affected was the thrombin time [13].

CLSI considers either glass or plastic tubes to be acceptable, provided that the inside surface is non-activating [1]. Nevertheless, before changing from one tube type to another, it is advisable for laboratories to evaluate the effects, if any, on their coagulation assays by conducting crossover studies using specimens with normal and abnormal results.

2.4 Collection Techniques

Several options are available for collecting blood specimens for coagulation testing, including venipuncture blood collection systems into evacuated tubes, winged needles and tubing, syringes, vascular access devices (VADs), and capillary specimens. Of these, the recommended method is venipuncture collection directly into tubes containing anticoagulant [1]. The other methods all have potential problems that may affect specimen quality. Winged collection systems have a length of tubing whose dead-space may result in under-filled tubes or activation of clotting factors or

platelets if not properly managed. Syringe draws may result in activation of clotting factors when there is a delay in transferring blood from the syringe to the anticoagulated specimen tube. Syringe draws may also cause hemolysis if blood is drawn into or expelled from the syringe with too much force. VADs may lead to heparin contamination if the line is heparin-imbedded, has been flushed with heparin, or has had heparin administered through it. VADs may also lead to specimen dilution or partially clotted blood if the dead space is not managed appropriately. Capillary specimens are subject to activation of clotting factors if blood is not flowing freely, dilution with extracellular fluid if the skin puncture site is squeezed too vigorously, and hemolysis if the puncture site is overly "milked."

Historically, a discard sample (or "pilot tube") was collected prior to the specimen for coagulation testing, due to concern about activation of clotting factors or platelets by the venipuncture procedure. Studies have shown that this practice is not necessary for PT and PTT testing [1, 14, 15], although discard samples have been recently advocated for PT/INR testing on patients receiving long-term warfarin therapy [16]. Smock and colleagues demonstrated the acceptability of omitting a discard sample for special coagulation tests, including fibrinogen, D-dimer, protein C, protein S, antithrombin, and factors VII, VIII, IX, X, and XI [17]. Raijmakers and colleagues reached the same conclusion in a separate study [18]. Two caveats apply to the omission of a discard sample. First, with winged collection sets, the long tubing contains a dead space that must be cleared of air to avoid under-filling the specimen tube. A discard sample may be drawn to fill the dead space with blood, but the discard sample itself need not be of any particular volume [1]. Second, the dead space of VADs may contain IV fluids, flush fluids, heparin, micro-clots, bacteria, and other undesirables. For this reason, the use of VADs for coagulation specimen collection should be discouraged. In addition, collecting coagulation specimens through a VAD that has ever had heparin in it should be avoided, if possible. When a VAD must be used, it should be flushed with at least 5 mL saline, then 5 mL or 6 dead-space volumes should be drawn and discarded prior to specimen collection [1]. Discard samples should be collected in non-additive or coagulation tubes or in non-additive syringes. Other additives may contaminate the collection device and affect coagulation test results.

Needle gauge should be based on the amount of blood to be drawn, age of patient, and vein size [1]. Needles larger than 16-guage should be avoided to prevent hemolysis caused by turbulence. Small gauge needles on syringes create a temptation to use excessive force when drawing blood into or expelling blood from a syringe, leading to hemolysis. Small gauge needles on winged collection sets may result in slow blood flow through the tubing, with resultant activation of clotting factors and platelets. However, with proper technique, needle gauge need not be an impediment to collection of suitable specimens.

The order in which blood collection tubes should be filled has been defined to avoid problems with cross-contamination of additives between tubes. Cross-contamination of additives can result from drawing additive tubes before non-additive tubes resulting in spurious test results (e.g., contamination of a serum tube with

Table 2.3 Specimen collection techniques and cautions

Method	Dead-space issues	Discard sample	Other limitations and cautions
Venipuncture collection system	None	None	
Winged collection system	Dead-space air needs to be cleared prior to specimen collection	Needed only to clear air from the tubing	Slow blood flow through tubing may lead to activation of clotting factors or platelets
			Air leaks in connections may cause hemolysis or under-filling
Syringe with hypodermic needle	None	None	Excessive force when drawing into or expelling from syringe may cause hemolysis
			Blood must be transferred from syringe to anticoagulant tube within 1 min of collection
			Air leaks in connections may cause hemolysis or under-filling
Vascular access device	Dead space may contain IV fluids, micro-clots, heparin, etc.	After VAD is flushed with saline, discard 5 mL or 6 dead-space volumes, whichever is greater	Due to high risk of specimen contamination, use of VADs should be discouraged
			VADs that have ever had heparin in them should not be used
			Air leaks in connections may cause hemolysis or under-filling
Capillary	None	Do not use the first drop of blood	Free flow of blood is needed to avoid activation of clotting factors or platelets
			Excessive squeezing or "milking" may cause hemolysis or contamination with extracellular fluid

potassium EDTA will falsely increase the serum potassium measurement and falsely decrease the serum calcium measurement). The recommended order of draw is [19]:

- Blood culture tube
- Coagulation tube (e.g., blue closure)
- Serum tube with or without clot activator, and with or without gel separator (e.g., red closure, gold closure)
- Heparin tube, with or without gel separator (e.g., green closure)
- EDTA tube (e.g., lavender closure, pink closure)
- Glycolytic inhibitor (e.g., gray closure)

A summary of coagulation specimen collection techniques, issues, and cautions is provided in Table 2.3.

2.5 Specimen Preparation

The accepted goal of centrifugation of coagulation specimens is to produce platelet-poor plasma with a platelet count $<10\times10^9$/L ($<10\times10^3$/μL) [1]. This may be accomplished by centrifuging specimens at 1,500 g for 15 min or longer at room temperature. Other centrifugation protocols may be used, but should be validated to assure the production of platelet-poor plasma and comparable results on coagulation assays.

Some coagulation tests are less sensitive to the presence of platelets than others. PT, PTT, and thrombin time (TT) test results are not significantly affected by platelet counts up to 200×10^9/L in fresh specimens [1]. However, this does not hold true for specimens containing heparin [20]. Tests for lupus anticoagulant, antiphospholipid antibodies, and heparin monitoring require platelet-poor plasma. Data are not available for other tests. Specimens to be frozen must be platelet-poor; otherwise, the disruption of platelet membranes during the freeze-thaw process may affect coagulation test results.

Given the possibility that other tests may be added to specimens initially tested for PT, PTT, or TT and given that laboratories are often unaware of the presence or absence of therapeutic heparin, particularly in specimens from hospitalized patients, it seems prudent to retain the goal of producing platelet-poor plasma whenever coagulation specimens are centrifuged.

Laboratories need processes to manage citrated specimens that are not intended for platelet-poor-plasma-based testing. Specimens for platelet aggregation studies need to be centrifuged to produce platelet-rich plasma. Increasingly, citrated specimens are used for whole blood testing for thromboelastography (TEG), platelet function analysis (e.g., PFA-100), and antiplatelet therapeutic monitoring (e.g., VerifyNow). Laboratories need processes to prevent these specimens from being centrifuged because testing is not valid on resuspended specimens.

2.6 Specimen Stability

Specimen stability varies with the test to be performed, type of specimen required, presence of cells, presence of heparin, and temperature. Stability may also be affected by test methods. Stability data for routine and special coagulation tests are shown in Table 2.4. Laboratories should verify the stability data on their own test systems and adjust their standard operating procedures accordingly.

2.7 Problem Detection and Actions

As the preceding sections indicate, a number of serious problems can (and do!) occur with coagulation specimens. It is important to have procedures for detecting and addressing problems that may affect the integrity of test results.

Table 2.4 Specimen stability for coagulation tests

Test	Specimen type	Storage conditions			
		Ambient (18–24 °C)	Refrigerated (2–4 °C)	Frozen (–20 °C)	Frozen (–70 °C)
ACT	Whole blood	1 min	Unacceptable	Unacceptable	Unacceptable
Anticardiolipin antibodies	Serum	48 h	2 weeks	1 year	1 year
Antithrombin activity	Plasma (citrate)	4 h	8 h	1 month	1 year
Anti-Xa heparin assay	Plasma (citrate)	4 h	8 h	1 month	6 months
APC resistance	Plasma (citrate)	4 h	Unacceptable	3 months	6 months
D-dimer	Plasma (citrate)	8 h	8 h	1 month	2 years
Factor IX	Plasma (citrate)	4 h	4 h	2 weeks	2 years
Factor V	Plasma (citrate)	4 h	4 h	2 weeks	1 year
Factor V Leiden mutation	Whole blood (EDTA)	3 days	1 week	Unacceptable	Unacceptable
Factor VII	Plasma (citrate)	4 h	4 h	2 weeks	2 years
Factor VIII	Plasma (citrate)	4 h	4 h	2 weeks	6 months
Factor X	Plasma (citrate)	4 h	4 h	2 weeks	1 year
Factor XI	Plasma (citrate)	4 h	4 h	2 weeks	6 months
Factor XII	Plasma (citrate)	4 h	4 h	2 weeks	2 years
Fibrinogen	Plasma (citrate)	4 h	24 h	1 month	18 months
Lupus anticoagulant	Plasma (citrate)	4 h	4 h	3 months	18 months
PFA	Whole blood (citrate)	4 h	Unacceptable	Unacceptable	Unacceptable
Plasminogen activity	Plasma (citrate)	4 h	Unacceptable	3 months	6 months
Protein C activity	Plasma (citrate)	4 h	Unacceptable	3 months	18 months
Protein S activity	Plasma (citrate)	4 h	4 h	3 months	6 months
Prothrombin	Plasma (citrate)	4 h	4 h	2 weeks	2 years
Prothrombin mutation	Whole blood (EDTA, ACD, citrate, heparin)	72 h	1 week	Unacceptable	Unacceptable

(continued)

Table 2.4 (continued)

Test	Specimen type	Storage conditions			
		Ambient (18–24 °C)	Refrigerated (2–4 °C)	Frozen (–20 °C)	Frozen (–70 °C)
PT/INR	Plasma (citrate)	24 h[a]	Not recommended[b]	2 weeks	1 year
PTT	Plasma (citrate)	4 h[c]	4 h[c]	2 weeks	2 years
TEG®	Whole blood	6 min	Unacceptable	Unacceptable	Unacceptable
	Whole blood (citrate)	2 h			
Thrombin time	Plasma (citrate)	2 h	4 h	2 weeks	18 months
VerifyNow (ASA, P2Y12)	Whole blood (citrate)	4 h	Unacceptable	Unacceptable	Unacceptable
VWF antigen	Plasma (citrate)	4 h	Unacceptable	3 months	1 year
VWF activity (ristocetin cofactor)	Plasma (citrate)	4 h	Unacceptable	3 weeks	6 months
VWF multimers	Plasma (citrate)	4 h	Unacceptable	3 months	6 months
VWF sequencing	Whole blood (EDTA, ACD, citrate, heparin)	72 h	1 week	Unacceptable	Unacceptable

Sources: Clinical and Laboratory Standards Institute [1]; ARUP Laboratories. Laboratory Test Directory, http://www.aruplab.com (Accessed 29 Mar 2014)
Abbreviations: *ACT* activated clotting time, *APC* activated protein C, *PFA* platelet function analysis, *PT* prothrombin time, *INR* international normalized ratio, *PTT* partial thromboplastin time, *TEG®* thromboelastograph, *VWF* von Willebrand factor
[a]Long stability is for unopened tubes at room temperature, either centrifuged or uncentrifuged
[b]Refrigeration is not recommended due to cold activation of factor VII
[c]Specimens containing heparin should be centrifuged within 1 h of collection

- *Underfilled tubes*. At the time of receipt in the laboratory, the fill level of each tube should be assessed for adequacy. Many laboratories find it helpful to have a chart or marked tube for comparison. If more than one size of tube is used in the institution or by laboratory clients, then it will be necessary to have a comparison chart or tube for each size. Underfilled tubes should be rejected and new specimens should be collected.
- *High hematocrit*. After centrifugation, the height of the cell fraction should be used to estimate hematocrit. It is helpful to have a chart or marked tube for comparison. A supplemental approach is to have the hematology laboratory notify the coagulation laboratory of patients with high hematocrits. When the hematocrit is too high, a new specimen should be obtained with a different blood-to-citrate ratio, as described in the anticoagulant section of this chapter. If the hematocrit is borderline high and the coagulation tests are within the reference range, it may be unnecessary to obtain a reduced-citrate specimen.
- *Heparin contamination*. A markedly prolonged PTT with a normal or slightly prolonged PT is the most common clue of heparin contamination, particularly with specimens collected by nursing staff or through VADs. Even if clinicians claim that a patient is not on heparin and that the VAD has never had heparin in it, suspicious results should be evaluated for heparin contamination before a major workup is initiated for factor deficiency or coagulopathy. The presence of heparin may be presumptively made if (1) a heparin assay indicates a concentration of heparin consistent with the test results, (2) test results revert to normal after the specimen is treated with a heparinase or a heparin filtration procedure, or (3) results are normal with a new specimen collected by venipuncture.
- *Clots in specimen*. After centrifugation, each specimen should be observed for the presence of clots and, if present, testing should not be performed.
- *Lipemia*. Lipemia may interfere with optical clot detection methods. If a specimen is severely lipemic or the analyzer indicates interference, the specimen should be retested by a method with mechanical or electromechanical clot detection. Alternatively, the specimen may be ultracentrifuged and the supernatant retested.
- *Icterus*. Icterus may also interfere with optical clot detection. If this occurs, the specimen should be retested by a method with mechanical or electromechanical clot detection.
- *Hemolysis*. After centrifugation, each specimen should be observed for hemolysis. Hemolysis may be either an *in vitro* or *in vivo* phenomenon. *In vitro* hemolysis indicates a problem with specimen collection or handling, and the specimen should not be used because of possible activation of clotting factors [1]. The *in vitro* nature of the hemolysis may be inferred if other types of specimens from the same patient do not show visible hemolysis. A new specimen is required for coagulation testing. *In vivo* hemolysis may occur with hemolytic anemia, disseminated intravascular coagulation, crush injuries, and other conditions. Coagulation testing may be informative, even though the specimen shows visible hemolysis. Communication with the ordering clinician may be useful for determining whether to proceed with testing. If the level of hemolysis interferes with optical clot detection, then mechanical or electromechanical clot detection must be employed to obtain valid results.

- *Incorrect anticoagulant*. The specimen type must be verified at the time of receipt in the laboratory. Aliquoted specimens create a special challenge for reference laboratories, because the tube type cannot be verified by the manufacturer's label or color of the tube closure. Laboratories need a procedure for confirming the suitability of the specimen type. Algorithms to use chemistry tests have been published [21]. They work very well for identifying EDTA plasma and less well for distinguishing heparinized plasma or serum from citrated plasma, but may be a useful adjunct to clot-based tests.
- *New oral anticoagulants*. New oral anticoagulants, including rivaroxaban, apixaban, dabigatran, and others, affect routine and special coagulation tests to varying degrees, but their presence or concentration cannot be conclusively demonstrated at this time in most laboratories. A high index of suspicion and close communication with clinicians are necessary to identify new oral anticoagulants as the cause of "abnormal" test results.

2.8 Coagulation Testing on Specimens Other than Citrated Plasma

Some coagulation tests require specimen types other than citrated plasma. The activated clotting time (ACT), thromboelastography (TEG), and point-of-care PT/INR tests use fresh whole blood without anticoagulants. Specimens for these tests are usually collected by skin puncture, syringe venipuncture or from VADs. TEG may also be run on citrated whole blood. Antiplatelet therapeutic monitoring tests and other platelet function tests often use citrated whole blood. All the cautions described above apply with the additional note that the presence of hemolysis or clots is not likely to be detected because the specimens are not centrifuged. Accordingly, specimen collection techniques need to be even more pristine. In addition, manufacturers' instructions require a discard specimen be collected first for TEG and VerifyNow assays.

Antiphospholipid antibody testing, including anticardiolipin antibodies, uses serum. Routine specimen collection procedures are appropriate.

Genetic tests, including factor V Leiden mutation and prothrombin G20210A mutation, require EDTA-anticoagulated whole blood, although ACD, citrate, or heparin anticoagulants may also be acceptable. It is important that personnel understand to retain these specimens as whole blood even though they fall in the domain of the coagulation laboratory.

2.9 Common Pitfalls

The potential for problems with coagulation specimens is great. Common hazards that may lead to inaccurate test results include:

- Specimen tubes with different citrate concentrations are used within an institution.
- Specimen tubes near or beyond their expiration date do not fill adequately due to partial loss of vacuum force.
- Laboratory criteria for rejecting under- or overfilled tubes are unclear or inconsistently applied.
- New tube types are introduced without validation studies to assess their equivalence with existing tubes.
- Phlebotomy or nursing staff are not adequately trained in collection techniques.
- Specimens are collected through lines that have had heparin in them.
- Discard volumes for line collections are insufficient.
- Specimens are contaminated with other tube additives or anticoagulants through improper order of draw.
- Centrifugation protocols do not produce platelet-poor plasma with all specimens.
- Changes in centrifuge settings go unnoticed.
- Specimens intended for whole-blood testing get centrifuged.
- Incorrect specimen types, including serum, urine, non-citrated plasma, and under- or over-citrated plasma, are placed in aliquot tubes for reference lab testing.

2.10 Summary and Key Points

Coagulation testing is particularly sensitive to deviations from standard practices regarding anticoagulant concentration, container materials, collection technique, centrifugation, and storage. Some key points for the director to keep in mind are:

- Specimen integrity is vital for accurate coagulation test results. Accordingly, standard operating procedures need to be established for specimen collection and handling as well as for specimen acceptance and rejection.
- Specimen problems are commonplace and serious and, when unrecognized, lead to inaccurate or invalid test results. It is vital for laboratories to establish processes for detecting and responding to specimen problems.
- Well-founded recommendations exist and, if followed, will prevent many problems. The data behind the recommendations also provide the laboratory director with guidance for dealing with non-ideal specimens.

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Instrumentation for the Coagulation Laboratory

3

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A broad spectrum of coagulation analyzers is available for purchase on the market today. Laboratories can choose from manual, semi-automated or fully automated, moderate or high-throughput analyzers with narrow or broad assay menus depending upon clinical and test volume requirements. These laboratory-based instruments require plasma prepared from spun, anticoagulated whole blood for analysis. In addition, a number of point of care (POC) devices designed to analyze fresh whole blood are available for use on hospital patient care units, in clinics and doctors' offices, and even in patients' homes. For a current summary of marketed devices, the reader is referred to the annual College of American Pathologists summary list of laboratory-based coagulation analyzers and their attributes published on the College's web site (www.cap.org). A separate list of point of care analyzers can also be found on the site.

3.1 Laboratory Instruments

3.1.1 Test Methodologies

Coagulation test methodologies available on current laboratory analyzers vary according to the analyte or process being measured. Routine clot-based assays include the prothrombin test (PT)/International Normalized Ratio (INR), the activated partial thromboplastin test (PTT), the thrombin time (TT), the activated clotting time (ACT), coagulation factor assays (including fibrinogen), and lupus anticoagulant tests.

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Chromogenic assays, tests based on the production of a chromophore secondary to the intrinsic enzymatic activity of the analyte (e.g., antithrombin, proteins S and C, factor VIII), or the inhibition of that activity (e.g., heparin anti-factor Xa activity) are available on many instruments. Finally, immunologic-based testing is also available on some platforms (e.g., D-dimer, von Willebrand factor, protein S). Most laboratory analyzers have an open reagent system that allows use of reagents not produced by the instrument manufacturer. However, the vendor may indicate that the system is optimized for their reagents, and therefore will not guarantee results using other vendors' reagents. Regardless of the source of reagents, each individual reagent-instrument test combination must be validated due to the known variability of test results between reagents – even for different lots of the same reagent, and from analyzer to analyzer - even of the same make and model. In the U.S. market, most coagulation instruments don't provide the capability for calibration of clot-based assays.

3.1.2 Detection Methods

A key factor in assessing the clinical utility of a coagulation analyzer is a consideration of the detection modes available (Table 3.1). Detection methods in laboratory instruments can be classified into two general categories: photo-optical and electro-mechanical. Clot-based, chromogenic, immunologic and chemiluminescent assays may all utilize a photo-optical detection method, while electro-mechanical detection of clot formation is obviously limited to clot-based assays. Photo-optical detection of clot formation involves measurement of light that has emanated from a source (generally monochromatic) and has passed through, been absorbed by, or scattered from a reaction vessel containing a mixture of reagent, patient sample and fibrin clot. In the case of the PT, PTT, TT and ACT tests, the time required to reach a pre-defined, optical endpoint determines the clotting time. Any factor, other than fibrin clot, that diminishes the relative amount of light passing through the test mixture can adversely affect the result. This includes interfering colored substances (e.g., hemoglobin, bilirubin) that may absorb light at defined frequencies, or suspended particles such as lipoproteins that scatter light. The same substances may also interfere with chromogenic assays that use spectrophotometric detection, as well as immunologic methods that employ turbidimetry. Therefore, as noted in Chap. 2, lipemic, hemolyzed or icteric specimens may interfere with assays employing photo-optical detection methods.

Table 3.1 Automated laboratory coagulation analyzers

Manufacturer	Instrument	Clot detection method	Compensation for interfering substances
Instrumentation laboratories	ACL series	Photo-optical	Interrogating wavelength
Siemens healthcare diagnostics	Sysmex series	Photo-optical	Blank measurement
	BCS	Photo-optical	Blank measurement
			Second wavelength
Diagnostica Stago	STA series	Mechanical	Not applicable
	Destiny series	Mechanical or photo-optical	Mechanical mode

The simplest approach to dealing with samples containing interfering substances is to re-collect the specimen to avoid the interference (e.g., avoid hemolysis, avoid post-prandial lipemia). However, this may be either impractical (e.g., patient availability) or impossible (e.g., hyperlipidemic or hemolytic disease). Ultracentrifugation of samples to remove lipoprotein particles is a common approach to dealing with lipidemia. Use of a blank measurement to zero out the interference, measurement at a less susceptible wavelength, or measurement at multiple wavelengths are other potential approaches to modify photo-optical methods to compensate for interferences. An alternative approach is to use a mechanical clot detection system.

Mechanical detection methods employed in automated laboratory analyzers detect clot formation through changes in viscosity (movement of a metal ball), and are generally not considered to be susceptible to interference from colored solutes or suspended particles. However, it is worth noting that large concentrations (>3 g/dL) of dissolved hemoglobin, in the form of hemoglobin-based oxygen carriers, can cause artificial prolongation of clotting times when employing a mechanical-based detection method [1]. In addition, high concentrations of fibrin degradation products act to impede fibrin strand polymerization, and may result in falsely low fibrinogen measurements [2, 3].

3.2 Point of Care Instruments

Portable coagulation analyzers have been developed primarily to meet the need for whole blood monitoring of out-patient warfarin therapy (PT/INR) and moderate- to high-dose heparin anticoagulant management (ACT) during invasive procedures in the hospital setting. Over time, the manufacturers have extended the test menus of the professional instruments to encompass more of the routine coagulation assays available from the Laboratory (e.g., PTT, TT, fibrinogen, heparin). In addition, portable monitors that measure the viscoelastic properties of clot formation have been developed primarily for the management of intra-, and post-operative hemostasis. More recently, analyzers designed for platelet function analysis have entered the market and undergone clinical outcome trials.

3.2.1 Waived PT/INR Instruments

POC monitors intended primarily for warfarin management (Table 3.2) provide whole blood PT/INR testing, and are used primarily for clinic and home monitoring. PT/INR monitors employ unit-use reagent strips that contain thromboplastin reagents that are assigned an International Sensitivity Index (ISI) based on a comparison between a whole blood device and a reference plasma method. Theoretically, this indirect assignment (plasma to whole blood) could present difficulty in comparing results between plasma and whole blood tests using reagents with comparable ISI assignments. However, comparability is generally quite good when the ISI values are similar [4]. The preferred specimen type is fresh capillary whole blood, though untreated venous blood collected in a plastic syringe is also acceptable for some analyzers. These instruments detect clot formation through electrochemical (impedance) or mechanical

Table 3.2 CLIA waived PT/INR monitors

Instrument	Manufacturer	ISI	Endpoint detection	Integrated QC with analysis	Automatic download
CoaguChek XS, XS Plus, XS Pro	Roche diagnostics	1.0	Amperometric	Yes	Available
Coag-Sense	CoaguSense, Inc.	1.0	Mechanical	No	No
INRatio	Alere	1.0	Electrochemical	Yes	Available
ProTime	International Technidyne Corporation	1.0	Microcapillary flow	Yes	Yes

(cessation of movement of iron particles; change in rate of blood/reagent mixture movement) methods (Table 3.2). Patients who have tested positive for a lupus anticoagulant should not be monitored with these devices due to the potential for interference with the test. In general, patients who are being treated with heparin should also not be monitored with these devices. However, the Roche Coaguchek assays incorporate heparinase into the test and can neutralize low dose heparin therapy.

Published comparisons of INR values produced by PT monitors and laboratory coagulation analyzers on the same specimens generally demonstrate good agreement in the therapeutic range [4]. POC INR values above 3.0 often have a positive bias relative to the laboratory instrument value, and the degree and spread of disparity usually increases with increasing POC INR. This positive bias may be more common in patients who are not stably anticoagulated on warfarin (e.g., new patients, noncompliant patients) due to differential sensitivities of recombinant versus tissue-extract thromboplastins to FVII levels [5]. Some clinicians and/or laboratories may decide to confirm POC INR values by the laboratory method at values above an INR of 4.0. This approach seems prudent since the laboratory analyzer is expected to be more precise, and the POC value is generally more susceptible to sample collection error. However, published data support the safety of managing patients solely by POC INR [4, 6–8]. Nonetheless, POC coagulation analyzers should be validated against the laboratory reference method, and clinicians should be made aware of the analytical relationship between the methods, since patients may be tested by different modalities during the course of their routine care. In addition, periodic comparisons against the laboratory reference method are advisable [9].

Prior generations of waived instruments lacked the capability for automatic download of results into an information system. This posed a significant risk for transcription errors. Fortunately, most waived POC PT/INR instruments now have download capability.

3.2.2 POC Analyzers Designed for Coagulation Monitoring During Invasive Procedures

The primary clinical application of these instruments is real time monitoring of temporary heparinization during invasive procedures. They may also be used to direct fresh frozen plasma transfusion therapy during surgical procedures.

Table 3.3 Non-waived analyzers designed for monitoring during invasive procedures

Instrument	Vendor	Test menu	Endpoint detection
i-Stat®1	Abbott	PT/INR, ACT	Amperometry
Actalyte Mini II	Helena	ACT, Max ACT	Magnet rotation
Actalyke XL	Helena	ACT, Max ACT.	Magnet rotation
Cascade POC	Helena	PT/INR, APTT, ACT	Photomechanical
GEM PCL plus	Instrumentation laboratory	PT, APTT, ACT, ACT low range	Fluid movement
Hemochron response	ITC	ACT, heparin response test, protamine response test, protamine dose assay	Magnet displacement
Hemochron signature+	ITC	PT, PTT, ACT	Fluid movement
Hemochron signature elite	ITC	PT, APTT, ACT, ACT low range	Fluid movement
ACT plus	Medtronic	ACT, ACT low range, heparinase test cartridge	Plunger motion
HMS plus	Medtronic	ACT, heparin dose response, heparin protamine titration	Plunger motion

Abbreviations: *PT* prothrombin time, *APTT* activated partial thromboplastin time, *ACT* activated clotting time, *INR* international normalized ratio, *ITC* International Technidyne Corporation

Real-time heparin management is employed in a variety of settings including cardiac bypass surgery, percutaneous coronary therapeutic interventions, radiological procedures and extracorporeal membrane oxygenation. Instruments used to monitor heparin are generally less specialized than the PT monitors, frequently offering the PT, the PTT and the activated clotting time ACT tests on the same device. Fresh whole blood is the predominant sample employed by these analyzers. They detect clot formation by mechanical methods (detection of cessation of movement of fluid; detection of displacement or rotation of a magnet; measurement of resistance to movement of a plunger through the sample), and in one case, by electrochemical (amperometric) detection (Table 3.3).

POC PTT tests can be used to monitor low- to intermediate-intensity heparinization (0.2–1.0 U/mL) or to detect the presence of low levels of residual heparin after cardiovascular surgery. The POC PTT test is a one-stage test, unlike the two-stage test employed in laboratory instruments. Correlations between POC and laboratory instruments have generally been reported to be low enough that results cannot be interpreted interchangeably. This is particularly true for patients treated with heparin. Therefore, POC-specific cutoffs and ranges should be established for clinical use [4].

The primary test employed to manage heparin therapy is the ACT test, since this test, unlike the PTT, can be designed to be sensitive to a wide range of blood levels of unfractionated heparin (0.5–8.0 U/mL). Test reagents use either celite or kaolin to activate clotting, and clotting times vary depending upon the activator in the reagent. Celite-based tests generally produce longer clotting times than kaolin-based tests. Clotting times may also vary between ACT analyzers manufactured by

different (or the same) vendors depending upon the source of the activator, the formula of the activator, the amount of activator relative to the sample volume, the mixing method, the response to hypothermia and hemodilution, and the response to therapeutic drugs (e.g., aprotinin) administered to the patient [10]. Analyzers generally achieve a level of precision less than 10 % (coefficient of variation); however repeat values in individual patients can vary considerably, particularly at higher ACT values [11]. Like the PTT, ACT levels are highly variable in their correlation with heparin concentration (anti-factor Xa levels). Published correlations range from approximately r = 0.2–0.9, depending upon the device, the heparin level and the clinical indication for heparin therapy [12]. Therefore, there is no reference method that can be used to validate the ACT test. Instrument-specific protocols must be established and validated for each type of clinical procedure, and revalidated if a new ACT system is put into use [4].

3.2.3 Viscoelastic Analyzers

Analyzers that measure the viscoelastic properties of fibrin polymerization in whole blood have been developed to address the criticism that standard coagulation tests (PT, PTT, and platelet count) are artificial, non-cellular, surrogate measures of an *in vivo*, cell-based (platelet) hemostatic process. In different variations of this type of analyzer (TEG®, ROTEM®, and the Sonoclot®), blood is allowed to clot in a cup or cuvette with a pin or hollow probe suspended into the patient whole blood sample. The cup (TEG®) or pin (ROTEM®) is rotated through a narrow angle (~4°), or the probe oscillates 1.0 μM up and down (Sonoclot®), and the viscoelastic forces generated between the clot and the pin or probe are recorded as a tracing. The shapes of the tracings represent the initial formation of fibrin, the strengthening and stabilization of the clot, and in the case of the Sonoclot®, clot retraction. Clot lysis can also be recorded in hyperfibrinolytic states.

TEG® and ROTEM® analyses produce five comparable routine parameters that are derived from the tracing: R and CT reflect the latent period between initiation of the test and initial clot formation; K and CFT measure the time from initial clot formation to a defined level of clot strength; α is a measure of the rate of clot strengthening; MA and MCF are a measure of maximum amplitude of the tracing and are related to clot strength; and finally, CL30 and LY30 are a measure of clot lysis 30 min after maximum amplitude (MA). Some, but not all studies have found small but significant correlations between these five parameters and traditional measures of coagulation such as the PTT (R/CT, K/CFT and α), fibrinogen concentration (R/CT, MA/MCF and α), platelet count (MA), and clot lysis time (euglobulin lysis time) [11]. Consequently, protocols for the transfusion of platelets, cryoprecipitate and fresh frozen plasma have been developed around these tracing parameters. Though it might be expected that standard TEG® analysis, and specifically the

MA parameter, would be sensitive to changes in platelet function, TEG® has been shown to be insensitive to the effects of antiplatelet therapy [13] and unable to differentiate the *in vitro* effects of added platelets versus platelet fragments [14].

A number of modifications have been developed to standard TEG® analysis. Activators can be added to the whole blood sample to accelerate clotting (e.g., celite) or to eliminate the effect of heparin (e.g., heparinase). Addition of tissue factor dramatically shortens R/CT decreasing the test turn-around-time. Addition of reptilase and FXIII to a heparinized sample (TEG® Platelet Mapping assay) allows for monitoring of antiplatelet therapy, though two analyzers are required to perform the assay [13].

The Sonoclot® tracing yields five primary parameters: the onset time (time to initial clot formation), the R1 slope (the rate of fibrin formation), the R2 slope (reflecting further fibrin production and polymerization plus platelet-fibrin interaction and initial clot retraction), time to peak amplitude (overall rate of fibrin production), and the R3 slope (a result of clot retraction, and clot lysis in cases of hyperfibrinolysis). In the presence of sufficient clot activator, the onset time behaves like an ACT test. The coagulation tests that have been correlated with the Sonoclot® parameters are the PT (R1, R2), the PTT (ACT, R1), and the fibrinogen concentration and platelet count (R1 and peak amplitude). Like thromboelastography, Sonoclot® analysis is insensitive to the platelet-effects of aspirin, but can detect GPIIb/IIIa receptor inhibition. Gender and age have been identified as significant contributing factors to some Sonoclot® parameters [15].

3.2.4 Platelet Function Analyzers

Light transmission aggregation analysis on platelet rich plasma remains the gold standard for the evaluation of platelet function; however it is generally only available in reference laboratories. The template bleeding time has served as a surrogate evaluation of platelet function for many years. The recognition of the bleeding time's poor reproducibility, sensitivity and specificity [16] has led to the availability of several platelet-function POC analyzers (Table 3.4). As noted above, viscoelastic analyzers can be used to monitor some, but not all platelet function defects.

Table 3.4 Point of care platelet function analyzers

Device	Endpoint	High shear flow measurement
PFA-100®	Closure of membrane aperture	Yes
Plateletworks®	Platelet counts	No
VerifyNOW™	Platelet aggregation	No
TEG® PlateletMapping	Clot formation	No
Multiplate® Analyzer	Platelet aggregation	No

3.3 Platelet Aggregometry

3.3.1 Light Transmission Aggregometers (LTA)

These devices detect platelet aggregation in response to various platelet agonists (activators) by measuring turbidity (light transmission) of platelet rich plasma. This methodology is not amenable to bedside analysis and requires large volumes of blood to prepare the platelet rich plasma for analysis. Though fully capable of detecting acquired platelet function disorders, these analyzers are primarily used to detect inherited disorders of platelet function (see Chap. 6).

3.3.2 Multiplate® Analyzer

The Multiplate® is a whole blood impedance aggregometer. The disposable reaction cell detects platelet aggregation as an increase in electrical resistance as activated platelets aggregate on the sensor wires. Testing requires much smaller blood samples and obviates the need for the sample processing required for LTA. The Multiplate® can be implemented in a laboratory or at the point of care.

3.3.3 PFA-100®

The PFA-100® is the most studied of this class of analyzers. It is designed to assess platelet function under conditions of high shear. Blood is aspirated through a capillary in a test cartridge that contains a membrane coated with collagen plus epinephrine (CEPI) or collagen plus ADP (CADP). The membranes induce platelet adhesion and aggregation resulting in occlusion (closure) of the central aperture. Results are reported as closure times (CT) in seconds. The analysis requires < 1.0 mL of citrated whole blood for each cartridge tested, and pneumatic tube sample transport is not recommended due to platelet activation. Closure times are not affected by deficiencies of coagulation factors; however, the CT is highly dependent upon the interaction between von Willebrand factor (VWF) and platelet membrane GPIb and GPIIb/IIIa. Consequently, the PFA-100® lacks specificity in that it can't distinguish between a decrease in VWF levels (i.e., von Willebrand disease) and platelet function disorders. In addition, the PFA-100® is insensitive to mild forms of inherited platelet disorders and von Willebrand disease. Therefore, the International Society on Thrombosis and Haemostasis has determined that the PFA-100® has insufficient sensitivity and specificity for use in screening patients for platelet disorders [17].

Aspirin ingestion by normal individuals prolongs the CEPI CT, but not the CADP CT, presumably allowing differentiation of drug-induced platelet dysfunction from other causes. However, the CEPI CT is not consistently prolonged in patients taking aspirin for coronary or peripheral vascular disease. GPIIb/IIIa inhibitors prolong both closure times, while the two closure times are not particularly sensitive to the effects of either clopidogrel or ticlopidine [18].

Patient and pre-analytical factors that can prolong the CT include thrombocytopenia ($<80 \times 10^9$/L), anemia (hematocrit < 30 %), blood type O (due to decreased VWF levels), and collection in 3.8 % citrate versus 3.2 % citrate anticoagulant. Closure times are shortened in newborns [18, 19], and in the morning versus the evening. Flavenoid-rich foods prolong the CEPI CT. Inconsistency of results between different cartridges lots has been reported to be an issue [20].

3.3.4 Plateletworks®

The Plateletworks® assay is based upon the comparison of electronic impedance platelet counts in an EDTA anticoagulated sample versus samples containing one of two platelet agonists: collagen or ADP. The decrease in platelet count in the agonist-containing tubes is directly proportional to the degree of aggregation induced by the agonist. The test output includes a complete blood count including platelet counts, % aggregation and % inhibition. Plateletworks® collagen and ADP aggregation results correlate with standard platelet aggregometry. The analyzer is designed for evaluation of platelet function in patients undergoing cardiovascular procedures.

3.3.5 VerifyNow™

The VerifyNow™ system measures platelet-mediated aggregation of fibrinogen-coated microparticles induced by a platelet agonist. Aggregation is measured as an increase in light transmittance, and results are reported in platelet aggregation units. The test specimen is anticoagulated whole blood. The test cartridges are designed to measure the *in vivo* effect of specific drugs or drug classes on platelet function through the use of appropriate platelet agonists. Current test (agonist) cartridges available assess the platelet function effects of aspirin (arachidonic acid), GPIIb/IIIA receptor blockade (thrombin receptor activating peptide), and the thienopyridine class of drugs that includes clopidogrel (ADP plus prostaglandin E1). Analysis of a pre-drug administration sample is only required for the GPIIb/IIIa test. A limitation of these tests is a lack of evaluation of the effects on test results of platelet counts less than 100,000/μL, and the presence of inherited platelet disorders.

3.4 Summary and Key Points

There are a variety of instruments available for coagulation testing in the laboratory and at the point of care. Sensitivity to interfering substances and the levels at which interference occurs are important considerations when selecting a laboratory analyzer. POC instruments that measure the PT/INR, the PTT and the ACT have been integrated into routine patient monitoring during invasive procedures and in the clinic setting. Viscoelastic analyzers are being used in many operating rooms as the basis for transfusion protocols. The role of POC platelet function analyzers in

routine clinical practice is still under investigation. The clinical utility of the POC platelet function analyzers and the viscoelastic analyzers is discussed further in Chap. 7.

- Mechanical detection is less susceptible to commonly encountered interfering substances than photo-optical detection.
- Waived POC PT/INR monitors are suitable for use in oral anticoagulant clinics and, with careful selection of patients, for home monitoring.
- Instrument-specific ACT protocols must be established for each category of invasive procedure requiring heparinization.
- Viscoelastic analyzers are used primarily to direct intra- and post-operative transfusion practice. Their role in platelet function analysis has not yet been defined.
- POC platelet function analyzers have the potential for targeted clinical applications.

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Validation of Hemostasis Assays, Analyzers, and Reagents

4

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A key responsibility of the laboratory director is to assure that information and services provided by the laboratory meet the needs of its users and conform to established regulations and standards even before clinical specimens are tested and results are generated for patient care. The process of providing this assurance is commonly referred to as validation.

4.1 Validation Overview

In general terms, validation is the process of assessing whether something meets expectations. In a more formal sense, validation is the documented process of showing that a system meets its pre-determined specifications and attributes [1]. The need for validating assays, analyzers, and reagents in the hemostasis laboratory is obvious, yet there are no universal approaches to or standards for validation. Ultimately, it is the laboratory director who is responsible for the validity of test systems and laboratory processes. The laboratory director has much latitude and must exercise judgment in establishing validation procedures and assessing results of validation studies. This chapter provides guidelines for validation of hemostasis test systems and references for additional information.

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PT Reagent Lot Validation Checklist

Analyzer Model(s) & Serial No(s). _____

Reagent, Lot No., & Exp. Date _____

Complete	N/A	Task
_____	_____	Manufacturer-performed validation completed
_____	_____	QC tolerance limits established
_____	_____	QC limits entered and verified in analyzer(s)
_____	_____	QC limits entered and verified in LIS
_____	_____	Reference intervals established
_____	_____	Reference intervals entered and verified in analyzer(s)
_____	_____	Reference intervals entered and verified in LIS
_____	_____	Critical value limits entered and verified in LIS
_____	_____	ISI obtained from thromboplastin package insert
_____	_____	ISI entered and verified in analyzer(s)
_____	_____	Geometric MNPT calculated
_____	_____	MNPT entered and verified in analyzer(s)
_____	_____	PT/INR crossover studies performed
_____	_____	Analyzer-to-analyzer comparability studies performed
_____	_____	INR calculations verified for accuracy
_____	_____	PT/INR results
_____	_____	Standard operating procedures updated and approved
_____	_____	Testing personnel trained
_____	_____	Lab client services notified of significant changes
_____	_____	Account managers notified of significant changes
_____	_____	Clinicians/other lab clients notified of significant changes
_____	_____	Implementation date selected (Date_____)
_____	_____	Post-implementation plan prepared
_____	_____	Applicable documentation attached

Approval _____

Fig. 4.1 Example of a validation checklist

4.2 Validation Tools

Validation needs to be thorough and systematic. Each laboratory should create or obtain a set of tools to assist in this process. Checklists are effective tools for assuring that all important issues are considered [2, 3]. Checklists may also be used to document the results and the laboratory director's approval of validation studies. An example of a checklist for a hemostasis laboratory validation study is shown in Fig. 4.1.

Forms, spreadsheets, and statistical software are additional tools that help assure consistent recording and analysis of data.

4.3 Validation Studies

In the hemostasis laboratory, validation studies encompass the establishment of analytical performance expectations; calibration and calibration verification; determination of quality control ranges, reference intervals, and therapeutic ranges; crossover studies; inter-analyzer comparisons; verification of analyzer and computer set-up; evaluation of reports; verification of implementation readiness of laboratory staff and clinicians; and a "green light" from the laboratory director. This set of studies may be organized in a variety of ways and varies to some extent by the type of assay, number of components involved, and level of familiarity with the assay, analyzer, and reagents. A generic set of validation studies is presented in this section.

4.4 Validation Plan

A written validation plan should be developed and approved by the laboratory director or designee prior to the initiation of validation studies. A written plan helps assure that the extent of validation testing has been defined, expectations are clear for personnel who conduct validation studies, and the laboratory director will have the information necessary to confidently approve the implementation of the new test or method at the conclusion of validation testing.

4.5 Analyzer Set-up and Utilities

The first step in validation is to assure that the analyzer is set up, configured, and functioning according to manufacturer's specifications [4]. Most manufacturers have an installation procedure to verify performance of mechanical, electrical, fluidic, and optical components, as well as software function and configuration. Adequacy of power sources and ambient temperature control are vital and, if deficient, should be rectified before proceeding further with assay validation [4].

4.6 Calibration and Calibration Verification

Calibration and calibration verification have long been considered the domain of the chemistry laboratory. In the hemostasis laboratory, clot-based methods and platelet function assays do not involve calibration. Fibrinogen and clotting factor assays require standard curves, but not calibration in the classical sense. On the other hand, many hemostasis assays, including quantitative D-dimer, protein C, protein S, von Willebrand factor, and others, are calibrated and directly measure the concentration

or activity of an analyte using chromogenic, immunoturbidimetric, or enzyme immunoassay methods [5]. For these assays, it is important to conduct calibration and calibration verification prior to other validation studies. Indeed, proper calibration is requisite for other validation studies to be meaningful.

- Calibrate the assay.
  Calibration should be performed in accordance with the manufacturer's instructions at the frequency defined by the manufacturer or when indicated by calibration verification results or other quality metrics.
- Verify the calibration.
- Calibration verification using samples that are different from the calibrators used in the calibration step may be prescribed by manufacturers as the final step in the calibration process. More often, calibration verification is performed as an independent process to assess the stability and suitability of the existing calibration. Calibration verification is typically required at a defined frequency, usually every 6 months, or with events that pose a risk of invalidating the current calibration, *e.g.*, reagent lot changes, analyzer repairs or major service of analyzers, or that may indicate assay instability, *e.g.*, drifting QC or shift in aggregate patients' results [5, 6] Failed calibration verification may indicate the need to recalibrate, after checking for possible instrument and reagent issues.

4.7 Quality Control Ranges

Validation of quality control ranges consists of verifying that quality control range studies are appropriately conducted, control ranges (or control limits) are properly selected, and control ranges are correctly entered into the analyzers, laboratory information system (LIS), or quality control (QC) application, as applicable.

- Establish quality control ranges.
  Some guidelines for establishing quality control ranges are as follows:
  – Create a data set of control values. Ideally, obtain a minimum of 20 measurements made on separate days [7]. When this time frame is not feasible, test control material a minimum of 20 times distributed over at least 3 days. Performing measurements over a greater number of days will provide more robust control limits. It is inadvisable to generate the entire data set of control values in a single run or on a single day because the variance of the assay will be underestimated, perhaps grossly underestimated, resulting in control ranges that are unrealistically tight. It is also advisable to simulate the working environment rather than ideal conditions when conducting control range studies. For example, if a control sample may be used for 24 h after preparation, control studies should use control samples from throughout their 24-h "lifespan" rather than using only newly prepared samples. Similarly, control studies should encompass the lifespan of reagents.

- Review a run chart of values to evaluate for trends or outliers. Reviewing a histogram of results may also help highlight outliers or precision problems. If trends are obvious or there are other indications of instability in the assay, the assay should be evaluated and stabilized, then the control range study should be repeated. Caution should be exercised in classifying values as outliers. Some good working definitions of an outlier are [8]:
  1. An observation whose difference from its closest observation is more than 1/3 of the range of all observations.
  2. An observation that exceeds the 3rd quartile (75th percentile) by more than 1.5 times the interquartile range (*i.e.*, the difference between the 75th and 25th percentiles) or falls below the 1st quartile by more than 1.5 times the interquartile range.
- Calculate the mean and standard deviation for each control, and compare these statistics with the manufacturer's information, if any. The mean and standard deviations should also be compared with historical values. Substantial deviations from target means or excessive imprecision may indicate problems with the analyzer, reagents, control materials, or testing procedures, and should be evaluated.
- Select control limits. Parametric methods are usually employed. For example, the control limits are often set at the mean plus or minus 2 standard deviations [9]. Other multiples of the standard deviation may be suitable based on the precision of the assay and clinical tolerance for bias and variance in test results [9]. When fewer than 40 results are used to establish a control range, it is a good idea to recalculate control limits later with a larger number of results.
- Establish a QC plan that includes frequency and decision rules, based on applicable regulations, accreditation standards, performance characteristics of the assay, number of tests performed by the laboratory, medical applications of the test, and clinical impact of an erroneous result [10, 11].

  See Fig. 4.2 for a sample report for evaluating control range studies and selecting control ranges.

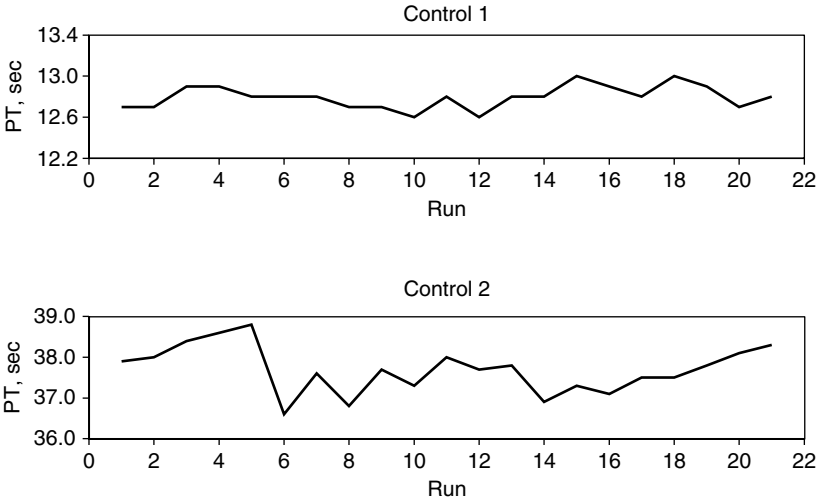
  A rigorous discussion of quality control may be found in Clinical and Laboratory Standards Institute (CLSI) documents C24-A3 and EP23-A [7, 10].

- Verify that control ranges and decision rules are correctly entered into the analyzers, LIS, and QC application.

  When analyzers have QC capabilities, laboratories may find it convenient to use the analyzers to alert testing personnel of out-of-range results, document corrective actions, prepare run charts, and calculate summary statistics. Many laboratories use the LIS for QC management to consolidate QC from throughout the laboratory and to implement more complex decision rules. Some laboratories use stand-alone applications for QC, either in place of or as a supplement to the LIS. It is not uncommon for QC to be managed with a combination of applications. It is vital that control ranges be entered correctly and consistently in all applications used.

PT Control Ranges Study
April 2014

Method: Analyzer, Thromboplastin, Lot No. 55555, Exp. 5/2015
Control 1: Normal Control, Lot No. 11111, Exp. 2/2015
Control 2: Abnormal Control, Lot No. 22222, Exp. 2/2015



Control	Mean	SD	CV	N	Control Range
Control 1	12.80	0.11	0.9 %	21	12.6–13.0
Control 2	37.70	0.58	1.5 %	21	36.5–38.9

Approval _____

Fig. 4.2 Example of a report for evaluating quality control results and assigning control ranges

4.8 Reference Intervals

Reference intervals are the principal means by which clinicians interpret test results. Valid reference intervals help assure appropriate triage, therapy, and additional diagnostic studies. Inappropriate reference intervals have been identified as a source of misguided therapy and over-utilization of laboratory tests and other medical services [12–14]. Validation of reference intervals consists of verifying that reference interval studies are appropriately conducted, reference intervals are properly selected, and reference intervals are correctly entered into the analyzers and LIS.

- Establish reference intervals.

  Several important steps are required to establish valid reference intervals for hemostasis tests. Here are some guidelines:

  – Select an equal number of female and male reference subjects. Some laboratories also obtain an equal number of subjects above and below age 45. Full statistical validity may require 120 subjects or more [8], but a practical minimum number is 20 subjects [9]. Using 40 or more subjects provides a higher level of confidence in the reference intervals. Laboratories that are unable to obtain a satisfactory number of reference subjects may find it valuable to obtain reference specimens from another laboratory, although a dose of caution is in order for tests such as the PTT that may be unreliable with frozen specimens unless they are prepared and handled meticulously.
  – The reference population is presumed to be those individuals with "normal" hemostasis. Because of relatively high prevalence of acquired defects, including prescription and non-prescription medications that affect components of the hemostatic system, use of exclusion criteria with potential reference subjects is critical. Exclusion criteria should include a history of bleeding or clotting disorders, systemic inflammatory conditions, pregnancy, hormonal contraceptives (oral, implant, or patch), and acute illness. Medications should be closely scrutinized for potential effects or, alternatively, exclusion criteria may include the use of any medication. Vigorous exercise in the preceding 18–24 h is also grounds for exclusion [9].
  – Review a histogram of the reference values (i.e., test results from the reference subjects). Be cautious about classifying values as outliers, as discussed above.
  – Calculate the mean and standard deviation of the reference values, and calculate the 2.5th and 97.5th percentiles. Compare these statistics with historical values, published reference intervals, and information from the manufacturer. Significant discrepancies from comparable methods and populations should provoke an evaluation of the reference subjects, specimen collection and handling procedures, and assay.
  – Select reference intervals. Either parametric or non-parametric methods may be used, depending on the distribution of test results from the reference subjects.

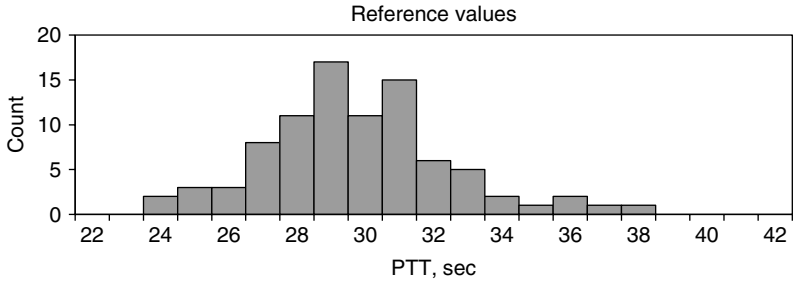
    See Fig. 4.3 for a sample report for evaluating reference intervals.

    An approach for establishing reference intervals that may be overlooked is that of transference. Transference involves adopting or "transferring" reference intervals from another laboratory's or manufacturer's reference interval study when the analytical systems and test subject populations are sufficiently comparable [8]. Transference is particularly valuable for small laboratories that have difficulty finding an adequate number of reference subjects or mobilizing resources for an extensive study. Transference enables a laboratory to perform a limited validation study to confirm the suitability of the candidate reference intervals.

    An excellent discussion of reference intervals may be found in CLSI document EP28-A3C [8].

PTT Reference Interval Study
April 2014

Method: Analyzer, Reagent, Lot No. 666666, Exp. 5/2015



Mean	SD	2.5th %ile	97.5th %ile	N	Parametric interval	Non-parametric interval
29.3	2.7	24.6	36.0	88	23.9–34.7	24.6–36.0

Approval

Fig. 4.3 Example of a report for establishing reference intervals

- Verify that reference intervals are correct in analyzers and LIS.

  Some laboratories find it useful to have the analyzer display reference intervals, when the analyzer has this capability. The analyzer may then be used to flag abnormal results and display reference intervals on analyzer-generated reports. The LIS provides reference intervals on reports and may transmit reference intervals to other interfaced information systems. If interfaced information systems maintain their own reference interval tables, then these tables also need to be verified. It is critical that reference intervals be entered correctly and consistently in the analyzers, LIS, and other information systems, as applicable.

4.9 Performance Characteristics

Knowing the performance characteristics of an assay is good laboratory practice and, in some countries, required by regulation. Performance characteristics include analytical accuracy and precision, interferences, analytical sensitivity and measurement range, and methodological limitations or issues such as carryover.

- Verify accuracy.

  Accuracy is vital for test results to meet clinical requirements. Detailed procedures have been developed for accuracy verification [15–18], but for most

purposes the combination of calibration verification and analytical measurement range verification will provide laboratories with sufficient evidence of accuracy.

- Determine precision.

  Information about precision helps laboratories assess whether differences in replicate results on a specimen indicate problems with the assay. Information about precision helps clinicians assess whether differences in test results on a given patient indicate a change in the patient's status. Detailed methods are available for determination of precision [17–19], but for most purposes, laboratories can gain adequate information about precision from manufacturer's information and quality control statistics.

- Determine interferences.

  Determining interferences with an assay is important to avoid spurious results, particularly when the interferences are not detected by the assay system and a result is produced. For most purposes, laboratories can adequately determine interferences from the medical literature and manufacturer's information.

- Verify the analytical measurement range.

  Analytical measurement range verification is required to establish the range over which analyses are acceptably accurate. For some assays, including the prothrombin time (PT), International Normalized Ratio (INR), partial thromboplastin time (PTT), activated clotting time (ACT), and thrombin time (TT), the concept of analytical measurement range is not applicable because there is no standard of "trueness." However, for many other coagulation tests, standards do exist and the analytical measurement range needs to be verified [5].

  Here are some guidelines for analytical measurement range verification:

  – Obtain standards of a suitable matrix with target values or assayed values that cover the range of values over which testing is desired.
  – Test each standard in duplicate and calculate the average of each set of duplicates. A higher number of replicates is useful for imprecise assays.
  – Calculate the absolute and relative (percent) deviations of the averages from the target values.
  – Compare the deviations with defined tolerance limits. Tolerance limits may be defined by the manufacturer, regulations, or published data. In their absence, tolerance limits may be defined by the judgment of the laboratory director.
  – The analytical measurement range is verified at and between those levels at which the deviations are within the tolerance limits.

  It is generally considered that if an assay has a theoretical measurement range beyond that for which standards are available, the working analytical measurement range should be limited to that covered by the standards.

- Determine reagent stability.

  All reagents degrade over time, potentially leading at some point to incorrect test results. In many cases, laboratories may safely use manufacturer's information or published studies to determine reagent stability. When information does not exist or is not credible, it is incumbent on the laboratory to determine the period of reagent stability.

Here are some guidelines for one approach:

- Prepare fresh reagent and place on the analyzer.
- Prepare and test fresh controls in duplicate initially and at defined intervals. The chosen interval is influenced by the expected stability and intended use of the reagent. For example, if a reagent is expected to be stable and intended to be used over a period of, say, 12 h, then a suitable interval for testing fresh controls might be 1 or 2 h. If a reagent is expected to be stable and intended to be used over a period of several days, then an interval of 8 or 12 h might be chosen.
- Prepare a run chart of the averages of duplicates.
- The maximum stability is defined by the last time point before any value exceeds control limits or a clear and significant drift in results is evident.
- Repeat the above steps with several preparations of fresh reagent. Depending on the analyzer, it may be possible to evaluate multiple preparations simultaneously.

• Determine specimen stability.

As with reagents, specimens also degrade over time, leading at some point to incorrect test results. In most cases, laboratories may safely use manufacturer's information or published studies to determine specimen stability. When information does not exist or is not credible, or the laboratory wishes to extend the period during which specimens may be tested, it is incumbent on the laboratory to determine the stability period.

Here are some guidelines for one approach:

- Sequentially or randomly select patients' specimens after completion of ordered testing. Hold specimens under defined storage conditions (*e.g.*, ambient or refrigerated).
- Retest at defined intervals.
- Assess the change in results as a function of time from collection.
- The maximum stability is defined by the last time point before any value exceeds defined limits or a clear and significant drift in results is evident.

• Determine clotting factor sensitivities.

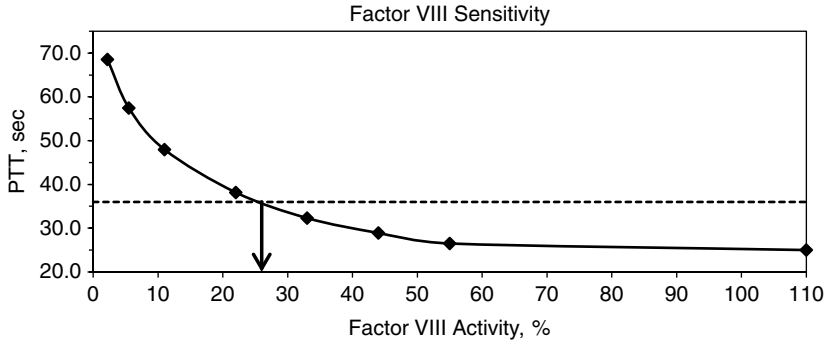
To meet clinical requirements, it is important to know the sensitivity of PT and PTT assays to various factor deficiencies. Often, laboratories can gain adequate information about factor sensitivity from manufacturer's information and published studies. When information does not exist or is suspect in some regard, it may be necessary for a laboratory to determine the sensitivity of an assay to one or more clotting factor deficiencies.

Here are some guidelines for one approach [9]:

- Prepare a set of specimens with a range of factor levels by mixing different ratios of factor-deficient plasma and normal plasma. Normal plasmas with assayed factor levels are commercially available and readily suited for this type of study. A suitable set of test specimens will have target factor concentrations of 100, 50, 40, 30, 20, 10, 5, and 2.5 %. The actual values may vary from these targets if the factor concentration in the normal plasma is not exactly 100 %.
- Test each specimen in duplicate.

PTT Factor VIII Sensitivity Study
April 2014

Method: Analyzer, Reagent, Lot No. 666666, Exp. 5/2015



F.VIII %	110.0	55.0	44.0	33.0	22.0	11.0	5.5	2.2
PTT 1	25.0	26.6	28.6	32.2	38.3	48.4	57.8	68.8
PTT 2	25.0	26.4	29.2	32.4	38.0	47.5	57.1	68.3
Mean	25.0	26.5	28.9	32.3	38.2	48.0	57.5	68.6

Reference Interval

Upper Limit, sec	Sensitivity
36.0	26 %

Approval _____

Fig. 4.4 Example of a report for factor sensitivity determination

  - Prepare a scatter plot of test results as a function of predicted factor concentration and fit a smooth curve to the data points.
  - Identify the factor concentration at which the result exceeds the upper limit of the reference interval. This is the factor sensitivity of the assay.

  See Fig. 4.4 for a sample report for identifying the factor sensitivity of an assay from this type of study.
- Determine lupus anticoagulant sensitivity.

  PTT reagents differ in their sensitivity to lupus anticoagulants. No consensus exists about the appropriate level of sensitivity; some laboratory directors favor insensitive reagents while others prefer more sensitive reagents. In laboratories that perform lupus anticoagulant testing, it is feasible to store frozen plasma aliquots from patients with lupus anticoagulants. Specimens with a range of strengths of lupus anticoagulant should be tested with new PTT reagents or reagent lots [20]. It is particularly important to include specimens with weak lupus anticoagulants to detect changes in sensitivity. Laboratories without ready access to lupus anticoagulant specimens may be able to obtain information from manufacturers about relative sensitivities.

- Determine heparin sensitivity.

  The importance of laboratory monitoring in heparin therapy requires that laboratories assess heparin sensitivity of new PTT reagents and reagent lots. This may be achieved by comparison with existing reagents or lots or by comparison with an anti-Xa assay [5]. Refer to the Unfractionated Heparin section in Chap. 10 for a detailed discussion.
- Carryover determined

  Many analyzers use a single probe for aspirating all specimens. Carryover from one specimen to another may produce inaccurate test results, so it is important to verify that significant carryover does not occur. Carryover of heparin neutralizers from PT reagents may artificially shorten PTT values in specimens containing heparin. Carryover of heparin may artificially prolong PT, PTT, or TT results.

  Here are some guidelines for one approach to assessing carryover:
  - Establish a baseline PTT or TT on a normal specimen. The TT is considerably more sensitive to heparin and will detect low levels of carryover.
  - Test a specimen spiked with a high concentration of heparin.
  - Again test the normal specimen and evaluate for prolongation of the result compared to the baseline.

  A more rigorous procedure has been described by Adcock and colleagues [20].

The procedures outlined in this section are suitable for and can be performed in most settings. Rigorous statistical methods for determining performance characteristics may be found in CLSI documents EP05-A2, EP09-A32, EP10-A3, EP15-A2, and EP21-A [15–19].

4.10 Method Comparisons

Before a laboratory implements a methodological change to an assay, it is important to assess how the change will affect patients' results. A methodological change may be as minor as changing a reagent lot or as major as bringing up a different manufacturer's analyzer and reagent. In either case, there is a potential for patients' results to change. Method-comparison studies help assess the potential for clinical impact from the change.

- Perform a crossover study between the new and current methods.

  A crossover study, comparing the new method with the current method, will help answer the question of whether clinicians will see a change and, if so, the magnitude of the change.

  Here are guidelines for crossover studies:
  - Test 40 or more patients' specimens over several days by the current and new methods. Select specimens that cover the range of results typical for the laboratory. A smaller number of specimens may be acceptable if the test results are approximately uniformly spread over a broad range.

- Review scatter plots of results. Be cautious about classifying values as outliers.
- Calculate the correlation coefficient of the pairs of results. Most spreadsheet programs have built-in functions for calculating correlation coefficients.
- Calculate the orthogonal regression line. Orthogonal regression is preferred over simple least squares regression because it assumes random error is present in the results of both methods. See Fig. 4.5 for an illustration of orthogonal regression. Unfortunately, most spreadsheet programs do not have built-in functions for orthogonal regression, but the calculations are relatively straightforward. The slope of the orthogonal regression line is calculated as follows: Given two data sets $\{x_1, x_2, x_3, \ldots, x_n\}$ and $\{y_1, y_2, y_3, \ldots y_n\}$, $slope = u + \sqrt{u^2 + 1}$, where $u = \dfrac{\sum (y_i - \bar{y})^2 - \sum (x_i - \bar{x})^2}{2\sum (x_i - \bar{x})(y_i - \bar{y})}$ and $intercept = \bar{y} - slope * \bar{x}$.
- In addition, most spreadsheet programs contain functions that can be used to simplify the orthogonal regression calculations. For example, in Microsoft Excel (Microsoft Corp., Redmond, WA) the value of u in the equation above can be calculated using the following formula:

$$= \left(\mathrm{VARP}(\mathrm{Y}) - \mathrm{VARP}(\mathrm{X})\right) / \left(2 * \mathrm{COVAR}(\mathrm{Y}, \mathrm{X})\right)$$

where X and Y are spreadsheet ranges.

Passing-Bablok regression is an acceptable alternative to determine slope and intercept [21].

- Evaluate the results for systematic shifts. Difference plots, also known as Bland-Altman plots, provide better visual representation of bias than scatter plots [22].
- Evaluate the results for concordance of clinical classifications relative to the reference intervals. This will provide an early warning if clinicians are likely to see a substantive change in the frequency of abnormal results. In addition, low concordance may signal the need for a reassessment of reference interval limits.

Ideally, there will be no significant shifts in results or changes in clinical classifications, so the change is invisible to clinicians. However, if there are changes, information from crossover studies is invaluable to clinicians as the laboratory implements the new method.

See Fig. 4.6 for a sample report of a crossover study.

- Perform a comparison of the secondary analyzer(s) versus the primary analyzer.

A second type of method comparison is between analyzers in the same laboratory to assure that results with the new method will be equivalent between analyzers. If the results are not equivalent, the analyzer comparison studies provide information to guide the implementation of steps to prevent a negative impact to patient care.

- Follow all the steps of the method comparison study described above.
- Assess whether results are sufficiently equivalent. No universal criteria exist for accepting results as equivalent. Criteria may vary with the analytical capabilities

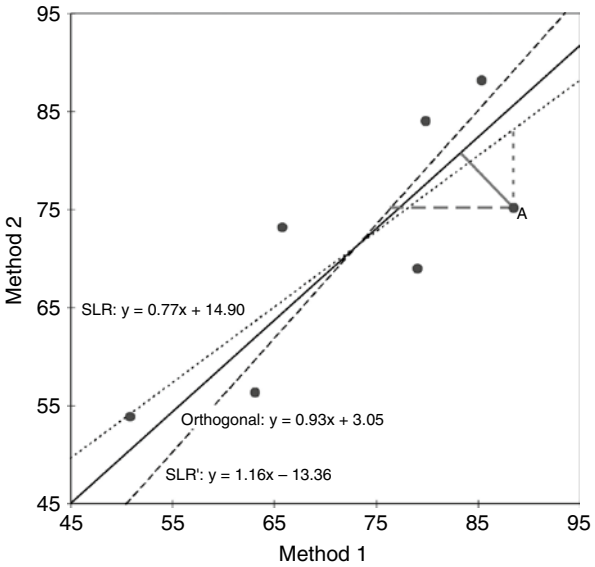


Fig. 4.5 Illustration of orthogonal regression versus simple least squares (SLR) regression. The SLR line (– – – –) is calculated under the assumption that the x-values are known without error and the y-values contain random error. The alternate SLR line, SLR' (— — —), is calculated under the assumption that the x-values contain random error but the y-values do not. The orthogonal regression line (———) is calculated under the assumption that both the x- and y-values contain random error. The line segments from point A illustrate that SLR minimizes the sum of the squared vertical distances between the data points and regression line, SLR' minimizes the sum of the squared horizontal distances between the data points and regression line, and orthogonal regression minimizes the sum of the squared perpendicular (or orthogonal) distances between the data points and the regression line. Note that orthogonal regression yields a line between the SLR and SLR' lines

of the analyzers, clinical tolerance for differences, and preferences of the laboratory director. Thresholds of 3, 5, 10, and 15 % are often used in practice.

– If results are not equivalent but are highly correlated, use the regression equation to "adjust" the secondary analyzer's results, then re-evaluate the comparison. This step may be used to assess whether the secondary analyzer's results should be adjusted mathematically to make them equivalent to the primary method's results.

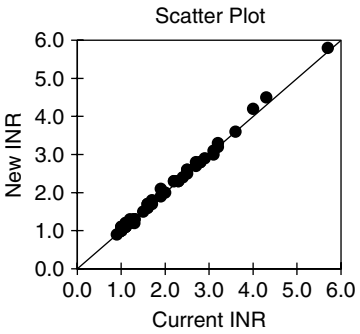
See Fig. 4.7 for a sample report of a study comparing a secondary analyzer with a primary analyzer.

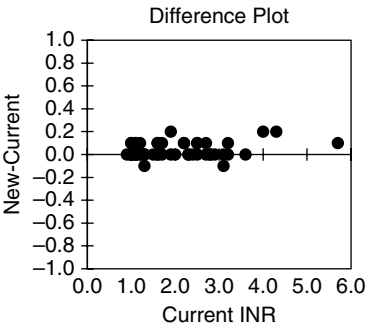
4.11 INR

The INR standardizes PT values for warfarin anticoagulation therapeutic monitoring. The INR is calculated from the PT using the thromboplastin's ISI (international sensitivity index) and the laboratory's MNPT (mean normal prothrombin time).

INR Crossover Study
April 2014

Current: Thromboplastin, Lot 55554, Exp. 5/2014, ISI=1.32, MNPT=13.1
New: Thromboplastin, Lot 55555, Exp. 5/2015, ISI=1.25, MNPT=13.5





Correlation (r^2)	Slope	Intercept	N
0.997	0.998	0.037	52

Current Class.	New Classification		
	Low	Therapeutic	High
Low	28	1	0
Ther.	0	14	0
High	0	1	8

Agreement = 96.2 %

	Predicted	
Current	New	Difference
1.00	1.04	3.5 %
2.00	2.03	1.6 %
3.00	3.03	1.0 %
4.00	4.03	0.7 %
5.00	5.03	0.5 %
6.00	6.02	0.4 %

Acceptance Criteria _____

Approval _____

Fig. 4.6 Example of a report for method comparison studies comparing a new reagent lot with a current lot

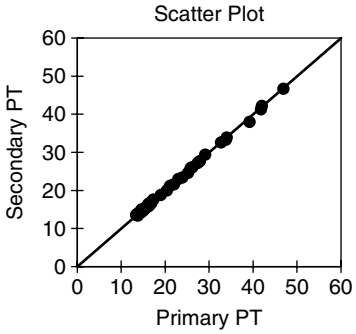
Refer to Chap. 10, for a more detailed discussion of the INR. Validation of the INR involves verifying that the correct ISI and MNPT values are used and that calculations are performed correctly.

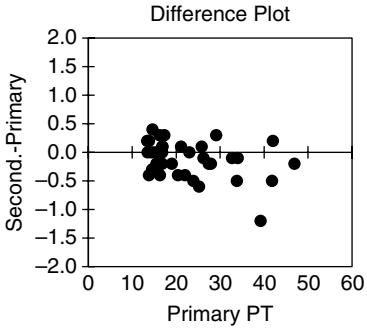
- Verify that the ISI and MNPT values are correct in the analyzers and LIS.

  Some laboratories use the coagulation analyzer to perform the INR calculations, others use the LIS to do the calculations, and some use both. Advantages of using the analyzer for INR calculations are that INRs are

PT Interanalyzer Comparison Study
April 2014

Primary: Analyzer 1
Secondary: Analyzer 2
Reagent: Thromboplastin, Lot 55555, Exp. 5/2015, ISI=1.25, MNPT=13.5





Correlation (r^2)	Slope	Intercept	N
0.999	0.990	0.078	40

Primary PT	Secondary PT Low	Normal	High
Low	0	0	0
Normal	0	5	0
High	0	1	34

Agreement = 97.5 %

Predicted Primary	Second.	Difference
13.0	13.0	–0.4 %
15.0	14.9	–0.5 %
20.0	19.9	–0.6 %
30.0	29.8	–0.7 %
50.0	49.6	–0.8 %
75.0	74.3	–0.9 %
100.0	99.1	–0.9 %

Acceptance Criteria _____

Approval _____

Fig. 4.7 Example of a report for method comparison studies between primary and secondary analyzers

viewable on the analyzer at the time of analysis and INRs continue to be calculated when the LIS is down. An advantage of using the LIS to do the calculations is that the ISI and MNPT may need to be entered only once for multiple analyzers. The ISI and MNPT values must be verified in the analyzers and LIS, as applicable.

- Verify that INR values are calculated correctly by the analyzers and LIS.

INR Calculation Verification

Analyzer Serial No. _____

Reagent, Lot, & Exp. _____

ISI and MNPT _____

Result	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
PT					
Expected INR					
Analyzer INR					
LIS INR					

Acceptance Criteria _____

Approval _____

Fig. 4.8 Example of a form for documenting verification of INR calculations

Even if the ISI and MNPT are input correctly, it is possible for the calculations to be set up incorrectly. It is critical that calculations made by the analyzers and LIS be checked against manual or spreadsheet calculations over a range of values.

Fig. 4.8 shows a sample form for documenting the verification of calculations.

Another aspect of INR validation is the verification of its accuracy [23]. Most laboratories do not have access to reference thromboplastins or reference test methods to verify INR accuracy, so the recommended approach is to test INR certified plasmas, if available for the analyzer, reagent, and locale. A minimum of three INR certified plasmas spanning the INR therapeutic range should be used [24]. INR certified plasmas may be intended for use with a specific manufacturer's analyzers and reagents or on multiple manufacturers' platforms. Follow the INR certified plasmas' manufacturer's instructions regarding preparation, handling, testing, data analysis, and acceptability criteria. When INR certified plasmas are not an option, laboratories should at least verify the comparability of INR values with previous methods or other laboratories. Refer to the Method Comparisons section of this chapter for additional discussion.

4.12 Heparin Therapeutic Ranges

Laboratory monitoring plays an important role in the efficacy and safety of unfractionated heparin (UFH) therapy. The PTT assay is used in virtually every laboratory that supports facilities administering UFH therapy. The anti-Xa UFH assay is also available in many laboratories. Laboratory monitoring of low-molecular-weight heparin (LMWH) therapy with the anti-Xa LMWH assay is important for selected patient groups.

- Verify that the standard curve for the anti-Xa UFH assay has been established. Refer to Chap. 10 for more information about the anti-Xa assay.
- Verify that the PTT therapeutic range has been established for UFH therapeutic monitoring.
- The therapeutic range for UFH needs to be established for each PTT reagent/analyzer combination used in the laboratory. With each reagent lot change, the range needs to be verified or re-established. Refer to the Unfractionated Heparin section in Chap. 10 for a more detailed discussion.
- Verify that the standard curve for the anti-Xa LWMH assay has been established.
- Depending on the manufacturer, distinct sets of samples are needed to establish the LMWH and UFH curves or a single set of samples may be used. Refer to Chap. 10 for additional discussion.

4.13 Instrument Interfaces

When in place, instrument interfaces form the communication link between the LIS and the analyzers. The LIS transmits orders and patient information to the analyzer; the analyzer transmits results back to the LIS. If the interface is not functioning properly, orders may be missed or results may go unreported.

- Verify that instrument interfaces are working as expected.

  Verification of instrument interfaces involves ordering tests on the LIS, tracing these orders to the analyzer, running the tests on the analyzer, and then tracing results back to the LIS. It is wise to challenge both arms of the interface. The ordering arm should be challenged by sending a variety of order types, for example, PT only, PT and PTT, fibrinogen only, fibrinogen and D-dimer, etc. The resulting arm should be challenged with normal and abnormal results, results with a > or < sign, and so forth.

4.14 Reports

Laboratories exist essentially to provide information, so reports are the laboratory's product. Reports may be issued in a number of ways, including by electronic display, print, or fax. Validation of reports involves not only test values but also

reference intervals, high/low or abnormal flags, comments, and any other information provided in reports that may influence the interpretation of test values.

- Verify that results display correctly in the LIS and interfaced information systems.
  Electronic display is the primary means for the conveyance of laboratory results in many institutions. The displays on interfaced systems need to be carefully reviewed for their handling of reference intervals, > and < signs, comments, high/low flags, and modified results. When mappings of patient identification numbers or test codes are involved, these also need to be validated.
- Verify that results display correctly on reports printed from the LIS.
  Many LISs provide a variety of report options and formats. Each type of report that is used should be validated.
- Verify that results display correctly on reports faxed from the LIS.
  Some LISs have the capability of directly generating faxes, rather than requiring faxing from a printed report. The output of these faxes should be validated for content and readability.
- Verify that results display correctly on reports printed from the analyzer(s).
  Analyzers may be used to generate reports when the LIS is down or in laboratories without an LIS. The displays on interfaced systems need to be carefully reviewed for their handling of reference intervals, > and < signs, comments, high/low flags, and modified results.
- Verify that therapeutic guidelines display correctly on all reports.
  Many laboratories include target therapeutic ranges or other guidelines for warfarin and heparin therapy on reports of PT/INR, PTT, and anti-Xa tests [5]. The presentation of this information should be reviewed for all report types and modalities.

4.15 Implementation Readiness

Once an assay is validated, several additional activities need to occur before it is implemented.

- Validate standard operating procedures.
  Methodological changes often require modifications to standard operating procedures (SOPs). For example, reference intervals and therapeutic ranges may need to be updated. For major changes, SOPs may need to be largely or entirely rewritten. Each modified SOP needs to undergo a technical validation to assure that following the SOP produces the desired result. SOP changes need to be approved prior to implementation of the assay changes.
- Notify and train lab personnel.
  Laboratory personnel are the key to a smooth change. They should be notified of the upcoming change far enough in advance to prepare for the change. Preparation may involve only becoming aware of minor changes or, for major changes, may involve significant hands-on training. In the validation phase, the important point is to verify that lab personnel are ready to implement the change.

- Notify clinicians.

  Clinician notification is vital for any changes that may alter the interpretation or use of results, such as new reference intervals, a systematic shift in results, a change in heparin sensitivity or therapeutic ranges, changes in factor sensitivity, or changes in lupus anticoagulant sensitivity. Laboratory personnel may not be aware of existing clinical protocols or decision support routines that use specified laboratory values as targets or triggers, such as heparin dosing algorithms. Clinicians should be notified far enough in advance to have time to update protocols prior to the laboratory's implementing a change.

  Fig. 4.9 shows an example of a clinician notification letter.

To: <<Facility>> Physicians and Nurse Managers

From: <<Pathologist, Lab Manager, or Coagulation Supervisor>>
Clinical Laboratory

Date: <<Date>>

Subject: **PT and PTT Coagulation Testing Changes**, Effective <<Starting Date>>

Please note the following information and share it with your staff.

On **<<Starting Date>>**, the Clinical Laboratory will be changing analyzers and reagents for prothrombin time (PT) and activated partial thromboplastin time (PTT) testing. Our studies comparing the new test methods with the current test methods show the following:

Reference Ranges
The reference ranges will change for both the PT and PTT.

Test	New Reference Range	Old Reference Range
PT	nn.n – nn.n seconds	nn.n – nn.n seconds
PTT	nn – nn seconds	nn – nn seconds

Therapeutic Ranges
Therapeutic ranges for the INR (for monitoring coumadin therapy) will not change. PTT values correspond to unfractionated heparin concentrations of 0.3 and 0.7 U/mL anti-Xa activity.

Test	To Monitor	Therapeutic Ranges
INR (PT)	Warfarin	2.0 – 3.0 (moderate intensity) 2.5 – 3.5 (high intensity) Individual patients may require higher or lower ranges.
PTT	Unfractionated Heparin	nn – nn seconds

Comparison of Results
With the new methods, testing for most patients is expected to show the following trends (individual patients may show larger or smaller differences):

- PT results will ….
- INR results will ….
- PTT results will ….

If you have questions or comments about these changes, please contact <<local contact>>.

Fig. 4.9 An example of a clinician notification letter

4.16 Approval

Before implementation of a change, validation studies and documents need to be reviewed and approved by the laboratory director or designee.

- Obtain approval of the laboratory director or designee.
  Some laboratory directors like to see only summary data and others prefer more detail. Validation processes and documentation may be tailored to the laboratory director's preferences, but the laboratory director retains accountability for the completeness and suitability of the validation process.

4.17 "Full" Versus "Partial" Validations

The scope of validation testing depends on the scope and potential ramifications of the change [5]. Implementing a completely new system obviously requires more extensive validation than changing a reagent lot. It is wholly appropriate for the laboratory director to exercise judgment in setting the scope of the validation. For example, in a reagent lot change, if crossover studies do not show any significant differences in results, it may not be necessary to conduct reference interval studies, therapeutic range studies, or heparin sensitivity studies.

4.18 Other Considerations

Introduction of a new assay or modification of an existing assay may have other ramifications than those discussed in this chapter [20, 25]. For example, proficiency testing orders may need to be altered, client manuals may need to be updated, or regulatory agencies may need to be notified. These items may be added to the laboratory's validation checklist or handled another way deemed appropriate by the laboratory director.

4.19 Common Pitfalls

Although the concept of validation is intrinsically understood, in practice it may be difficult to remember everything that needs to be done unless a systematic approach is developed and followed. Some of the more common problems with validation are:

- One of more of the validation studies are overlooked and not performed.
- Validation studies are inadequately designed.
- Calculations are not validated.
- Information system validation is not performed.
- Changes are not communicated effectively to clinicians.

4.20 Summary and Key Points

Validation is the process of showing that a system is stable and performs according to expectations. In the hemostasis laboratory, validation encompasses establishing analytical performance expectations, determining limits for quality control, reference intervals, and therapeutic monitoring, assessing the clinical effects of methodological changes, verifying the setup of computer systems and analyzers, verifying the content and readability of reports, and assuring readiness for implementation. Some key aspects of this process are:

- Validation is critical to assure intended results.
- "Validation" means different things to different people. Defined procedures and analytical tools help assure the director's definition of validation is met.
- Checklists help "error-proof" the validation process.

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Hemostasis Screening Assays

5

George M. Rodgers and Christopher M. Lehman

5.1 Hemostasis Screening Assays: Use and Interpretation

The cornerstone assays of coagulation testing are the prothrombin time (PT) and partial thromboplastin time (PTT). When combined with results of the platelet count obtained from the complete blood count (CBC), differential diagnoses can be generated to assist in the evaluation of patients with bleeding disorders. This chapter will summarize methodologies for the PT and PTT assays, as well as other clinically useful tests that many hospital laboratories provide. An approach to diagnosing bleeding disorders using results of the PT and PTT assays (and the platelet count) will also be presented. Lastly, limitations of these assays will be discussed in the context of evaluating patients for bleeding. All assays discussed in this chapter have FDA approval.

5.2 The CBC

The CBC is discussed in this chapter because the platelet count component of the CBC is a useful parameter in the evaluation of hemostasis. Details of platelet count testing are discussed in Chap. 7. Typically, EDTA-anticoagulated blood is obtained

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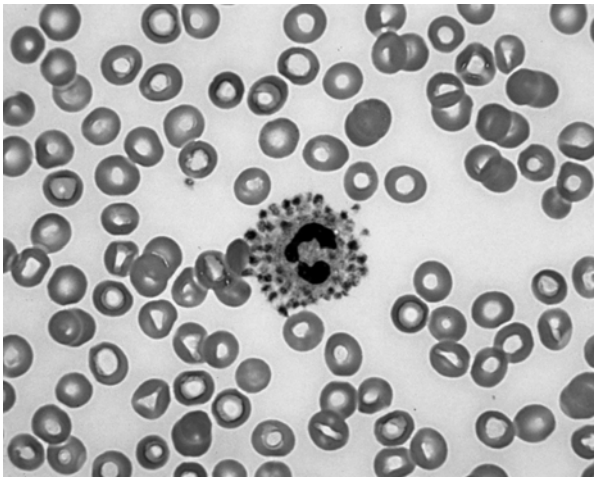

Fig. 5.1 Platelet satellitism and platelet clumping in a blood smear from a patient with EDTA-pseudothrombocytopenia (Courtesy Sherrie L. Perkins, MD, University of Utah Medical Center [Wright's stain, oil immersion]) (From Rodgers [1], with permission)

for analysis in an automated particle counter. The reported platelet count is usually quite precise (CV ~5 %). In asymptomatic patients in whom thrombocytopenia is reported, the possibility of pseudothrombocytopenia or EDTA-induced thrombocytopenia should be considered, especially in patients without a history of bleeding. This phenomenon occurs in 0.1–1 % of normal people; it results from EDTA modifying platelet membrane proteins which then react with preexisting antibodies present in patient blood that recognize the modified platelet proteins, producing platelet clumping or satellitism (Fig. 5.1). It should be routine laboratory policy for technical personnel to review peripheral blood smears of patients with newly diagnosed thrombocytopenia to determine whether the thrombocytopenia is true or spurious. If EDTA-induced thrombocytopenia is suspected, the CBC should be repeated using blood collected in a citrate or Acid-Citrate-Dextrose collection tube (see Chap. 7). Figure 5.2 illustrates an algorithm that suggests one strategy to evaluate thrombocytopenia.

In terms of hemostasis evaluation, one limitation of the CBC is that even though it is usually a reliable indicator of the platelet count, the CBC does not measure platelet function. The bleeding time test was originally thought to perform this function, but as discussed below, is not uniformly reliable in assessing platelet function.

5.3 The PT Assay

The PT assay has two purposes: to screen for inherited or acquired deficiencies in the extrinsic and common pathways of coagulation (Fig. 5.3) and to monitor oral anticoagulant therapy. The PT is affected by decreased levels of fibrinogen, prothrombin, factors V, VII, or X. Since 3 of the 5 coagulation factors measured by the PT are vitamin K-dependent proteins (prothrombin, factors VII and X), the PT assay is useful in detecting vitamin K deficiency from any cause including liver disease, malnutrition, or warfarin therapy. The PT does not measure factor XIII activity or components of the intrinsic pathway [3].

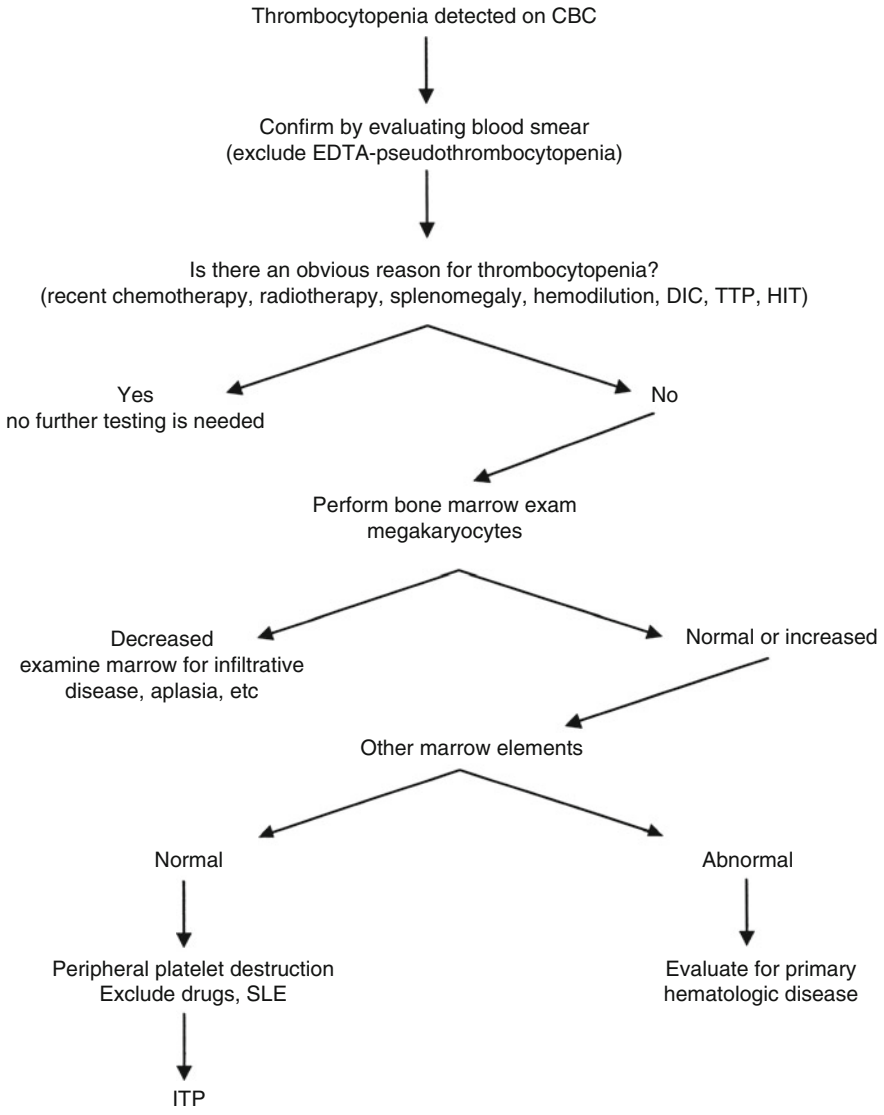

Fig. 5.2 A strategy to evaluate thrombocytopenia. Algorithm for evaluation of thrombocytopenia. *Abbreviations*: *DIC* disseminated intravascular coagulation, *TTP* thrombotic thrombocytopenic purpura, *SLE* systemic lupus erythematosus, *HIT* heparin-induced thrombocytopenia, *ITP* idiopathic thrombocytopenic purpura. The laboratory evaluation for DIC, TTP, etc. is discussed in Chaps. 8 and 9 (Modified from Rodgers [2], with permission)

The PT assay is performed by mixing patient plasma with thromboplastin. Thromboplastin is a commercial tissue factor/phospholipid/calcium preparation, which is derived either from animal tissue or from recombinant methods. Tissue factor in the thromboplastin preparation binds factor VII in patient plasma to initiate coagulation. The clotting time is measured in seconds using instruments with mechanical or photo-optical endpoints that detect fibrin formation [4].

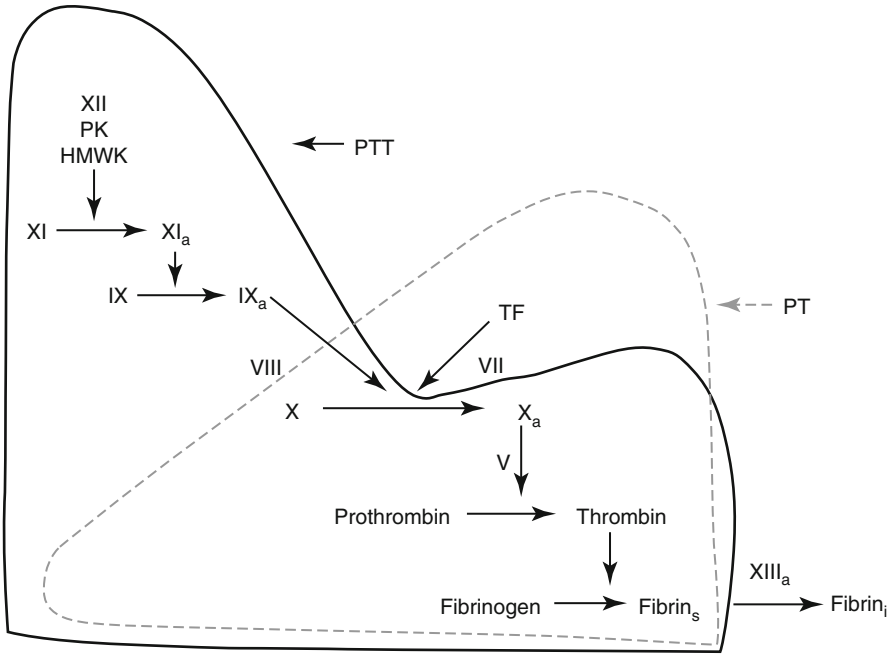

Fig. 5.3 The coagulation mechanism as measured by the PT and PTT assays. The PT assay measures the extrinsic (tissue factor) and common pathways, and is performed in the laboratory by mixing patient plasma with a commercial preparation of tissue factor and calcium (thromboplastin). This results in tissue factor-factor VII activation of factor X, then factor X_a – mediated conversion of prothrombin to thrombin. This reaction requires factor V as a cofactor. Thrombin converts fibrinogen to soluble fibrin, which polymerizes into fibrin strands, the endpoint detected by the coagulation instrument. Factor $XIII_a$ cross-linking of fibrin is not measured by the PT assay. The PTT assay measures the intrinsic and common pathways, and is performed by adding patient plasma to the PTT reagent (contact activator). This preincubation step initiates contact activation of plasma in which factor XII and factor XI are activated in the presence of prekallikrein and high-molecular-weight kininogen. Factor XI_a then activates factor IX to IX_a. Calcium is then added to the sample. This results in factor IX_a – mediated activation of factor X in the presence of the cofactor, factor VIII, with subsequent activation of prothrombin and fibrinogen are described above. As with the PT assay, the endpoint of the PTT assay is generation of polymerized fibrin strands, so that factor XIII activity is not measured by the PTT assay (*Abbreviations*: *PK* prekallikrein, *HMWK* high-molecular-weight kininogen, *TF* tissue factor, *fibrin$_s$* soluble fibrin, *fibrin$_i$* insoluble fibrin) (Modified from Rodgers [2], with permission)

Thromboplastin preparations can vary in their sensitivities, resulting in different clotting times. This is discussed further in Chap. 10. A typical PT reference range is 10–15 s. Most PT assays are automated, with the instrument adding reagents and patient plasma samples per protocol. Manual finger stick methods to measure PT/INR values are available and are discussed in Chap. 3.

In general, the PT assay is more sensitive in detecting low levels of factors VII and X than low levels of fibrinogen, prothrombin or factor V. In particular, different thromboplastin reagents may exhibit variable sensitivities to these factor deficiencies. Mild factor deficiency (i.e., 40–50 % of normal) may not be detected by many

thromboplastin reagents. The PT assay is less affected by heparin than is the PTT assay, but therapeutic doses of unfractionated heparin will usually prolong the PT by a few seconds unless a heparin neutralizer is present in the PT reagent [3, 4].

Shortened PT values may result from either a poor quality venipuncture (activated sample), chronic disseminated intravascular coagulation (in vivo activation), or cold activation of the sample (in vitro activation from factor XII activation of factor VII) that occurs if the plasma sample is stored at cold temperatures (above freezing) for several hours. Administration of recombinant factor VII_a also will decrease the PT.

5.4 The PTT Assay

The PTT assay is useful for three reasons – as a screening test for inherited or acquired deficiencies of the intrinsic pathway (Fig. 5.3), to detect the lupus anticoagulant, and to monitor heparin therapy. The PTT is affected by decreased levels of intrinsic pathway components (factors VIII, IX, XI, XII, prekallikrein, and high-molecular-weight kininogen), as well as decreased levels of common pathway components (fibrinogen, prothrombin, factor V and X). Factors VII and XIII are not measured by the PTT assay [3].

To perform the PTT assay, patient plasma is preincubated with the PTT reagent (crude phospholipid and a surface-activating agent such as silica or kaolin). This preincubation initiates contact activation (intrinsic pathway activation) in which factors XII and XI are activated in the presence of cofactors, prekallikrein and high-molecular-weight kininogen. Factor XI_a then converts factor IX to IX_a. Calcium is then added to the preincubation mixture; this results in factors IX_a/VIII activation of factor X, then factor X_a/V-mediated activation of prothrombin to thrombin followed by conversion of fibrinogen to soluble fibrin, the endpoint of the PTT assay [3, 4]. As with the PT assay, most PTT assays are automated.

The PTT is more sensitive to deficiencies of factors VIII and IX than to deficiencies of other intrinsic factors. Mild factor deficiency (i.e., 30–50 % of normal) may not be detected by most PTT reagents, but deficient levels of 10–20 % should be detected. The usual PTT reagent is less sensitive to factor IX than to factors VIII, XI, and XII. The ability to detect mild factor deficiency is reagent-dependent, and this is an important consideration when choosing the laboratory PTT reagent (see Chap. 4). For example, a hospital laboratory that evaluates a large population of bleeding disorder patients may prefer a PTT reagent that is more sensitive to detection of factor deficiency than to detection of lupus anticoagulants. In contrast, a hospital laboratory that evaluates large numbers of obstetrical patients may prefer a PTT reagent that has the opposite characteristics. The PTT may be affected by high levels of factor VIII, an acute-phase response protein; high factor VIII levels may mask co-existing mild intrinsic coagulation deficiencies. A typical PTT reference range is 25–36 s.

Shortened PTT values may result from a poor-quality venipuncture (activated sample), chronic disseminated intravascular coagulation (*in vivo* activation), or increased factor VIII levels.

It should be emphasized that the PT and PTT assays are screening tests, that normal PT/PTT results do not exclude a bleeding disorder, and that many patients with mild factor deficiency will have normal results for these assays [3].

5.5 The Mixing Test (Inhibitor Screen)

Abnormal PT or PTT results are due either to deficiency of a factor measured by the assay, or by an inhibitor such as an antibody or heparin. Uncommon inhibitors include fibrin degradation products and certain paraproteins. The mixing test is useful to distinguish between deficiency vs inhibitor, and mixing test results usually suggest subsequent test ordering. The most common mixing test protocol mixes patient plasma with normal plasma in a 1:1 ratio, followed by repeat assay of the PT or PTT immediately after mixing and repeated 1–2 hours later. Sample mixes that correct to normal at both intervals suggest that the original abnormal result was due to factor deficiency, while sample mixes that fail to correct to normal at one or both intervals suggest the presence of an inhibitory substance [3]. If heparin is suspected as the inhibitor, screening tests for the presence of heparin can be done with the thrombin time assay and reptilase assay (discussed below) or by direct assay of heparin or low-molecular-weight heparin using anti-factor X_a methods (see Chap. 10).

The mixing test is most useful for evaluating prolonged PTT results. Almost all prolonged PT samples result from factor deficiency, and the mixing test is less useful in this circumstance.

5.6 The Thrombin Time Assay

The thrombin time (TT) measures the conversion of fibrinogen to fibrin. It is performed by the addition of purified thrombin to patient plasma; the resulting clotting time is a function of fibrinogen concentration and activity. The TT is a screening test for the presence of heparin in a plasma sample. Other causes for a prolonged thrombin time include quantitative deficiency of fibrinogen, qualitative abnormality of fibrinogen (dysfibrinogen), elevated levels of fibrin degradation products (FDP), the presence of certain paraproteins, and markedly increased levels of fibrinogen (>5 g/L) [3].

If heparin is suspected as the cause of a prolonged TT, confirmation of heparin presence can be made using heparin assays (see anti-X_a assays in Chap. 10) or by using the reptilase clotting time that also measures the conversion of fibrinogen to fibrin. The reptilase clotting time is not affected by heparin. Therefore, a plasma sample with a prolonged TT, but a normal reptilase clotting time indicates the presence of heparin.

For hypofibrinogenemia to prolong a TT, the fibrinogen value will usually be ≤0.7–1 g/L. If the thrombin concentration in the TT assay is more than 3 U/mL, the assay will be less sensitive in detecting abnormalities.

5.7 Fibrinogen Assays

Fibrinogen is a heterodimeric molecule, with each half of the molecule composed of three polypeptide chains – Aα, Bβ and γ. It is acted upon by thrombin to produce fibrin monomers that polymerize to form fibrin strands, and ultimately a fibrin clot. Circulating fibrinogen molecules are structurally heterogeneous, and not all fibrinogen molecules are capable of participating in clot formation. Therefore antigenic assays and clot-based assays may return different results depending upon the composition of fibrinogen molecules in a specific patient sample [5]. Only clottable fibrinogen is of interest for the purpose of hemostasis screening.

Fibrinogen assays available on current automated coagulation analyzers include the Clauss and PT-derived methods. The Clauss method is a modified thrombin time. To initiate clotting, excess thrombin is added to patient plasma that has been diluted with buffer. The clotting time is proportional to the fibrinogen concentration. The dilution with buffer decreases the effects of interfering substances (e.g., heparinoids, FDPs) on the Clauss reaction, relative to the thrombin time assay. However, most manufacturers only claim to inhibit interference from heparin up to a concentration of 1–2 U/mL. Higher concentrations may result in falsely low fibrinogen measurements. FDPs generally result in decreased fibrinogen estimates by the Clauss method, as well, though the degree of interference varies by manufacturer. Some manufacturers include FDP inhibitors in their reagents [5, 6]. Since Clauss assays have excess thrombin in the reagents, carryover of thrombin to subsequent tests is a potential problem that should be addressed during instrument validation.

In the PT-derived assays, the fibrinogen concentration is proportional to the total change in optical signal observed during the PT assay. PT-derived assays have the advantage that a fibrinogen level can be derived directly from the PT assay without additional time or expense. In addition, FDPs generated by thrombolysis do not interfere with these assays [5, 6]. However, these assays are not recommended for routine use in the clinical laboratory since they have lower precision than the Clauss assays, and suffer from decreased accuracy at low or high fibrinogen concentrations, or when turbid plasma is tested [6].

In most circumstances, the PT, PTT and platelet assays are sufficient for screening bleeding patients. However, in cases where fibrinogen levels may drop precipitously (e.g., disseminated intravascular coagulation [DIC] in obstetric patients), a fibrinogen assay is an essential screening test, since the PT and PTT are relatively insensitive to low levels of fibrinogen (see Chap. 12).

5.8 D-dimer Assays

D-dimer is formed through the proteolytic action of plasmin on polymerized fibrin that has been cross-linked by factor XIII (Fig. 5.4). The presence of D-dimer in the circulation is evidence that clot has formed and has been cleaved by plasmin. As such, D-dimer is an effective screening assay for two conditions: DIC and venous thromboembolism (VTE). In DIC, excessive thrombin is formed resulting in clot

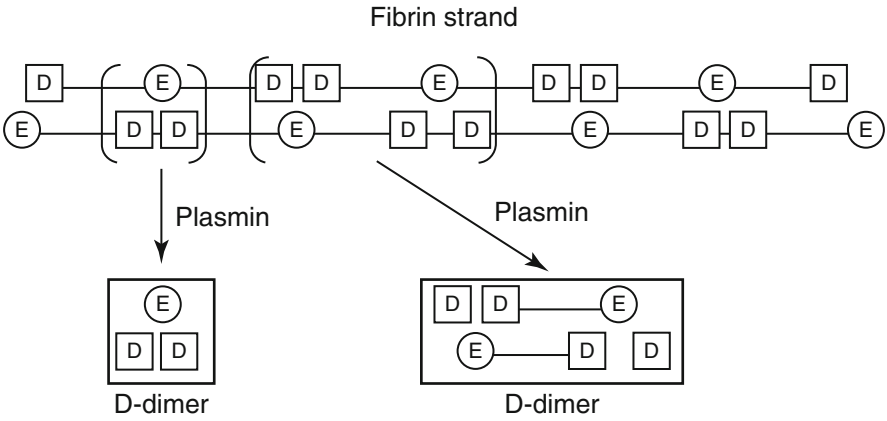

Fig. 5.4 Proteolytic action of plasmin on cross-linked fibrin polymer producing heterogenous D-dimer molecules. *Abbreviations*: *D* D domain of fibrinogen, *E* E domain of fibrinogen

formation and activation of the fibrinolytic system that, in turn produces D-dimer (see Chap. 8). In VTE, clot forms in the deep venous system of the pelvis and/or proximal, lower extremities secondary to pre-disposing conditions (Virchows triad). Clots may then embolize to the lungs. Plasmin cleaves cross-linked fibrin polymers in the clot releasing D-dimer into the circulation.

D-Dimer assays are immunoassays with different antibody specificities for the heterogeneous D-dimer fragments produced by the action of plasmin on cross-linked clot (Fig. 5.4). Therefore, D-dimer levels produced by different assays are generally not interchangeable. The sensitivity of D-dimer assays is judged relative to reference ELISA assays performed in microtitre wells. The manual agglutination assays are the least sensitive tests, while the automated ELISA and immunoturbidimetric assays are the most sensitive, rapid assays. Any of the available assays is suitable for screening for DIC. Many patients, particularly hospitalized patients, have low levels of plasma D-dimer that exceed the reference interval, but they do not have clinical evidence of DIC. Therefore, assay-specific cutoffs should be established to maximize sensitivity and specificity for the diagnosis of DIC [7].

Sensitive D-dimer assays have been demonstrated to have excellent negative predictive value for the diagnosis of VTE, when combined with a pre-test probability assessment [8]. Manual agglutination assays are insufficiently sensitive to be used to rule out VTE.

5.9 The Bleeding Time (BT) Test

The BT test was developed to provide information on platelet function and the likelihood of bleeding with surgery or invasive procedures. However, an extensive literature on the BT has evolved indicating that the test has minimal clinical utility, including a position paper by the College of American Pathologists and the American Society of Clinical Pathologists [3]. A clinical outcomes study published

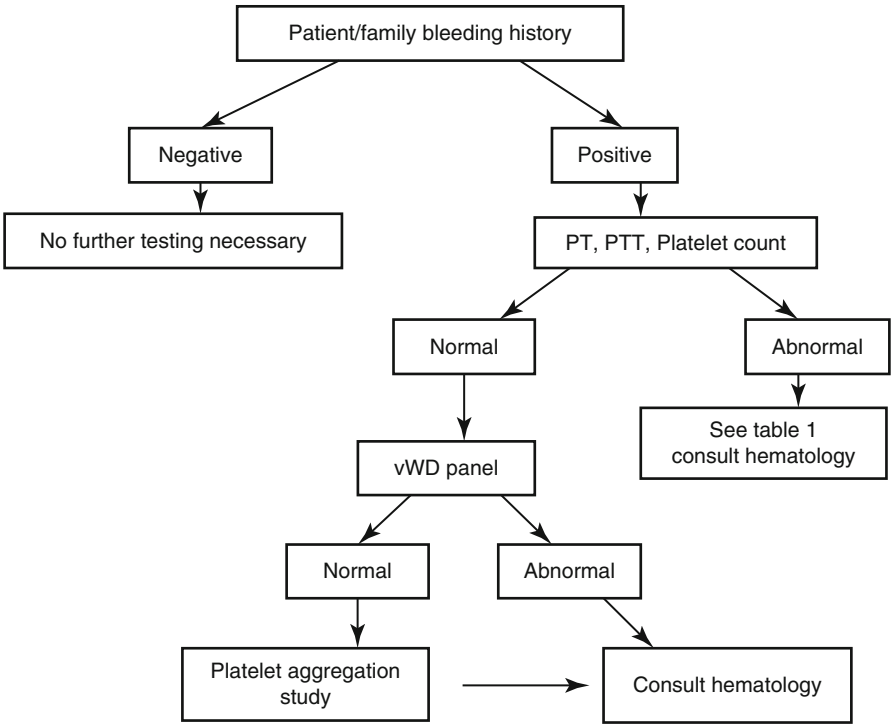

Fig. 5.5 An algorithm to evaluate patients for possible platelet dysfunction. The patient and family history are important in deciding whether to pursue laboratory evaluation. If the history is positive, the screening tests of hemostasis (PT, PTT, platelet count) are used for further evaluation. The vWD panel consists of factor VIII activity, von Willebrand factor antigen, and ristocetin cofactor activity. (*Abbreviations*: *vWD* von Willebrand's disease) (Modified from Lehman et al. [9])

in 2001 found that when the BT test was discontinued at a tertiary medical center, there were no negative impacts, including no increase in procedural or surgical bleeding [9]. The authors of that study concluded that appropriate evaluation for patients suspected of having platelet dysfunction should focus on the patient and family history of bleeding, as well as specific tests for platelet dysfunction, including laboratory evaluation of von Willebrand disease and platelet aggregation studies. Figure 5.5 summarizes an algorithm used to evaluate patients for platelet dysfunction. Other laboratory tests to evaluate platelet function are discussed in Chaps. 6 and 7.

5.10 Interpretation of Hemostasis Screening Tests in Patients with Bleeding Disorders

Table 5.1 summarizes the differential diagnosis and recommended subsequent laboratory evaluation for patients with a positive bleeding history using the three hemostasis screening tests – PT, PTT, and platelet count. As mentioned above, these are screening tests, and normal test results do not exclude a bleeding disorder [3].

Table 5.1 Interpretation of hemostasis screening tests in patients with bleeding disorders, and recommended subsequent evaluation

PT	PTT	Platelet Count	Differential Diagnosis	Recommended Tests
↑	–	–	Common	
			Acquired F VII deficiency (Liver disease, malnutrition, warfarin therapy)	Liver function tests Drug history
			Rare	
			Some cases of DIC	D-dimer
			Factor VII inhibitor	Mixing test and F VII assay
			Dysfibrinogenemia	Functional/antigenic fibrinogen levels
			Inherited F VII deficiency	F VII level
			Certain F X variants	F X level
–	↑	–	Common	
			Heparin	Mixing test, TT assay, heparin assay
			Deficiency or inhibitor of Factors VIII, IX, XI	Mixing test, assays for F VIII, IX and XI, VWD panel
			Rare	
			Lupus anticoagulant with platelet dysfunction	Mixing test, LA panel, platelet aggregation
			Certain F X variants	F X assay
↑	↑	–	Common	
			Vitamin K deficiency	Liver function tests
			Liver disease, warfarin, heparin	Drug history
			Rare	
			DIC	D-dimer
			Dysfibrinogenemia	Functional/antigenic fibrinogen levels
			LA with hypoprothrombinemia	Mixing test, LA panel, prothrombin level
			Primary fibrinolysis	Fibrinogen level, FDP levels (exclude DIC with D-dimer)
			Deficiency or inhibitor to factors X, V, prothrombin, or fibrinogen	Mixing test, factor levels

↑	↑	↓	Common	
			DIC	D-dimer
			Liver disease	Liver function tests
			Rare	
			Heparin-induced thrombocytopenia	Drug history, Heparin-PF4 ELISA
–	–	↓	Common	
			Decreased platelet production	Bone marrow
			Increased platelet production	Bone marrow
			Splenomegaly	Physical exam
			Hemodilution	History
			Rare	
			Certain inherited platelet disorders	Blood smear evaluation
			Bernard-Soulier syndrome, Wiskott-Aldrich syndrome	Platelet aggregation study
–	–	↑	Myeloproliferative disorders	Bone marrow Cytogenetics, PCR tests (BCR-ABL, JAK-2, etc.)
–	–	–	Common	
			vWD	vWD panel, multimeric analysis
			Acquired platelet dysfunction (uremia)	Renal function tests (platelet aggregation study)
			Rare	
			Inherited qualitative platelet disorder	Platelet aggregation study
			F XIII deficiency	F XIII assay
			Dysfibrinogenemia	Functional/antigenic fibrinogen levels
			Mild factor deficiency	Factor assays
			Disorders of platelet procoagulant activity	Prothrombin consumption assay
			Alpha$_2$-antiplasmin deficiency	Alpha$_2$-antiplasmin assay
			Vascular disorders	Physical exam

Modified from Rodgers [10], with permission
Consideration of patients with abnormal screening tests and negative bleeding histories is not included in this table
↑ = increased, ↓ = decreased, – = normal
Abbreviations: *F* factor, *DIC* disseminated intravascular coagulation, *TT* thrombin time, *vWD* von Willebrand disease, *LA* lupus anticoagulant, *FDP* fibrin degradation products, *PF4* platelet factor 4, *HHT* hereditary hemorrhagic telangiectasia

The category that may require the most extensive testing is the last one, in which all screening tests are normal. Although most patients will have common disorders such as mild von Willebrand disease or platelet dysfunction, some patients will require extensive testing before a diagnosis is made.

5.11 Key Points

- Screening tests of hemostasis (platelet count, PT, PTT) are useful in suggesting diagnostic possibilities in bleeding patients.
- The presence of normal values for platelet count, PT, and PTT assays does not exclude the possibility of a bleeding disorder. In fact, the most common bleeding disorders (von Willebrand's disease, platelet dysfunction) will usually present with this coagulation profile.
- Isolated, prolonged PT values suggest inherited or acquired (liver disease, malnutrition, warfarin therapy, DIC) factor VII deficiency.
- Isolated, prolonged PTT values in bleeding patients suggest either heparin therapy, factor deficiency or inhibitor to factors VIII, IX, or XI.
- Mixing studies should be performed to evaluate prolonged PTT values. Failure to correct to the normal reference interval with mixing suggests the presence of heparin, a lupus anticoagulant, or a specific factor antibody.
- Prolongations of both the PT and PTT suggest either inherited deficiency of a common pathway factor (uncommon), acquired multiple factor deficiency (DIC, vitamin K deficiency), or heparin or warfarin therapy.
- PT-derived fibrinogen methods are not recommended for routine use in the coagulation laboratory.
- D-dimer is a sensitive test for the diagnosis of DIC. However, many patients with low levels of plasma D-dimer do not have DIC, so assay-specific cutoffs must be established.
- Sensitive D-dimer assays, in combination with a pre-test probability assessment, have excellent negative predictive value for ruling out VTE. Manual agglutination assays cannot be used to rule out VTE.

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Testing for Inherited Bleeding Disorders

6

George M. Rodgers

Inherited bleeding disorders can be classified into three categories – those due to deficient platelet number or function, those due to deficient coagulation protein activity, and those due to vascular abnormality. The coagulation laboratory has a key role in evaluating patients in the first two categories, while disorders of vascular function are usually diagnosed by physical examination and certain genetic tests. Table 6.1 is a comprehensive listing of inherited platelet and coagulation disorders associated with bleeding, categorized by manner of genetic transmission. This chapter will focus on laboratory aspects of qualitative platelet disorders, von Willebrand's disease, and the coagulation disorders associated with bleeding. Unlike certain inherited thrombotic disorders that are associated with highly-conserved point mutations (e.g., factor V Leiden), the inherited bleeding disorders are polygenic and are best diagnosed with functional coagulation assays. All assays discussed in this chapter have FDA approval except for the quantitative factor XIII assay.

6.1 Qualitative Platelet Disorders

In these disorders, platelet dysfunction is associated with a normal platelet count. Normal platelet function results from several platelet activities. Platelet adhesion to subendothelium is the initial response to vascular injury, and is mediated by the plasma adhesion protein, von Willebrand's factor (vWF) and its platelet receptor, glycoprotein (GP) 1b. Platelet-platelet interaction (aggregation) is mediated by

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Table 6.1 Summary of inherited platelet and coagulation disorders associated with bleeding

Disorders associated with:	Thrombocytopenia	Platelet dysfunction (qualitative platelet disorders)	Coagulation disorders
	Autosomal recessive	Autosomal recessive	X-linked
	Bernard-Soulier syndrome	Bernard-Soulier syndrome	Factor VIII deficiency (hemophilia A)
	Congenital amegakaryocytic thrombocytopenia	Glanzmann's thrombasthenia	Factor IX deficiency (hemophilia B)
		Storage pool disorder	
	Gray platelet syndrome	Gray platelet syndrome	Autosomal recessive
	Thrombocytopenia with absent radius	Cyclooxygenase deficiency	Factor XI deficiency
		Thromboxane synthase deficiency	Prothrombin deficiency
			Factor V deficiency
	Autosomal dominant		Factor VII deficiency
	May-Hegglin anomaly	Scott's syndrome	Factor X deficiency
	Quebec platelet disorder		Factor XIII deficiency
	Epstein's syndrome		Afibrinogenemia
	Fechtner's syndrome		Hypofibrinogenemia
	Sebastian's syndrome		α_z Antiplasmin deficiency
	Montreal platelet syndrome		Plasminogen activator inhibitor deficiency
	Heredity macrothrombocytopenia		Combined deficiency of factors V/VIII
	X-linked		Autosomal dominant
	Wiskott-Aldrich syndrome		von Willebrand's disease
	X-linked thrombocytopenia		Dysfibrinogenemia
	GATA-1 mutation		

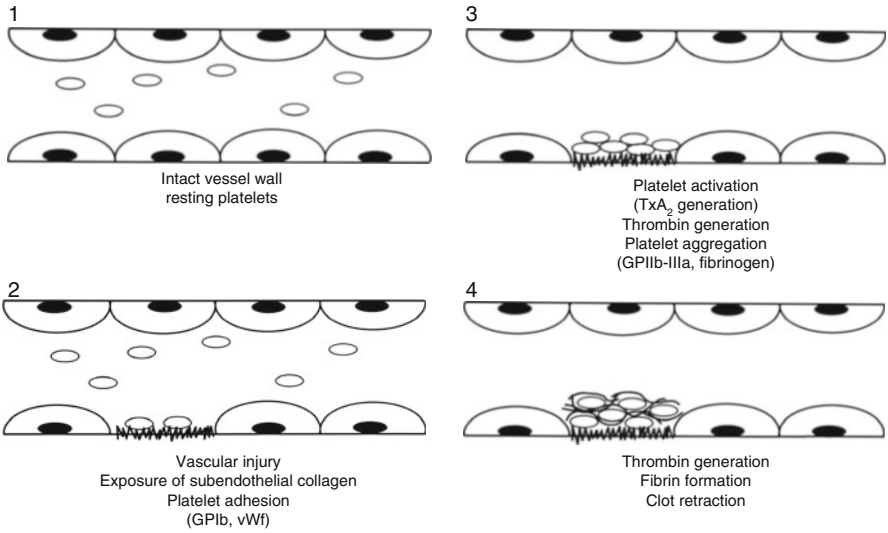


Fig. 6.1 Sequence of hemostatic events following vascular injury. *1* In the resting state, platelets are non-activated and the endothelium is intact. *2* Following vascular injury, subendothelial components are exposed to induce platelet adhesion, mediated by vWF. *3* Collagen exposure activates platelets leading to platelet secretion, thromboxane A_2 generation and formation of thrombin. These events lead to platelet aggregation. *4* Thrombin generation results in cross-linked fibrin that reinforces the platelet plug (From Rodgers [1], p. 306, with permission)

plasma fibrinogen and its platelet receptor, GP IIb-IIIa. Following platelet activation, secreted ADP amplifies platelet aggregation to generate the platelet thrombus [1]. These hemostatic events are summarized in Fig. 6.1. The platelet plug is consolidated (reinforced) by fibrin generated from the coagulation mechanism. All of these platelet functions (adhesion, aggregation, secretion) can be measured in the laboratory, and deficient platelet GP function can be inferred or quantitated. Screening tests for platelet function such as the bleeding time are not recommended and the utility of other screening tests such as the PFA-100 is unproven (see Chaps. 7 and 3).

6.2 Platelet Aggregation

The traditional assay for platelet function is platelet aggregation using platelet-rich plasma. Since countless prescription and over-the-counter medications and dietary supplements may impair platelet function, patients' medication lists must be reviewed by the coagulation laboratory prior to testing. Drugs containing aspirin must be discontinued 10–14 days prior to phlebotomy, while most other drugs should be stopped 4–7 days before phlebotomy. Citrated blood is obtained from the patient and a control subject (who also is taking no interfering medications), and platelet-rich plasma (PRP) is prepared by slow-speed centrifugation. The blood

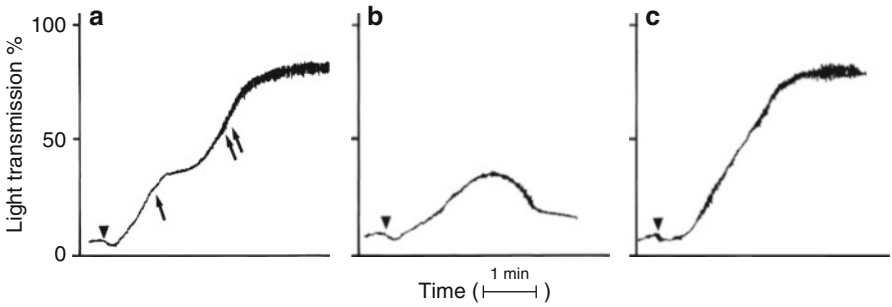


Fig. 6.2 Normal platelet aggregation patterns. (**a**) The primary and secondary waves of the platelet aggregation response. The primary wave (*single arrow*) results from agonist-induced platelet aggregation whereas the secondary wave (*two arrows*) results from platelet secretion of endogenous ADP and recruitment of additional activated platelets. (**b**) Low-dose agonist-induced primary wave aggregation. The amount of stimulus is insufficient to fully activate the platelet and induce secretion of stored ADP. (**c**) High-dose agonist-induced platelet aggregation in which the primary and secondary aggregation waves have merged into a single aggregation wave. Triangles indicate addition of the agonist (From Rodgers [12], with permission)

sample should not be refrigerated. The PRP specimens are mixed with patient platelet-poor plasma to obtain a final platelet count ~250,000/μL [2].

Platelet aggregation is based on spectrophotometric monitoring of the turbid PRP sample after addition of various agonists (platelet activating stimuli). Agonist-induced platelet aggregation results in decreased turbidity and increased light transmittance. Figure 6.2 illustrates normal platelet aggregation patterns with PRP. The agonists used in platelet aggregation studies commonly include ADP (low-dose 0.5–2 μg/mL; high-dose 10–20 μg/mL), dilutions of a collagen suspension, arachidonic acid, and ristocetin (low-dose 0.5 μg/mL; high-dose 1–2 μg/mL). Some laboratories also use epinephrine as an agonist. This panel of agonists will test various platelet functions, including adhesion, aggregation, secretion and activation [2].

Zhou and Schmaier have published platelet aggregation procedures to standardize performance and interpretation of platelet function testing [3]. They ask patients to avoid all medications for 10 days prior to assay, to avoid coffee on the day of testing, and to fast for at least 4 hours before phlebotomy.

These authors recommend that each aggregation run last for 5–10 minutes, to allow observation of both primary and secondary aggregation waves, as well as the phenomenon of platelet disaggregation which may occur in some patients with platelet dysfunction.

Interpretation of platelet aggregation patterns can be done in two ways: calculation of the extent (percent) of aggregation, or determination of the initial rate of aggregation per minute. Zhou and Schmaier have published normal values for platelet aggregation results; most agonists result in maximal extent (percent) of aggregation of 70–80 % [3]. Recommendations from a committee of the ISTH on standardization of light transmission aggregometry summarize pre-analytical variables, blood collection techniques, platelet preparation methods, agonist choice and concentration and data reporting [4].

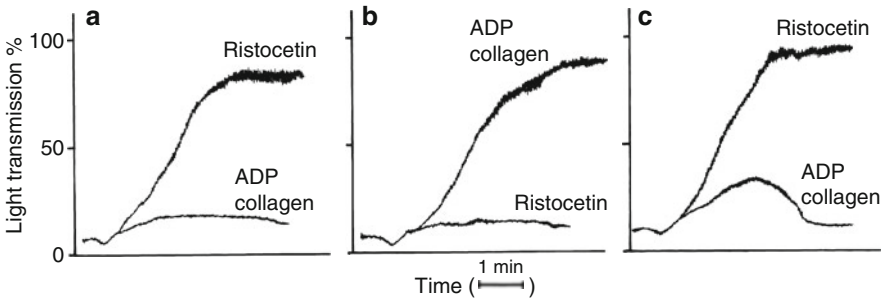


Fig. 6.3 Platelet aggregation profiles in vWD and the inherited qualitative platelet disorders. Typical responses to ADP, collagen, and ristocetin are shown. Three patterns of aggregation are shown: (**a**) Glanzmann's thrombasthenia. (**b**) Bernard-Soulier syndrome and vWD. Both diseases give similar aggregation results with decreased or absent response to ristocetin. The two diseases are distinguished by Bernard-Soulier patients having giant platelet morphology with thrombocytopenia and vWD patients having normal platelet morphology and decreased ristocetin cofactor activity. (**c**) Thrombopathies (secretion defects and abnormalities in prostaglandin synthesis) (From Rodgers [12], with permission)

Figure 6.3 summarizes platelet aggregation profile results in patients with Glanzmann's thrombasthenia, Bernard-Soulier syndrome, and storage pool/secretion disorders (thrombopathies). Glanzmann's thrombasthenia platelets have deficient GPIIb-IIIa receptors, so that aggregation dependent on this receptor is decreased or absent. Consequently, aggregation induced by ADP, collagen, arachidonic acid (all agonists except ristocetin) is abnormal. In contrast, patients with Bernard-Soulier syndrome have platelets deficient in GP1b receptors, so that aggregation dependent on this receptor is decreased or absent. Since ristocetin is the only agonist dependent on normal GP1b function, Bernard-Soulier syndrome platelets have normal aggregation responses to all agonists but ristocetin [2].

Platelets from patients with storage pool disorder or secretion defects will exhibit normal primary aggregation responses to all agonists, but since the defect is in platelet activation and/or secretion, secondary aggregation is defective to all agonists except ristocetin [2].

Platelet aggregation results can be reported quantitatively (percentage change of transmittance) or qualitatively (normal or abnormal), with a comment as to the type of abnormality and possible diagnosis. It is important to remember that numerous common medications can induce marked platelet dysfunction, and this potential confounding issue should be considered when interpreting an abnormal platelet aggregation study.

Table 6.2 summarizes the platelet aggregation profiles seen with the inherited qualitative platelet disorders. Although von Willebrand's disease (vWD) is best diagnosed with other tests, platelet aggregation can be used to diagnose vWD. Deficiency of vWF results in a profile where all agonist responses are normal, except for ristocetin, a pattern similar to that seen in Bernard-Soulier syndrome. In Bernard-Soulier syndrome, the defect is in the vWF receptor (GP1b); in vWD, the defect is absent vWF. The two disorders can be distinguished by assay of vWF

Table 6.2 Platelet aggregation profiles in the inherited qualitative platelet disorders and von Willebrand's disease

	Aggregation response				
	ADP		Collagen	Ristocetin	Comments
Disorders	Primary	Secondary			
Bernard-Soulier syndrome	N	N	N	A	Giant platelets and thrombocytopenia are present. vWF activity assay is normal
Glanzmann's thrombasthenia	A	A	A	N	Normal platelet morphology
Storage pool disorder	N	A	N	N	Platelet morphology by light microscopy is usually normal. Electron microcopy is abnormal
Secretion defect	N	A	N	N	Platelet morphology by light and electron microscopy is normal
vWD	N	N	N	A	Platelet morphology is normal. vWF activity assay is decreased

Source: Rodgers [2]; p. 331
N normal, *A* abnormal, *vWD* von willebrand's disease

activity with the ristocetin cofactor assay; levels are normal in Bernard Soulier syndrome and decreased in vWD (Table 6.2).

The key clinical distinction in evaluating patients for qualitative platelet disorders is to differentiate bleeding from a platelet disorder vs. vWD. Both disorders have similar clinical features, but are treated differently – vWD patients should receive a vWF replacement product, while patients with platelet dysfunction should receive platelet transfusions for significant bleeding.

Whole blood aggregation methods are also available that are less laborious than the PRP method. Certain whole blood assays also report platelet secretion data in addition to aggregation data, permitting direct identification of platelet dysfunction due to secretion defects.

6.3 von Willebrand's Disease

vWD is the most common inherited bleeding disorder, affecting ~1 % of the population. The disorder is characterized by deficiency or qualitative abnormality of vWF, the plasma adhesion protein necessary for primary hemostasis. vWF circulates in the blood in a complex with factor VIII, the plasma protein deficient in hemophilia A. *In vivo*, vascular injury results in exposure of subendothelial components that promote vWF-mediated platelet adhesion. This event is dependent primarily on the highest molecular weight component (multimers) of vWF. *In vitro* assays of platelet function using ristocetin mimic *in vivo* platelet adhesion; such assays (ristocetin-induced platelet aggregation, ristocetin cofactor activity) measure vWF function [2].

6.4 Assays for vWF

Several assays for vWF are available to assess patients with bleeding disorders for vWD. Most commonly, a vWD "panel" is obtained that measures antigenic and functional properties of the factor VIII – vWF complex. Panel components include vWF antigen, a quantitative assay of vWF, as measured by ELISA, Laurell immunoelectrophoresis, or immunoturbidimetric assay; vWF activity, as measured by a test called ristocetin cofactor activity; and factor VIII activity, as measured by coagulation or chromogenic substrate assay. Of these three panel components, ristocetin cofactor activity is frequently the most abnormal, but assaying all 3 components is recommended. If an abnormal vWD panel result is obtained, the patient's disorder is further classified by multimeric analysis, in which the patient's vWF is analyzed by immunoblot and characterized as type 1 (quantitative mild to moderate deficiency), type 2 [qualitative abnormality in which high-molecular-weight multimers are absent (type 2B), or high- and intermediate-molecular-weight multimers are absent (type 2A)], or type 3 (absence of vWF). This classification is clinically important since patients with type 1 vWD, but not types 2 or 3 vWD may respond to desmopressin, a non-transfusion therapy that can increase plasma vWF levels. Another assay that may be useful in diagnosing patients with vWD when the standard panel results are normal is ristocetin-induced platelet aggregation [2].

6.5 vWF Immunoassay

Historically, Laurell rocket immunoelectrophoresis has been the standard method to quantitate vWF antigen. This assay uses a precipitating polyclonal antibody to vWF in an electroimmunodiffusion technique, but this method is very labor-intensive. Current methods are based on ELISA techniques, which can be automated. Values less than 50 % of normal are usually diagnostic of vWD. vWF is an acute-phase response protein, and can be increased by stress, trauma, infection, inflammation, pregnancy, and female hormones. Consequently, a normal value does not exclude vWD, and repeat testing may be needed to establish the diagnosis [2].

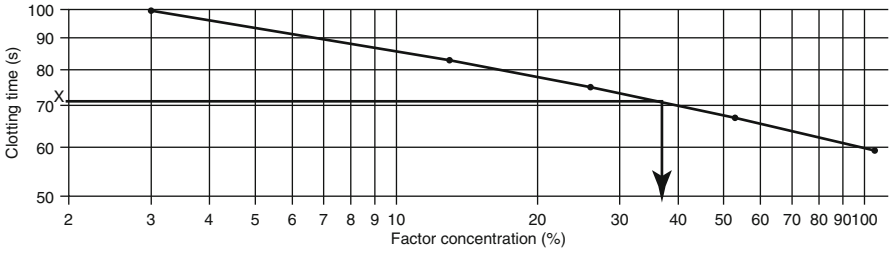
6.6 Ristocetin Cofactor Activity (vWF Activity) Assay

This is a functional assay of vWF that traditionally measures the ability of patient plasma to aggregate normal platelets in the presence of ristocetin, compared to normal plasma. This is a laborious technique that requires normal platelets, and is being replaced by ELISA methods in which a monoclonal antibody against a functional vWF epitope is used. Values less than 50 % of normal are usually diagnostic of vWD. As with the vWF antigen assay, acute-phase events, pregnancy, and female hormone use may confound the diagnosis of vWD [2].

6.7 Factor VIII Activity Assay

This assay is useful in evaluating patients for factor VIII deficiency due to hemophilia A or vWD, and in monitoring response to therapy. The standard coagulation assay principle is that dilutions of patient plasma deficient in factor VIII correct the prolonged partial thromboplastin time (PTT) of factor VIII-deficient plasma compared to normal plasma. For example, if a 1:40 dilution of normal plasma shortens the clotting time of deficient plasma to the same extent as a 1:10 dilution of patient plasma, the patient plasma has 25 % of normal factor VIII activity [5]. Figure 6.4 depicts a typical standard curve for this assay.

Dilutions of reference plasma (1:10, 1:20, 1:40, 1:80, 1:160, 1:320) and patient plasma (1:10, 1:20) are made in buffer (Owren's or imidazole). One part of diluted plasma is added to one part of factor VIII-deficient plasma and a PTT is performed. The 1:10 dilution of reference plasma is arbitrarily defined as 100 % activity, the 1:20 dilution as 50 %, etc. A line of best fit is obtained (Fig. 6.4) when clotting times are plotted against per cent factor VIII activity. The factor VIII activities that give the same clotting times as the 1:10 and 1:20 patient plasma dilutions are extrapolated from the graph. The 1:10 dilution value is the patient's actual factor VIII level; the 1:20 dilution value must be multiplied by two, and the mean of the two values is reported. Values less than 50 % of normal suggest factor VIII deficiency due to hemophilia A or vWD. As with vWF, factor VIII is an acute-phase response



Factor assay standard curve data

Factor concentration (%)	Normal plasma dilution	Clotting time (s)
105 %	1:10	59.1
53 %	1:20	67.2
26 %	1:40	75.2
13 %	1:80	82.7
3 %	1:320	97.4

Fig. 6.4 A typical factor assay standard curve. Dilutions of normal pooled plasma are made (1:10–1:320), and clotting times are measured. Results of clotting times vs. factor concentration are plotted on log-log paper, and the best-fit line is drawn. Patient plasma is diluted 1:10 and 1:20, and clotting times are measured. As indicated in the figure, the 1:10 dilution of patient plasma had a clotting time of 71 sec ("x"on the Y-axis). Interpolation from the standard curve yields a factor value of 37 %. These curves can be prepared manually, or fully-automated coagulation analyzers can be used to prepare dilutions, add reagents, measure clotting times, plot standard curves, and calculate patient results (From Rodgers [5], p. 351, with permission)

protein, and normal factor VIII levels may not exclude mild deficiency. A difficult phlebotomy (activated plasma sample) may also artifactually increase the factor VIII level [5].

6.8 vWF Multimeric Analysis

This is an immunoblot that is useful to distinguish quantitative vs. qualitative types of vWD. Patient and normal plasma are electrophoresed using dilute agarose (0.7–1 %). The separated plasma proteins are transferred to nitrocellulose paper, incubated with an antibody to vWF, followed by immunodetection using the avidin-biotin-peroxidase technique. The resulting immunoblot (Fig. 6.5) illustrates the full range of multimers in normal plasma and in plasma from patients with type 1 vWD, as well as absence of high-and/or intermediate-molecular-weight-multimers in patients with type 2 vWD, or complete absence of multimers as in patients with type 3 vWD [2].

6.9 Appropriate Test Ordering for Platelet-Type Bleeding Disorders

Patients with mucocutaneous bleeding, a normal platelet count, and a family history of excessive bleeding are appropriate candidates for testing of platelet-type bleeding disorders which include vWD and the qualitative platelet disorders. Given the

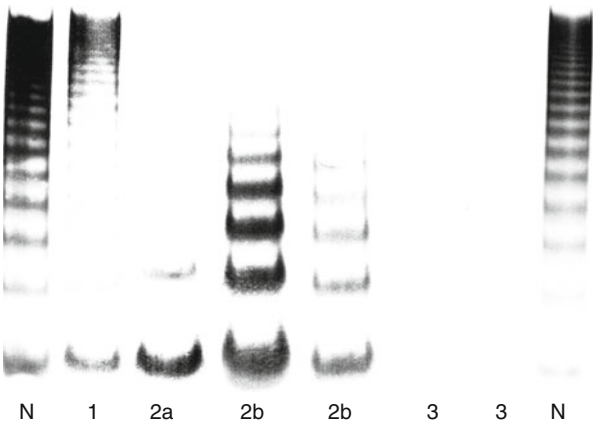


Fig. 6.5 Multimeric analysis of vWF. Plasma was obtained from a normal subject (N), and from patients with various types of vWD (1,2A, 2B, 3). Plasma was electrophoresed in an agarose gel, then vWF was identified using an immunoperoxidase method. The dark bands at the top of the gel (N) represent the high-molecular-weight multimers most important in platelet adhesion. Note the generalized decrease in band intensity in type 1, the loss of intermediate and high-molecular-weight multimers in type 2A, the loss of only high-molecular-weight multimers in type 2B, and the virtual absence of multimers in type 3 (From Rodgers [2], p. 339, with permission)

limitations of screening tests such as the bleeding time, it would be reasonable to initially evaluate patients for vWD since this disorder is much more common than the disorders of platelet function. An exception to this recommendation would be patients with abnormal platelet morphology on blood smear examination (giant platelets, gray platelets, etc.) that suggests a qualitative disorder for which platelet aggregation studies would be the appropriate initial test.

If the vWD panel is normal, platelet aggregation testing using a complete panel of agonists (collagen, ADP, arachidonic acid, ristocetin, all in various concentrations) is appropriate. It is important to ensure that all antiplatelet medications are discontinued prior to testing. Some patients with vWD will have normal panel results, but may be detected with ristocetin-induced platelet aggregation. If patients have normal results for both the vWD panel and platelet aggregation test, and the likelihood of the platelet-type bleeding disorder is thought to be high, repeat vWD testing is indicated, since factor VIII and vWF are acute-phase reactants, and many patients with mild vWD will have normal studies. For women who are on hormone therapy or pregnant, and in whom normal vWD panel results will not exclude the diagnosis, testing of symptomatic family members may be useful [2].

One genetic modifier of plasma vWF concentrations is the patient's ABO blood group; individuals with type O have ~25 % lower vWF concentrations than do those with a non-O blood type [6]. Correct interpretation of borderline-low vWF panel results should take into consideration the patient's blood type.

6.10 The Platelet Function Analyzer (PFA)-100®

The PFA-100® device is now available as a screening test for disorders of platelet function. An international consensus panel has recently evaluated the literature on this device in terms of its clinical utility [7]. These authors concluded that this assay should be considered optional in the evaluation of platelet function, based on a paucity of outcome data that has been published with the PFA-100® [7].

6.11 Inherited Coagulation Disorders

The qualitative platelet disorders and vWD described above result in defective primary hemostasis – formation of an ineffective platelet thrombus. The coagulation mechanism (secondary hemostasis) is necessary to generate fibrin to reinforce the platelet plug. The inherited coagulation disorders have in common deficient fibrin formation that frequently leads to delayed bleeding. Assays are based on the factor VIII activity method described above in which the ability of patient plasma to shorten prolonged clotting times (PT-or PTT-based) of commercially-deficient plasma is compared to normal plasma. For example, the factor IX activity assay uses factor IX-deficient plasma in a PTT-based assay. The factor VII activity assay uses factor VII-deficient plasma in a prothrombin time (PT)-based assay. Factors VIII, IX, XI, XII, prekallikrein, and high-molecular weight kininogen are assayed

Table 6.3 Results of PT and PTT assays in coagulation factor deficiency disorders

Factor deficiency	PT	PTT	Excessive bleeding?
XII	Nl	↑	No
Prekallikrein	Nl	↑	No
High-molecular-weight kininogen	Nl	↑	No
XI	Nl	↑	Yes
IX	Nl	↑	Yes
VIII	Nl	↑	Yes
VII	↑	Nl	Yes
X	↑	↑	Yes
V	↑	↑	Yes
Prothrombin	↑	↑	Yes
Fibrinogen	↑	↑	Yes
XIII	Nl	Nl	Yes

PT and PTT test results in patients with coagulation factor deficiency disorders are shown and classified as to whether excessive bleeding is seen with the disorder. Patients with mild bleeding disorders may have normal PT and PTT results, and identification of these patients may depend on PT and PTT reagent sensitivities. Although deficiency of factor XII, prekallikrein, and high-molecular weight kininogen is associated with increased PTT values, there is no excessive bleeding seen in these disorders

Abbreviations: *Nl* normal, ↑ increased, *PT* prothrombin time, *PTT* partial thromboplastin time

using PTT-based methods, while factors VII, X, V, and prothrombin are assayed using PT-based methods. Diagnosis of a specific coagulation-type bleeding disorder depends on specific coagulation factor assay results. Table 6.1 illustrates how abnormal PT and/or PTT results lead to recommended specific factor assays. Table 6.3 summarizes the effects on PT and PTT assays of coagulation factor deficiencies, classified further as to whether or not excessive clinical bleeding is present.

Tables 6.4 and 6.5 summarize pediatric reference intervals for coagulation tests [8–10]. Note that there is age-dependence for many analytes, including the PT, prothrombin, factors VII, VIII, IX, and X, alpha$_2$-antiplasmin, antithrombin and protein C.

6.12 Factor XIII (Clot Stability) Assay

The assay principle for factor XIII activity differs from other factor assays described above. The screening assay for factor XIII activity is performed by recalcifying patient and control plasma to produce a fibrin clot. Control and patient clots are suspended in solutions of strong denaturing agents (urea, trichloroacetic or monochloroacetic acid) for 24 hours. Clots that have been appropriately crosslinked by factor XIII$_a$ can withstand such conditions and no clot lysis is seen. Clots formed in the absence of factor XIII dissolve in the presence of denaturing agents [5]. Quantitative factor XIII assays can be performed to confirm the abnormal screening assay results. The quantitative assays are not routinely performed in coagulation laboratories; they measure transglutaminase activity.

Table 6.4 Summary of pediatric reference intervals

Age	7–9	10–11	12–13	14–15	16–17	Adult
N	245	164	164	164	150	120
PT (sec)	13.0–15.4	13.0–15.6	13.0–15.2	12.8–15.4	12.6–15.7	12.3–14.4
PTT (sec)	27–38	27–38	27–39	26–36	26–35	26–38
Factor II, %	78–125	78–120	72–123	75–135	77–130	86–150
Factor V, %	69–132	66–136	66–135	61–129	65–131	62–140
Factor VII, %	67–145	71–163	78–160	74–180	63–163	80–181
Factor VIII, %	76–199	80–209	72–198	69–237	63–221	56–191
Factor IX, %	70–133	72–149	73–152	80–161	86–176	78–184
Factor X, %	74–130	70–134	69–133	63–146	74–146	81–157
Factor XI, %	70–138	66–137	68–138	57–129	65–159	56–153
RCF, %	52–176	60–195	50–184	50–203	49–204	51–215
vWF Ag, %	62–180	63–189	60–189	57–199	50–205	52–214
Fibrinogen, %	198–413	197–410	215–378	204–392	208–438	211–441
Alpha$_2$-antiplasmin, %	88–147	90–144	87–142	83–136	77–134	82–133
AT, %	90–135	90–134	90–132	90–131	87–131	76–128
Plasminogen,%	76–116	74–117	66–114	71–124	75–132	71–144
Protein C, %	70–142	68–143	66–162	69–170	70–171	83–168
Protein S, % Male	66–140	69–139	72–139	68–145	77–167	66–143
Protein S, % Female	62–151	65–142	70–140	55–145	51–147	57–131

Source: Flanders et al. [8, 9]
PT and PTT results are expressed in seconds. All other analytes are expressed as percent of normal
Abbreviations: *PT* prothrombin time, *PTT* partial thromboplastin time, *RCF* ristocetin cofactor activity, *vWF Ag* von Willebrand factor antigen, *AT* antithrombin

Table 6.5 Summary of pre-term neonatal reference intervals

Age	<28 weeks	28–34 weeks
N	24	144
PT (sec)	14.5–20.9	13.9–20.6
PTT (sec)	27–64	30–57
Fibrinogen (%)	71–535	87–470

Source: Christensen et al. [10]
PT and PTT results are expressed in seconds. All other analytes are expressed as percent of normal
Abbreviations: *PT* prothrombin time, *PTT* partial thromboplastin time

6.13 Fibrinogen Activity Assay

This assay utilizes a thrombin time-based assay. Serial dilutions of a fibrinogen reference standard are prepared and a calibration curve is done. The clotting time is inversely proportional to the amount of fibrinogen in the sample. A typical reference

interval for fibrinogen in adults is 150–350 mg/dL [11]. Low fibrinogen levels are observed in inherited afibrinogenemia or hypofibrinogenemia, dysfibrinogenemia, primary fibrinolysis, and disseminated intravascular coagulation. Diagnosis of dysfibrinogenemia requires demonstration of a normal fibrinogen antigen level with a decreased functional level.

6.14 Alpha$_2$-Antiplasmin Assay

This assay measures functional activity of an enzyme associated with an uncommon bleeding disorder. This disorder is usually considered in patients with coagulation-type bleeding in whom other factor deficiencies have been excluded. Plasmin is added to the patient's plasma and residual plasmin activity is measured by a fluorescent or chromogenic substrate assay. Alpha$_2$-antiplasmin levels can also be measured using immunologic assays [5].

6.15 Assays for Factor VIII Antibodies (Inhibitor Assays)

Up to 30 % of patients with inherited factor VIII deficiency will develop antibodies to factor VIII. Identification of these antibodies is important since they are associated with increased bleeding, and will usually require specific treatment. Patients may acquire antibodies to factor VIII in the absence of inherited hemophilia in association with connective tissue or malignant diseases, or in certain non-malignant settings such as pregnancy.

The principle of these assays is based on the mixing study (inhibitor screen) that was discussed in Chap. 5. Normal plasma is incubated with patient plasma suspected of having a factor VIII antibody, and residual factor VIII activity of the mixed sample is measured. A screening assay for factor VIII antibodies is less labor intensive than the formal Bethesda assay that quantitates the factor VIII antibody level.

In the screening assay, one part of patient plasma is mixed with an equal part of normal pooled plasma that contains 100 % factor VIII activity. After a 2 hour incubation at 37 ° C, the mixture is diluted in saline (1:5) and assayed for factor VIII. If this assay result is 35 % or higher, no antibody is present, or if present, is too weak to be clinically important [5, 9].

Patient samples with factor VIII results <30–35 % should be further evaluated with a Bethesda assay to quantitate the factor VIII antibody level. Serial dilutions of patient plasma in buffer (1:2, 1:4, 1:8, etc.) are incubated with equal volumes of normal plasma for 2 hours at 37 °C. The residual factor VIII activity of the incubation mixtures is determined. The inhibitor level (in Bethesda units) is the reciprocal of the dilution of the patient plasma that results in 50 % inhibition of factor VIII activity. An antibody level <5 Bethesda units is labeled as a low-level inhibitor, while a level ≥ 5 Bethesda units is considered a high-level inhibitor [5].

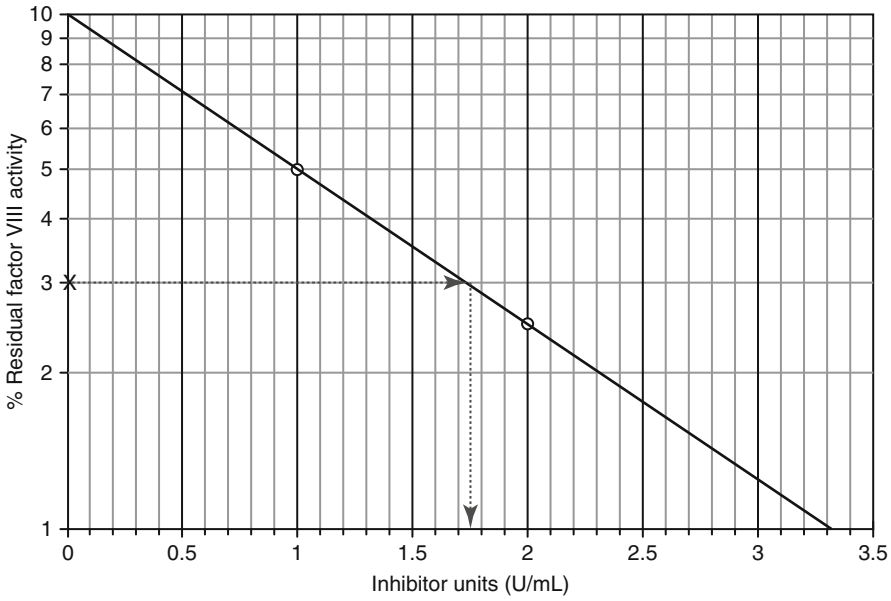


Fig. 6.6 A factor VIII Bethesda assay calibration curve. In this example, the residual factor VIII activity measured after incubating patient and reference plasma for 2 h was 30 % (small "x" on the Y-axis). Interpolation from the graph indicates that a factor VIII antibody is present at a level of 1.75 Bethesda units. If the patient sample was diluted prior to incubation with reference plasma, 1.75 would be multiplied by the dilution factor to obtain the final result

Laboratories that offer Bethesda assays should also provide similar assays against porcine factor VIII. These assays are similar to the above-mentioned method except that porcine factor VIII is substituted for human plasma. The anti-porcine factor VIII level provides useful clinical information in that if the patient has high antibody levels towards human factor VIII, but low levels against porcine factor VIII, porcine factor VIII may be a treatment option.

Figure 6.6 illustrates a factor VIII Bethesda assay calibration curve that converts residual factor VIII activity measured by incubating patient and reference plasma to Bethesda inhibitor units.

6.16 Key Points

- The gold standard assays for evaluating platelet function are platelet aggregation methods using either whole blood or platelet-rich plasma. Discontinuing patients' anti-platelet medications prior to testing is essential to appropriate interpretation of platelet aggregation results.
- The most common inherited bleeding disorder is von Willebrand's disease (vWD); diagnosis is best achieved with the vWD panel (factor VIII activity, von Willebrand factor antigen, ristocetin cofactor activity), with multimeric analysis used to classify the vWD type.

- With the exception of vWD, factor VIII and factor IX deficiencies, other inherited bleeding disorders are very uncommon. It may be most appropriate for reference laboratories to perform these esoteric tests.
- Pediatric reference intervals are now available and should be used to correctly classify younger patients with possible bleeding disorders.

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Testing for Acquired Platelet Disorders

7

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Acquired platelet disorders can be broadly categorized into disorders of platelet number and disorders of platelet function, though there can be overlap (e.g., myeloproliferative disorders). The hematology laboratory traditionally assesses platelet number using automated hematology analyzers, with targeted confirmation by manual counting. Platelet antibody assays may be performed by the immunology and/or coagulation laboratories, or by the transfusion service. Platelet function testing has traditionally been performed by special coagulation laboratories using platelet-rich plasma or whole blood platelet aggregation. However, new platelet function analyzers are being marketed as potential substitutes for traditional platelet aggregometry (see Chap. 3).

7.1 Platelet Counting Methods

7.1.1 Reference Method

Until relatively recently, the gold standard for platelet counting was the phase contrast manual count. Patient whole blood is diluted and loaded onto a hemocytometer and platelets are counted in a defined volume of sample. The raw counts are converted into a platelet concentration by a calculation that takes into account the dilution factor and the number and volume of the squares counted [1]. While this method is still utilized in the laboratory as a backup for automated methods, it

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suffers from poor precision and accuracy due to the small sample size that is evaluated. This created difficulties for manufacturers of automated hematology analyzers, since the reference method was less precise and accurate than the methods that they were attempting to validate. This problem was solved in 2001 when the International Council for Standardization in Haematology adopted a cytoflourometric-based platelet counting standard that labels platelets with two platelet-specific antibodies: anti-CD 61 and anti-CD 41 [2]. The ratio of platelet to red blood cell (RBC) events counted by cytoflourometry is multiplied by the RBC count produced by an automated hematology analyzer to derive the platelet concentration (counts/μL).

7.1.2 Automated Platelet Counters

Automated hematology analyzers use electromagnetic fields to count blood cells, including platelets. The oldest method, the impedance method, uses static electrical fields, and volume-dependent resistance to current produced by blood cells as they pass through an aperture to identify and size platelets. Different field frequencies can be overlaid to optimize an analyzer's ability to differentiate cell types. The impedance method is susceptible to interference from non-cellular (e.g., cryoglobulins) and cellular particulate debris (e.g., RBC and white blood cell fragments), as well as microcytic red blood cells and RBC ghosts that have a volume distribution that overlaps that of platelets. Overlap of the platelet distribution with microcytic RBCs generally limits the ability of impedance counters to detect large platelets. Sophisticated computer algorithms have been designed to minimize interferences with the impedance platelet count, including mathematical extrapolation to include counts of platelets larger than the actual measurement range [3].

A second approach to platelet counting uses light or fluorescence scatter detected at different angles to identify, count and size platelets [3]. Information obtained from optical detection methods provides better differentiation of platelets from microcytic RBCs allowing direct counting of large platelets [4]. Therefore, optical counting has been shown to be more accurate than impedance counting when specimen selection is weighted towards conditions that produce microcytosis (e.g., immune-mediated hemolysis), red cell fragmentation (e.g., thrombotic thrombocytopenic purpura) and large platelets (e.g., autoimmune thrombocytopenic purpura) [5]. However, comparisons involving random specimen selection, or thrombocytopenic specimens collected from oncology patients demonstrate excellent correlation of both optical and impedance methods with counts produced by the international cytoflourometric reference method [5, 6]. Of particular importance, current impedance and optical methods are sufficiently accurate to guide transfusion therapy at platelet count triggers of 15–20,000/μL [6, 7]. Transfusion triggers of 5–10,000/μL are probably beyond the capability of all current platelet-counting methods, with the possible exception of an immunologic assay [6, 8–10].

7.1.2.1 Pre-analytic Considerations

Pre-analytic factors must be considered when evaluating a low platelet count. Specimen clotting and platelet clumping due to insufficient mixing or platelet activation of the sample during phlebotomy must be ruled out by microscopy on any patient presenting with apparent thrombocytopenia by automated analysis. Hematology analyzers flag platelet clumps with variable sensitivity and specificity. A new specimen should be collected in the case of clotting or platelet clumping. Use of protocols to disaggregate platelet clumps in an attempt to produce an accurate platelet count should be used cautiously, and only after appropriate validation [11]. Pseudothrombocytopenia – *in vitro* platelet clumping due to anticoagulant-dependent (primarily EDTA), platelet autoantibodies – can also be ruled out by microscopy. Interference due to pseudothrombocytopenia can usually be overcome by collecting specimens to be analyzed for platelet counts in a sodium citrate or ACD tube. Counts from citrate or ACD tubes should be corrected for the dilutional effect of the liquid anticoagulant (10 % dilution for sodium citrate and 20 % for ACD).

7.2 Platelet Antibody Testing

Human platelets express a variety of platelet-specific antigens, as well as class I human leukocyte antigens (HLA) that are targets for alloimmunization. In addition, neo-antigens may be formed as a result of interactions between native platelet antigens and therapeutic drugs (e.g., heparin). Antibodies may be formed against these antigenic structures, as a result of infusion of therapeutic drugs, or exposure to non-self, platelet-specific antigens through pregnancy, transfusion of platelet-containing blood components or hematopoietic allo-transplantation (active immunization), or immunoglobulin administration (passive immunization). Finally, autoantibodies directed against glycoproteins common to all normal platelets may be produced after an acute infection, as a result of an autoimmune disease, or without a clear precipitating cause (idiopathic).

7.2.1 Immune-Mediated Thrombocytopenia

Immune thrombocytopenia results when antibody bound to a patient's platelets (allo- or auto-antibody) interacts with the reticulo-endothelial system and platelets are sequestered and destroyed. Alloimmune thrombocytopenia may occur as a result of active transfer by the placenta of platelet-specific (and possibly HLA) IgG antibodies into the fetal circulation and subsequent clearance of fetal platelets (neonatal alloimmune thrombocytopenia or NAIT); or as a result of binding of platelet-specific, alloantibody (produced secondary to prior pregnancy or platelet transfusion), to transfused platelets resulting in destruction of both transfused and native, bystander platelets (post-transfusion purpura or PTP). Alloimmune thrombocytopenia may also occur as a result of platelet-specific antibody production by passenger lymphocytes carried in a solid organ transplant, or after hematopoietic

allo-transplantation when the recipient immune system makes antibodies against the donor's platelet antigens. On the other hand, with the exception of rare cases of HLA-induced NAIT [12], production of HLA antibodies doesn't produce thrombocytopenia. Instead, HLA antibodies pre-dispose platelet transfusion-dependent patients to platelet transfusion refractoriness – poor increments in platelet count after transfusion of non-HLA matched platelet products.

Autoimmune thrombocytopenic purpura (AITP) results when patients produce antibodies against their own platelets resulting in rapid clearance of both the patient's and transfused platelets due to the fact that the antibodies are directed against platelet glycoproteins common to all normal platelets (GPIIb/IIIa and/or GPIb), rather than a platelet-specific antigen. A patient's own platelets may also be destroyed as a result of drug-induced, immune thrombocytopenia (DITP). This may result from an immune response against neoantigens formed as a result of drug-platelet interaction, or as a result of the production of non-drug dependent autoantibodies.

7.2.2 HLA Antibody Tests

Circulating HLA antibodies are routinely detected and identified using indirect methods including antibody-mediated cytotoxicity assays, cytofluorometric assays using manufactured microbeads coupled to purified HLA glycoproteins, or ELISA assays using immobilized, purified HLA glycoproteins bound to microtiter wells.

7.2.3 Platelet Antibody Tests

Testing for platelet antibodies has undergone significant evolution over the years [13]. Phase I tests were designed to detect the effect of serum/plasma platelet antibodies on control platelets after incubation. Test endpoints included platelet aggregation, platelet activation, platelet lysis, and release of platelet granule contents. These tests were primarily used to detect autoantibodies, but had poor sensitivity and specificity, and survive today as the serotonin release assay used for diagnosis of heparin induced antibodies, and aggregation methods for detecting the effects of cardiopulmonary bypass, platelet inhibitory drugs, and heparin dependent antibodies. Phase II assays measure platelet-associated immunoglobulins that associate with platelets both non-specifically and through specific binding to antigens. Total platelet associated immunoglobulin assays measure intracellular IgG released by platelet lysis, in addition to surface associated immunoglobulins. Since non-specific platelet binding of immunoglobulins is common in thrombocytopenic disorders other than ITP, these assays proved to be relatively non-specific, though very sensitive for ITP.

The development of phase III assays provided the opportunity to improve specificity for platelet autoantibody detection, as well as a platform for the identification of alloantibodies through the use of specific platelet glycoprotein targets. In the simplest form of these assays, the ability to type platelet glycoprotein antigens allows for screening of patients' serum or plasma against antigen-defined, donor

platelets with subsequent detection of bound antibody through immunofluoresence detected by cytofluorometry. This format can be modified to detect drug-dependent antibodies by incubating the platelets with the test drug prior to incubation with patient serum/plasma.

In the more sophisticated assays, antigenically defined, immunopurified or solubilized platelet glycoproteins are captured on a solid phase support (e.g., plastic wells, beads) for testing. A patient's serum/plasma or an eluate prepared from a patient's own platelets (autoantibody) or from sensitized donor platelets (auto- or alloantibody) is incubated with the glycoproteins, and binding of antibody is detected using labeled, anti-human antibody. Detection can be accomplished through radioactivity, standard ELISA methods, immunofluoresence or RBC agglutination. In a variation of this format, auto- or alloantibody is bound to patient or donor platelets, respectively, before platelet solubilization. The antibody-glycoprotein complex is then captured and the bound antibody is detected as already described. The advantage of this test format is that the glycoprotein epitopes are in their native state when antibody is bound, and not modified by the purification process.

7.2.4 Testing for Heparin-Dependent Antibodies

The administration of heparin to patients can result in the formation of heparin-dependent antibodies that are directed against a neoantigen produced by the association of heparin with platelet factor 4 (PF4). The production of heparin-dependent antibodies can result in heparin-induced thrombocytopenia (HIT) – a clinical syndrome characterized by thrombocytopenia and a risk for primarily venous, but also arterial thrombosis [14]. The College of American Pathologists recommends that reference laboratories validate more than one assay for the detection of heparin dependent antibodies to maximize sensitivity and specificity [15]. An immunologic assay and a functional assay are routinely employed in the evaluation of patient serum for heparin dependent antibodies.

The PF4 ELISA tests are immunoassays that employ PF4 complexed with either heparin or a polyanionic compound to bind heparin dependent antibodies [16]. Positive reactions can be confirmed by inhibiting antibody binding by adding excess heparin to the reaction. The PF4 ELISA assay is a sensitive assay ($\geq$90 %), but less specific ($\leq$90 %) than the serotonin release assay (SRA) discussed below. The particle gel immunoassay (PaGIA), another immunoassay, uses PF4-heparin complexes bound to colored particles to bind heparin-dependent antibodies that results in agglutination of the antibody bound particles. The particles are centrifuged through gel columns such that agglutinated particles remain at the top of the gel, while free particles migrate to the bottom of the column. These particles can also be adapted for use in a cytofluorometry-based assay. The PaGIA exhibits slightly lower sensitivity than the ELISA assays, but is closer to the SRA in specificity [16]. Three automated assays, a latex agglutination assay and two chemiluminescent assays, have entered the market and are awaiting FDA approval. These assays appear to have excellent sensitivity and negative predictive values [17].

Due to less complicated test formats, the PF4 immunoassays are frequently used as screening tests for patients suspected of a diagnosis of HIT. PF4 immunoassays that detect only IgG antibodies demonstrate better specificity, without loss of sensitivity [18]. A strong positive result has greater predictive value for a diagnosis of HIT than a weak positive, and the British Committee for Standards in Haematology (BCSH) [18], the International Society on Thrombosis and Haemostasis [19] and the American College of Chest Physicians (ACCP) [14] recommend reporting the optical density value of a test as well as the cutoff value for a positive result. In addition, the BCSH recommends reporting the degree of inhibition of positive results by high dose heparin, however, false negative inhibition studies may occur with samples containing strong antibodies that produce very high optical density values [19].

The SRA is a functional assay that identifies heparin dependent antibodies through induction of ≥20 % serotonin release from donor platelets in the presence of low concentrations of heparin (0.1–0.3 U/mL), and ≥50 % inhibition of that release in the presence of high concentrations of heparin (100 U/mL). SRA test sensitivity reportedly ranges from 90 to 98 % depending upon the expertise of the laboratory, and specificity is generally ≥95 % except when the decrease in platelet count occurs 5 or more days after heparin exposure [13]. Due to the high specificity of this test, it is considered the "gold standard" method. However, the requirement for fresh platelets susceptible to heparin dependent antibodies, the need to wash the platelets to maximize sensitivity, and the use of radioisotopes to detect serotonin release makes this assay impractical for most laboratories. Consequently, it is generally used as a confirmatory test [14].

One laboratory has reported a high rate of false-negative results on the initial specimens collected from patients suspected of having HIT [16]. In a retrospective review, one third of negative results by immunologic assay turned positive on subsequent specimens collected from patients with continuing suspicion of HIT. Therefore, in cases where clinical suspicion for HIT is high, retesting of additional specimens collected after a negative result may be indicated. However, increased sensitivity will be accompanied by decreased specificity if routine repeat testing is performed [19].

Screening patients with a prior history of HIT for heparin-PF4 antibodies is indicated before treating them with heparin during subsequent operative procedures. The risk of recurrent HIT is low if the pre-operative EIA screen is negative and the patient receives heparin solely during the intra-operative period. Many patients will form recurrent, platelet activating heparin-PF4 antibodies after heparin re-exposure, but avoiding post-operative heparin administration significantly decreases the risk of HIT [20].

7.2.5 Clinical Utility of Platelet Antibody Tests

Platelet antibody testing is indicated for the detection of alloantibodies in cases of NAIT, post-transfusion purpura (PTP), and for heparin-dependent antibodies in heparin-induced thrombocytopenia (HIT). Even though the platelet count recovers

after withdrawal of heparin, confirmation of HIT is helpful in guiding decisions about further anticoagulation therapy [20]. Testing for other DITP antibodies is generally not necessary (or practical), since thrombocytopenia routinely resolves after withdrawal of the implicated drug, and non-cross-reacting drugs can generally be substituted for the original drug. Testing for autoantibodies in suspected cases of autoimmune thrombocytopenia is more controversial. Phase II tests for platelet associated immunoglobulins are too non-specific to be useful. Phase III tests that identify binding of autoantibody to platelet-specific glycoproteins appear to have improved positive predictive value, but poor negative predictive value. The American Society of Hematology does not recommend testing for autoantibodies in suspected cases of AITP [21].

7.3 Platelet Function Testing

Acquired platelet dysfunction may be produced by disease states or by administration of therapeutic drugs or interventions. Light transmission aggregometry employing platelet rich plasma is the gold standard for identifying platelet dysfunction, however, availability is limited to specialty laboratories. Consequently, devices more suitable for use in the standard laboratory or at the point of care have been developed and evaluated for routine use, including whole blood aggregometry (see Chap. 3, Instrumentation for the Coagulation Laboratory). Thromboelastography is often considered a measurement of platelet function (as well as global hemostasis) since platelets play an important role in clot formation; however, modifications to standard protocols are required to fully assess platelet function.

7.3.1 Myeloproliferative Disorders

Myeloproliferative disorders may be complicated by platelet dysfunction. Platelet function testing is not indicated in these disorders, however, since it is not predictive of clinical outcome [22].

7.3.2 Uremia

Renal failure predisposes patients to bleeding secondary to anemia due to decreased displacement of circulating platelets towards vessel walls by red blood cells, and due to uremia. Uremia leads to the overproduction and accumulation of nitric oxide in the circulation that directly inhibits platelet function [23]. Uremic patients are at increased risk for spontaneous bleeding as well as excessive bleeding as a complication of invasive procedures – most commonly renal biopsy. Dialysis or treatment with desmopressin acetate (DDAVP) significantly reduces the bleeding risk. Unfortunately, the level of uremia cannot predict the risk of bleeding. The bleeding time test has traditionally been used to assess bleeding risk, but the predictive value

of the test is poor [24]. Preliminary evaluations of two other methods have been reported. In one study, the PFA-100® failed to detect uremia-induced platelet dysfunction after the hematocrit of the samples had been normalized [25]. In a second study, thromboelastography demonstrated positive and negative predictive values of only 71 and 74 %, respectively, for post-biopsy, renal hemorrhage [26].

7.3.3 Antiplatelet Drugs

Interest in monitoring antiplatelet drugs has focused primarily on aspirin and the thienopyridines (principally clopidogrel). Clinical interest in measuring anti-platelet effects has been two-fold: to determine the potential for an increased risk of bleeding during and after invasive procedures, and to determine the degree of platelet inhibition in patients taking aspirin, clopidogrel or both. Resistance to the platelet inhibitory effects of aspirin, as documented by laboratory testing, has been clearly demonstrated, though the incidence of resistance is controversial due to differences in assays and cutoff values defining resistance. There is evidence that this "aspirin resistance" can result in treatment failure and increased thrombotic events [27–29]. However, identifying aspirin resistance through platelet function testing has not proven useful in predicting patient outcomes and routine testing is therefore not recommended [30, 31].

The increased number of patients taking aspirin for prevention of cardiovascular events means more patients present for invasive procedures with aspirin on-board. The ACCP and the Society of Thoracic Surgeons (STS) have published recommendations for continuation/discontinuation of antiplatelet therapy perioperatively [30, 32]. For patients continuing aspirin therapy in the perioperative period, platelet testing has not proven to be predictive of excess bleeding and routine testing is not recommended [30, 32].

In the case of clopidogrel, it has been suggested that there may be a therapeutic window between excessive bleeding and adequate anti-thrombotic effect [33]. Due to a risk of excessive bleeding, the STS recommends discontinuation of clopidogrel for a few days before cardiovascular operations, and even 1–2 days when patients require urgent operation. The ACCP recommends discontinuing clopidogrel therapy 5 days before coronary artery bypass surgery [30]. The STS considers it reasonable to assess platelet function to estimate the risk of bleeding due to anti-platelet activity, rather than using an arbitrary number of days off drug to determine the surgical delay [32]. The ACCP perioperative management guideline does not support platelet function testing for this purpose, because the assays don't adequately predict clinical outcomes [30].

As many as one-third of patients taking clopidogrel for the prevention of cardiovascular thrombotic events do not attain adequate inhibition of platelet function due to inadequate metabolism of the pro-drug into the active form. High platelet reactivity poses a significant risk for coronary stent thrombosis [33]. Several clinical trials have assessed whether platelet function testing can be used to optimize

thienopyridine therapy in response to high platelet reactivity on therapy either through dosage escalation (clopidogrel) or change to a thienopyridine that is not affected by metabolism (e.g., prasugrel) [33]. Unfortunately, none of the clinical trials to date has demonstrated a benefit from platelet function testing of patients at low risk for thrombosis. Therefore, routine platelet function testing is currently not recommended, though use in high-risk patients is under study and may ultimately prove useful.

7.3.4 Cardiovascular Surgery

Cardiopulmonary bypass (CPB) procedures result in a demonstrable, transient platelet function defect that has been correlated with post-operative bleeding. Proposed causes of this platelet dysfunction include the platelet effects of high-dose heparin administered before and during the procedure, activation of platelets upon exposure to the materials that make up the CPB circuit, hypothermia, and platelet effects of protamine administration post-procedure [34]. Recent ingestion of aspirin, clopidogrel or both, frequently adds to the deleterious effect of CPB on platelet function.

Measurement of these individual or combined effects on platelet function has been reported to predict blood loss associated with CPB surgery, and clinicians have applied this observation to the incorporation of platelet function testing into transfusion protocols for cardiac surgery. Many of these trials have reported a significant decrease in allogeneic transfusions and blood loss [35]. However, it is generally difficult to sort out the relative importance of the implementation of a strict transfusion protocol versus the utility of the rapid assessment of hemostasis and platelet function at the bedside in the improved outcomes. Cardiac surgery transfusion practice can be highly variable across surgeons and institutions [36, 37], and implementation of a strict protocol, with structured indications for blood component transfusion and surgical re-exploration, most likely contributes significantly to decreased transfusions. The availability of rapid assessments of hemostasis with excellent negative predictive value for ruling out coagulopathy as a cause of excessive bleeding guides the surgeon's approach to the differential diagnosis of excessive bleeding most likely resulting in earlier intervention to control surgical bleeding [38–40]. The relative importance of platelet function testing in these transfusion algorithms has yet to be determined.

7.4 Summary and Key Points

Excessive bleeding (and less commonly thrombosis) may result from acquired platelet disorders that include thrombocytopenia and/or platelet dysfunction. There have been significant improvements in platelet counting and antibody detection methods over the years. There has also been a concurrent development of automated platelet function analyzers that are designed for routine use in the laboratory or at

the patient's bedside. The clinical role for routine platelet function testing, if any, has yet to be defined.

- An immunologic-based reference method has been described for use by manufacturers and reference laboratories for the validation of automated platelet counters.
- Optical- and impedance-based platelet count analyses perform comparably in the general patient population, and can guide transfusion therapy at platelet count triggers of 15–20,000/μL.
- Testing for autoantibodies in suspected cases of AITP is not recommended.
- Tests for heparin-dependent antibodies are indicated for the diagnostic workup of HIT. Patients with a history of HIT may be anticoagulated with unfractionated heparin during subsequent cardiac surgery if the EIA screen is negative and postoperative heparin is avoided.
- Clinical indications for acquired platelet dysfunction testing have not been sufficiently defined, nor have outcomes been sufficiently validated to warrant routine use.

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Acquired Coagulation Disorders and TTP

8

George M. Rodgers

The most common causes of acquired clotting factor deficiencies associated with bleeding are decreased or abnormal synthesis of clotting factors caused by liver disease or disseminated intravascular coagulation (DIC). The latter is seen in many severe illnesses, including metastatic cancer and infectious diseases. Vitamin K deficiency is another common cause of bleeding especially in hospitalized patients. Uncommon causes of acquired coagulation bleeding disorders include antibodies to coagulation factors and abnormal fibrinolysis [1].

Thrombotic thrombocytopenic purpura (TTP) is encountered in clinical practice primarily as an acquired, thrombotic, autoimmune disorder directed against the ADAMTS-13 protease that is responsible for the processing of ultra-high-molecular-weight multimers of von Willebrand factor (UHMWM-vWF) to normal vWF [2].

All assays discussed in this chapter have FDA approval except for the ADAMTS-13 assay for TTP diagnosis.

8.1 Liver Disease and Vitamin K Deficiency

The liver is the major site of clotting factor synthesis, and bleeding can occur in patients with severe hepatitis or cirrhosis. In these disorders, coagulation protein synthesis is reduced (all clotting factors but factor VIII and von Willebrand factor). In addition to altered coagulation protein levels, other hemostatic defects exist with liver disease, including decreased clearance of activated clotting factors and

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increased levels of degradation products of fibrinogen and fibrin. Fibrin degradation products inhibit hemostasis by interfering with both platelet function and fibrin formation. Fibrinolysis also may be enhanced in liver disease [1].

Significant liver disease, with associated portal hypertension and splenomegaly, can result in mild to moderate thrombocytopenia. Splenomegaly results in splenic sequestration of platelets. Hepatoma and cirrhosis also are associated with synthesis of qualitatively abnormal fibrinogen (dysfibrinogen) [1].

When vitamin K is deficient, the vitamin K-dependent clotting factors do not bind calcium, and although they are synthesized in normal amounts, are inactive. Vitamin K deficiency can be seen in patients with hepatic or biliary disease, malabsorption, malnutrition, warfarin therapy, or antibiotic treatment [1].

Vitamin K deficiency is a clinical diagnosis. Hospitalized patients who have screening coagulation test results of an isolated, prolonged prothrombin time (PT) or prolongation of both the PT and partial thromboplastin time (PTT) values, and that have clinical associations as outlined above will likely have vitamin K deficiency; no further laboratory tests are usually necessary. For patients in whom the diagnosis is uncertain, a D-dimer test will rapidly evaluate DIC (see Chap. 5). The response to oral or parenteral vitamin K is also diagnostic of vitamin K deficiency with improvement or normalization of the PT and PTT within 24 h. If laboratory diagnosis of vitamin K deficiency is important, factor assays demonstrating low levels of the vitamin K-dependent coagulation proteins (prothrombin, VII, IX, X) with normal levels of the other coagulation proteins (e.g., factors V and VIII) confirm vitamin K deficiency.

8.2 DIC

The basis for DIC is excessive thrombin formation, which may result in thrombi in the microcirculation, and/or depletion of platelets and fibrinogen, leading to a bleeding tendency. Common causes of DIC include sepsis, burn or traumatic injuries, obstetrical complications, and cancer. The hallmark of all of these disorders is unregulated thrombin activity in blood [1].

Figure 8.1 illustrates the consequences of thrombin activity in blood: thrombin cleavage of fibrinopeptides A and B from fibrinogen generates fibrin monomers; polymerization of fibrin monomers form fibrin polymers; factor $XIII_a$ cross-linking activity generates insoluble fibrin polymers; and lastly, the fibrinolytic mechanism produces fibrin degradation products (FDPs), including D-dimer, a clinically-useful FDP whose presence in large amounts confirms the diagnosis of DIC (see Chap. 5).

8.3 Antibodies to Coagulation Factors

Rarely, patients with previously-normal hemostasis who develop prolonged PT and/or PTT assays and bleeding will not have either vitamin K deficiency or DIC. These patients will probably have acquired antibodies against a clotting factor, usually

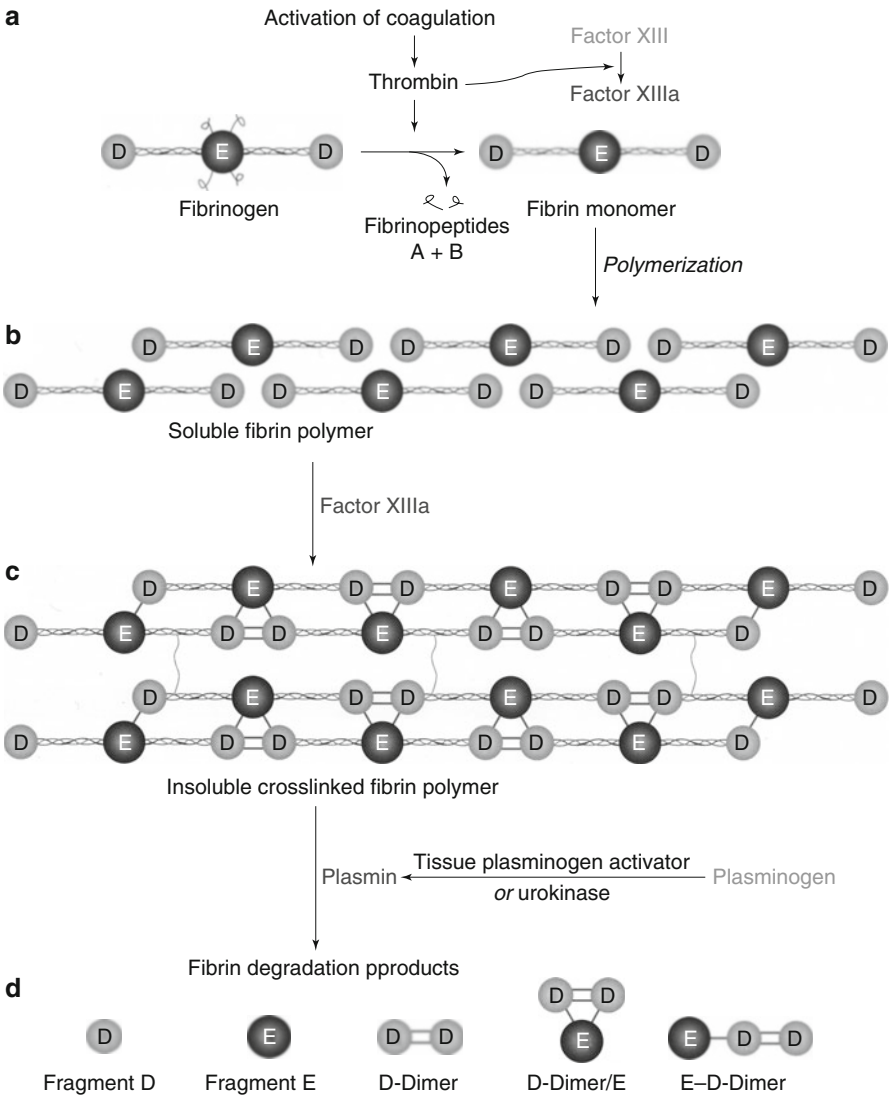

Fig. 8.1 Generation of cross-linked fibrin by thrombin and factor XIIIa following activation of coagulation, and consequences of plasmin degradation of cross-linked fibrin. (**a**) Following activation of coagulation, thrombin activates the transglutaminase, factor XIII to factor XIIIa, and cleaves fibrinopeptides A and B from fibrinogen to generate fibrin monomers. (**b**) Fibrin monomers align longitudinally and with adjacent monomers to form fibrin polymers. (**c**) Factor XIIIa cross-links the D-domains of fibrin monomers to form rigid fibrin polymers. (**d**) Plasminogen activators (TPA or UK) convert plasminogen to plasmin; plasmin degrades cross-linked fibrin to fibrin degradation products, including fragment D, fragment E, and D-dimer, in addition to other products

factor VIII or factor V [1]. The appropriate evaluation is to first perform mixing studies (see Chap. 5) to determine if an inhibitor (antibody) is present. Next, specific factor assays confirm the affected factor. Thus, for a patient with previously-normal PT and PTT values who develops bleeding with an isolated, prolonged PTT, failure to correct the prolonged PTT with a mixing study, followed by assay of factors VIII, IX, XI would diagnose this patient's acquired bleeding disorder. Antibodies to factor VIII are the most commonly seen.

For patients who have acquired prolonged PT and PTT values, performing mixing studies with both the PT and PTT assays is indicated, followed by assay of common pathway coagulation proteins—fibrinogen, prothrombin, factor V, and factor X. Of these clotting factors, antibodies to factor V are most commonly seen.

In addition to antibody inhibitors, heparin-like inhibitors may occasionally be seen, causing coagulopathy and bleeding in patients with malignant disease. Heparin-like inhibitors are suspected in bleeding patients who have prolonged PTT values that do not correct with mixing, normal PT values, markedly prolonged thrombin time values, normal reptilase assays, and positive results for heparin using an anti-factor X_a assay.

8.4 Abnormal Fibrinolysis

Abnormal fibrinolysis is an uncommon bleeding disorder in which excessive plasmin is generated. Plasmin is appropriately generated following initiation of coagulation and thrombin formation (secondary fibrinolysis). For example, patients with DIC have secondary fibrinolysis which is appropriate. In contrast, in abnormal or primary fibrinolysis, there is no laboratory evidence for thrombin formation (negative D-dimer). Examples of clinical conditions associated with abnormal fibrinolysis include inherited α_2-antiplasmin deficiency, tumors of the genito-urinary tract, gynecologic tumors, acute promyelocytic leukemia, liver disease, and fibrinolytic drugs [1].

The diagnosis of abnormal fibrinolysis is suggested by a low fibrinogen level, elevated FDP levels, a negative D-dimer assay, and an underlying disorder as outlined above. The presence of an elevated D-dimer level indicates that the abnormal fibrinolysis is secondarily due to excessive thrombin formation, probably DIC.

8.5 Thrombotic Thrombocytopenic Purpura

Thrombotic thrombocytopenic purpura usually presents as an acquired thrombocytopenic disorder in adults. The pathologic basis for TTP is autoimmune; patients acquire an auto-antibody to the protease responsible for processing ultra-high-molecular-weight multimers of von Willebrand factor to normal vWF. This protease is known as the vWF-cleaving protease or ADAMTS-13 [2]. Failure to process

UHMWM-vWF leads to disseminated platelet thrombosis and the clinical manifestations of TTP-microangiopathic hemolytic anemia, thrombocytopenia, neurologic abnormalities, fever, and renal dysfunction. TTP may rarely be inherited (Upshaw-Schulman syndrome); in these cases, the disorder presents in childhood in patients with mutations in the ADAMTS-13 gene [2].

TTP is primarily a clinical diagnosis because the fulminant nature of the disease requires emergent therapy. However, since the recognition of the basis of the disease, assays have been developed to more rapidly provide a diagnosis. Previous methods were based on demonstrating the presence of UHMWM-vWF in TTP patient plasma; this method required an immunoblot technique and took 1–2 days turn-around time. More recent methods quantitate ADAMTS-13 activity using a fluorogenic or chromogenic substrate (vWF73) that provides assay results in a few hours. Results are expressed in terms of ADAMTS-13 activity in normal, pooled plasma; most patients with classic TTP will have ADAMTS-13 levels <5–10 % of normal [3].

Even with the availability of ADAMTS-13 assays, results may take 1–2 days to be reported; however, clinical decisions on patient management (i.e., whether to use plasma exchange, or not) must be made urgently. One group of investigators reported that readily-available clinical data (platelet count, D-dimer, creatinine, indirect bilirubin, reticulocyte count) obtained at patient presentation can be used as a prediction score to rapidly identify which patients with thrombotic microangiopathy will be found to have severe ADAMTS-13 deficiency and respond to plasma exchange [4].

Patients with hemolytic-uremic syndrome (HUS) generally will have normal or only mildly decreased ADAMTS-13 levels; therefore, ADAMTS-13 activity assays and the above mentioned clinical prediction score provide a means to distinguish TTP from HUS [4, 5].

8.6 Summary and Key Points

- Acquired coagulation disorders are most commonly caused by vitamin K deficiency, liver disease, and DIC. The clinical situation, routine hemostasis tests (PT, PTT, platelet count) and a D-dimer assay will usually suggest the correct diagnosis.
- Uncommon acquired coagulation disorders include antibodies to clotting factors (usually factors VIII or V) and are suggested by results of the routine hemostasis tests (PT, PTT) and mixing tests.
- Abnormal fibrinolysis is suggested by the appropriate clinical situation, a low fibrinogen level, elevated FDP levels, and a normal D-dimer assay.
- The diagnosis of TTP remains primarily clinical; however, availability of assays for the ADAMTS-13 protease (FRETS-vWF73 assay) offers the possibility of a rapid laboratory confirmation of the diagnosis.

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Testing for Inherited and Acquired Thrombotic Disorders

9

George M. Rodgers

Virchow's triad, the major risk factors that predispose to thrombosis, include vascular injury, stasis, and hypercoagulability. Arterial and venous thrombosis have different pathogenic mechanisms. For example, arterial thrombosis primarily involves vascular injury (atherosclerosis) and platelet deposition. Venous thrombosis primarily involves stasis of blood flow and hypercoagulability, which is defined as altered blood composition associated with a thrombotic tendency. Fibrin deposition is the normal consequence of venous thrombosis.

The laboratory evaluation of inherited thrombosis is based on identifying abnormalities in regulatory proteins of coagulation; also known as natural anticoagulants, as well as mutations of other coagulation proteins. Over the past 20 years, major inherited etiologies of thrombosis including the factor V Leiden and prothrombin gene mutations have been identified. Acquired etiologies of thrombosis, including cancer, antiphospholipid antibodies, and hyperlipidemia are more common than inherited etiologies. This chapter will summarize the pathologic basis for thrombosis as related to laboratory testing for inherited and acquired etiologies. Methods for laboratory evaluation of thrombosis will be presented, and the utility of laboratory thrombosis testing will be discussed. All assays discussed in this chapter have FDA approval.

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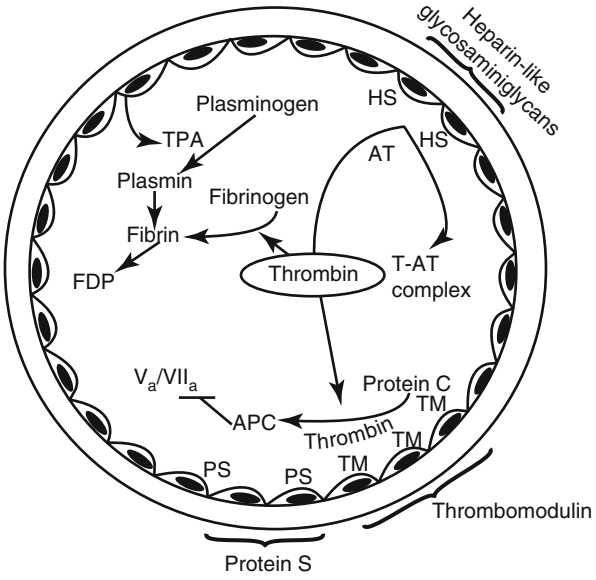

Fig. 9.1 Regulation of thrombin activity by vascular endothelium. The effects of major antithrombotic properties of the blood vessel wall are shown. Regulation of thrombin activity is important because thrombin is a major factor in thrombosis (platelet activation, fibrin formation). Abnormalities in regulation of thrombin activity may lead to hypercoagulability and an increased risk of thrombosis. Major antithrombotic properties include: heparin-like glycosaminoglycans (heparan sulfate, *HS*) on the luminal surface that catalyze antithrombin (*AT*) inhibition of thrombin, generating inactive thrombin-antithrombin (*T-AT*) complexes; thrombomodulin (*TM*), an endothelial cell receptor for thrombin. The thrombin-TM complex converts protein C to APC; protein S (*PS*) functions as a cofactor binding protein for APC, permitting inactivation of factors $V_a/VIII_a$, resulting in inactivation of coagulation; and endothelial cell secretion of tissue-plasminogen activator (*TPA*) that initiates fibrinolysis. Most of the coagulation components shown in this figure can be assayed by the laboratory to identify thrombosis risk (From Rodgers [2], with permission)

9.1 Pathophysiology of Thrombosis

Vascular endothelium plays a pivotal role in maintaining thromboresistance of the blood. The coagulation mechanism is modulated by several endothelial cell regulatory mechanisms [1]. Figure 9.1 summarizes the antithrombotic properties of endothelium that can be evaluated in the laboratory. A key regulatory mechanism is the protein C pathway, which consists of two vitamin K–dependent plasma proteins—protein C and protein S. Protein C is converted to an active form, activated protein C (APC); this activation is mediated by an endothelial cell receptor, thrombomodulin. Thrombomodulin forms a complex with thrombin; this complex activates protein C generating APC, which in turn inactivates factors Va and VIIIa. Protein S binds to the endothelial cell surface, providing a receptor for APC [1]. A factor V gene mutation called factor V Leiden results in thrombosis due to the inability of APC to

degrade the abnormal factor V_a molecule. This phenomenon is termed APC resistance and can be measured by the laboratory [3].

Antithrombin is a natural anticoagulant that irreversibly binds to and inactivates activated clotting factors such as factor Xa and thrombin. This inactivation is catalyzed by heparin-like glycosaminoglycans on the endothelial cell surface (see Fig. 9.1) or by therapeutic heparin [1].

The fibrinolytic mechanism components include plasminogen, tissue-plasminogen activator (TPA), plasmin, α_2 – antiplasmin, and plasminogen activator inhibitor (PAI-1). Fibrinolysis is initiated when vascular thrombosis triggers endothelial cell secretion of TPA. In the presence of TPA, plasminogen is converted to plasmin that degrades fibrin clots. Plasmin activity is regulated by α_2 – antiplasmin, while TPA activity is regulated by PAI-1. Either deficiency or excess of these components may occur leading to hypofibrinolysis and a thrombotic risk or hyperfibrinolysis and a bleeding risk [1].

9.2 The Inherited Thrombotic Disorders

Table 9.1 summarizes the inherited thrombotic disorders and describes their prevalence, inheritance patterns, and clinical features. Abnormalities of the protein C pathway (protein C, protein S, factor V Leiden, thrombomodulin) constitute most cases of inherited thrombosis [3]. Most inherited disorders are transmitted in an autosomal dominant manner, and venous thromboembolism is the usual clinical feature. The importance of inherited fibrinolytic disorders (TPA deficiency or excess PAI-1 activity) is uncertain.

Another common inherited thrombotic disorder is the prothrombin gene mutation. This mutation is associated with elevated plasma prothrombin levels, which may explain the predisposition to thrombosis [5].

Homocysteinemia is a metabolic disorder associated with thrombosis. Although pediatric patients present clinically with homozygous homocysteinemia (homocystinuria), adult patients heterozygous for homocysteinemia have primarily premature arterial disease (myocardial infarction, stroke, peripheral vascular disease). Heterozygous homocysteinemia may account for a significant number of patients with arterial vascular disease in the absence of traditional risk factors (e.g., smoking, hypertension, hyperlipidemia). Between 1 and 2 % of the general population have heterozygous homocysteinemia. Homocysteinemia is also associated with venous thromboembolism [6].

A recently described inherited risk factor for thrombosis is elevated levels of factor VIII activity. Although factor VIII is an acute-phase response protein, as many as 10–20 % of patients with recurrent venous thrombosis have elevated factor VIII levels as their only risk factor [3].

Increased levels of other coagulation factors, including fibrinogen, factor IX, and factor XI have also been associated with thrombosis. However, routine laboratory testing of these factors is not recommended by the College of American Pathologists Consensus Conference on Thrombophilia (see Table 9.2).

Table 9.1 Summary of the inherited thrombotic disorders

Classification and disorders	Inheritance	Estimated prevalence[a]	Clinical features
Deficiency or qualitative abnormalities of inhibitors to activated coagulation factors			
AT deficiency	AD	1 %	Venous thromboembolism (usual and unusual sites), heparin resistance
TM deficiency	AD	1–5 %	Venous thrombosis
Protein C deficiency	AD	1–5 %	Venous thromboembolism
Protein S deficiency	AD	1–5 %	Venous and arterial thromboembolism
APC resistance due to Factor V Leiden	AD	20–50 %	Venous thromboembolism
Abnormality of coagulation zymogen or cofactor			
Prothrombin mutation	AD	5–10 %	Venous thromboembolism
Elevated factor VIII	Unknown	20–25 %	Venous thromboembolism
Elevated factor IX	Unknown	~10 %	Venous thromboembolism
Elevated factor XI	Unknown	~10 %	Venous thromboembolism
Impaired clot lysis			
Dysfibrinogenemia	AD	1–2 %	Venous thrombosis >arterial thrombosis
Plasminogen deficiency	AD, AR	1–2 %	Venous thromboembolism
TPA deficiency	AD	?	Venous thromboembolism
Excess PAI-1 activity	AD	?	Venous thromboembolism and arterial thrombosis
Metabolic defect			
Homocysteinemia	AR	1 in 300,000 live births;	Arterial and venous thrombosis (homozygous patients);
		10–25 % of patients with recurrent thrombosis	Premature development of coronary and cerebral coronary and cerebral arterial thrombotic disease (heterozygous patients)

Source: Robetorye and Rodgers [4]
Abbreviations: *AT* antithrombin, *APC* activated protein C, *TPA* tissue plasminogen activator, *PAI-1* plasminogen activator inhibitor-1, *TM* thrombomodulin, *AD* autosomal dominant, *AR* autosomal recessive, *?* uncertain prevalence of abnormal fibrinolysis
[a]Prevalence data are estimated by pooling information from studies in which large groups of patients with thrombosis were screened for these disorders. Results are expressed in terms of a percentage that each disorder might constitute of the total patient population with inherited thrombosis (Assays for TM mutations are not widely available)

9.3 General Principles of Thrombosis Testing

1. Laboratory evaluation should be postponed until 2–3 months after the acute thrombotic event when the patient is clinically well and has not received anticoagulant therapy for 2 weeks. Thrombosis induces an acute-phase response that makes

Table 9.2 Summary of the College of American Pathologists' recommendations on laboratory testing for inherited thrombosis

Thrombotic disorder	Who should be tested?	Test method(s)	Comments
Factor V Leiden	First VTE at age <50 years Recurrent VTE First unprovoked VTE First VTE, unusual site First VTE, positive family history First VTE related to pregnancy or hormonal therapy Unexplained second or third trimester pregnancy loss	APC resistance assay using factor-V deficient plasma or DNA-based assay	Patients with relatives who are known to have FVL should be tested directly with DNA-based assays. Patients with positive APC resistance assays should have confirmatory DNA tests.
Prothrombin gene mutation	As above	DNA-based assay	Prothrombin activity assays should not be used
Homocysteinemia	Arterial vascular disease Controversial for VTE	HPLC or immunoassays	Genotyping for MTHFR mutations is not recommended. Fasting may not be necessary. Proper sample processing is necessary. Testing in VTE patients may be appropriate to identify and treat affected patients with vitamins.
Protein C deficiency	Infants with neonatal purpura fulminans VTE patient from a family with known PC deficiency	Chromogenic substrate assays are preferred	Avoid testing during acute thrombosis or anticoagulant therapy. Exclude causes of acquired PC deficiency. Consider age-dependent reference ranges.
	Asymptomatic female from a known PC-deficient family prior to hormonal therapy	Functional assays are useful Immunologic assays are discouraged	

(continued)

Table 9.2 (continued)

Thrombotic disorder	Who should be tested?	Test method(s)	Comments
Protein S deficiency	Patient with VTE from a family with known PS deficiency	Functional assay or Immunoassay for free PS Total PS antigen assays not recommended	Abnormal functional assay results should be confirmed with an immunoassay for free PS. Exclude acquired causes of PS deficiency. Avoid testing during acute thrombosis, anticoagulant therapy, and pregnancy. Consider age- and gender-dependent reference ranges.
Antithrombin deficiency	Patient with VTE from a family with known AT deficiency Asymptomatic female from a known AT-deficient family prior to hormonal therapy	Chromogenic substrate assays are preferred AT antigen assays not recommended	Exclude acquired causes of AT deficiency. Avoid testing during acute thrombosis or anticoagulant therapy.
Elevated factor VIII levels	Controversial	Factor VIII activity assay	Test 6 months after thrombosis. Avoid anticoagulant therapy.
Dysfibrinogenemia	Not recommended		
Heparin cofactor II	Not recommended		
Factor XIII polymorphisms	Not recommended		
Plasminogen activator inhibitor-1	Not recommended		
Plasminogen deficiency	Test in non-DVT patients with ligneous conjunctivitis		

Source: Greer et al. [7], with permission
From the College of American Pathologists' Consensus Conference on Thrombophilia [8], with permission
FVL factor V Leiden, *VTE* venous thromboembolism, *APC* activated protein C, *HPLC* high performance liquid chromatography, *MTHFR* methylenetetrahydrofolate reductase, *PC* protein C, *PS* protein S, *AT* antithrombin

interpretation of coagulation-based tests difficult. Reliable data for assays such as antithrombin, protein C, and protein S activities are best obtained in the absence of anticoagulant therapy. If anticoagulants cannot be discontinued in the affected patient, then surrogate testing of symptomatic family members who are not receiving anticoagulants can be done. However, if DNA-based assays (for the factor V Leiden or the prothrombin gene mutations) are performed, these results will not be affected by acute-phase changes of thrombosis or anticoagulant therapy. Similarly, homocysteine testing will not be affected by acute-phase changes or anticoagulant therapy. If factor VIII activity testing is to be done, it should be deferred until 6 months after the thrombotic event [3].

2. The probability of obtaining positive thrombosis testing results is increased if the patient population being investigated is restricted to young patients (<50 years of age) with recurrent thrombosis or patients with a single event and a positive family history for thrombosis [3].
3. Functional coagulation assays are recommended over immunologic assays for evaluation of antithrombin, protein C, or protein S deficiencies. Functional assays detect both quantitative deficiency and qualitative abnormality of the protein. On the other hand, functional assays are affected by anticoagulant therapy, and interpretation of abnormal functional assay results must take into account whether the patient is receiving anticoagulants [3].
4. Assay for common etiologies first (factor V Leiden/APC resistance, prothrombin gene mutation, homocysteinemia) [3].
5. Heterozygous homocysteinemia should be considered as a cause for thrombosis in middle-aged patients with premature vascular disease as well as a cause of venous thrombosis [3].

9.4 Laboratory Testing Strategy for Inherited Thrombosis

There are two testing strategies for inherited thrombosis-arterial and venous etiologies. Most cases of arterial thrombosis are not inherited, but acquired, including disorders such as diabetes, hyperlipidemia, and other causes of atherosclerosis, plus other etiologies such as vasculitis, myeloproliferative disorders, etc. Inherited etiologies for arterial thrombosis include elevated levels of PAI-1, homocysteinemia, and some patients with protein C or S deficiencies.

In contrast to the limited etiologies for arterial thrombosis, inherited venous thrombosis testing is more detailed. Figure 9.2 depicts one suggested strategy for testing in the venous thrombosis setting [10]. Assay of the most common etiologies is done initially (factor V Leiden/APC resistance, prothrombin gene mutation, homocysteinemia). If these tests are normal, then evaluation of uncommon causes of venous thrombosis are done (antithrombin, protein C, protein S). If the patient is to be evaluated for elevated factor VIII levels, this should be deferred for at least 6 months.

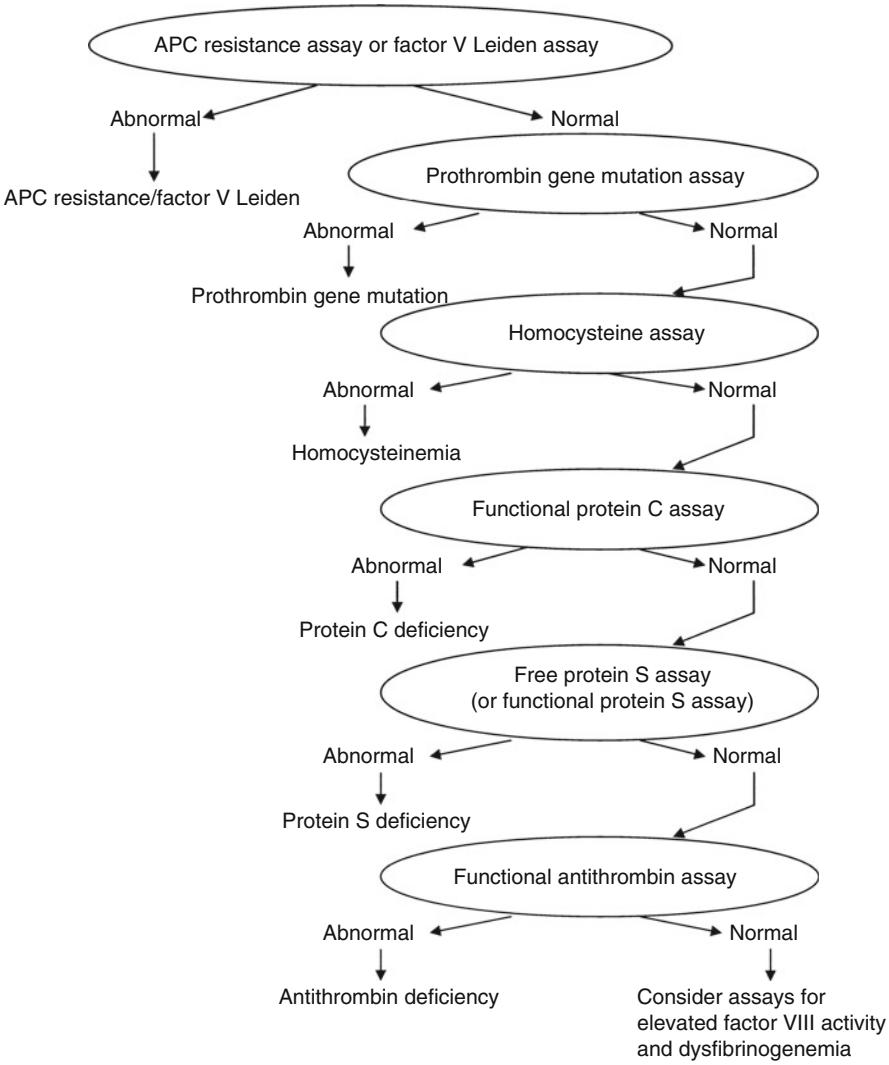

Fig. 9.2 An algorithm approach for the evaluation of patients with inherited venous thrombosis. It is assumed that patients have been appropriately selected and that certain assays (antithrombin, protein C, protein S) will not be performed until anticoagulation has been completed. The CAP Consensus Panel recommends confirmation of positive results for APC resistance assays with the factor V Leiden DNA test. Testing for elevated factor VIII levels is controversial, but if this is to be done, testing should be deferred until 6 months after the thrombotic event when anticoagulation has been discontinued (From Rodgers and Chandler [9], with permission)

9.5 Utility of Inherited Thrombosis Testing

The two major reasons to evaluate patients for inherited thrombosis are: (1) to screen family members for whom an inherited diagnosis may change management, and (2) to identify an inherited thrombotic disorder in the patient that may affect

their management (e.g., intensity or duration of anticoagulation) [11]. There is justification for the first reason to test, because for example, if a patient has female siblings or children who might also inherit a thrombotic disorder, this would potentially affect their treatment with oral contraceptives, or change counseling regarding pregnancy. In contrast, there is limited justification to test patients for the second reason (a thrombosis test result affects patient management). Several clinical trials in the past few years have produced results suggesting that positive test results for inherited thrombotic disorders do not predict recurrence of thrombosis; therefore, a positive result would not change patient treatment. Also, there is no data that patients with an inherited thrombotic disorder require a different intensity of anticoagulation than thrombosis patients without an inherited disorder [11]. One conclusion from this data is that testing should primarily be done if there are potentially affected female family members. It should also be noted that comprehensive testing can be expensive – between $1,000 and $2,000 for a single patient evaluation.

9.6 Laboratory Tests for Inherited Thrombosis

This section will briefly describe the principles of interpretation of common assays used to evaluate etiologies of inherited thrombosis. Most assays are available as commercial kits with standard methodologies that can be automated.

9.7 Activated Protein C Resistance Assay

APC resistance due to the factor V Leiden mutation is a very common cause of inherited venous thrombosis, especially in Caucasian populations. Test options include a clotting-based assay or a DNA-based test [12, 13].

APC is an anticoagulant that will prolong the clotting time of normal plasma, but patients with the factor V Leiden (or certain other) mutations will exhibit clotting times that are less prolonged (i.e., these plasmas exhibit APC resistance). In the APC resistance assay, two determinations are made, one with and one without the addition of APC. A ratio is obtained [e.g., Partial Thromboplastin Time with APC/Partial Thromboplastin Time without APC] and compared with that of a normal population. The ideal APC resistance assay uses either prothrombin time (PT) or partial thromboplastin time (PTT)-based assays in which patient plasma is diluted in factor V-deficient plasma. Using factor V-deficient plasma makes the test useful in patients who are receiving heparin or warfarin therapy, or who have lupus anticoagulants. If a normalized assay is done (the APC ratio of the patient is divided by the APC ratio of a control pooled plasma sample from the same test run), this method can distinguish normal vs. heterozygotes vs. homozygotes with the factor V Leiden mutation. If the laboratory has test samples from large numbers of patients receiving heparin or warfarin, and the local assay method does not use factor V-deficient plasma dilutions, then uninterpretable results may occur. In this situation, it may be preferable to perform factor V Leiden DNA testing.

9.8 Factor V Leiden DNA Test

With the discovery of the factor V Leiden mutation and the prothrombin gene mutation, each due to highly conserved point mutations, the utility of molecular diagnostic testing for thrombosis was enhanced.

For diagnosis of factor V Leiden, the original method used the restriction enzyme *Mnl* to digest a 267-bp amplified fragment of patient DNA. Digestion of normal patient DNA results in three fragments. A variety of molecular methods are available (summarized in the CAP Consensus Conference report) [13], including fluorescent detection of real-time PCR products, as well as non-PCR based methods.

Although DNA-based assays will be more expensive than the APC resistance assays, there will be no interferences, and the result should unequivocally be either normal, or heterozygous or homozygous for the factor V Leiden mutation. Negative test results for the factor V Leiden mutation will not exclude APC resistance due to other genetic defects (~5 % of patients with APC resistance).

9.9 Prothrombin Gene Mutation Assay

As with the factor V Leiden mutation, the prothrombin gene mutation is mostly seen in Caucasian populations. Although many patients with this mutation will have elevated levels of prothrombin activity, it is recommended that patients be tested for the specific DNA abnormality [5].

Multiple molecular diagnostic methods are available for assay, including restriction endonuclease digestion, automated fluorescence detection, or real-time fluorescence detection. Results are reported as normal, heterozygous, or homozygous for the prothrombin gene mutation [5].

The typical prothrombin gene mutation is G20210A. A variant prothrombin gene mutation has been reported in African-American patients – C20209T. This latter mutation may be under-recognized because standard PCR assays for the G20210A mutation may not detect the variant mutation. Laboratories that evaluate large numbers of African-American patients for thrombosis testing should consider assays for the C20209T mutation.

9.10 Homocysteine Assays

Unlike the other coagulation analytes discussed in this chapter that are linked to thrombosis, homocysteine is an amino acid whose levels can be elevated in inherited or acquired circumstances. It is usually assayed in the chemistry laboratory. Elevated levels of homocysteine may be associated with arterial or venous thrombosis, but consensus opinion suggests focusing on evaluation of patients with arterial thrombosis (Table 9.2) [6].

There are also molecular diagnostic tests available to identify mutations in the methylene tetrahydrofolate reductase (MTHFR) gene; however, consensus opinion is that testing for the MTHFR mutation should not be performed [6].

Earlier data suggested obtaining fasting samples for homocysteine measurement; this is no longer recommended. Either plasma or serum samples can be used, but collected blood samples should be placed on ice and red cells promptly separated. Quantification in the past was done by high-performance liquid chromatography, but the availability of fluorescence-based immunoassays has expanded the use of homocysteine measurement in smaller laboratories.

9.11 Protein C Assay

Unlike the highly conserved point mutations of factor V Leiden or the prothrombin gene mutations, deficiencies of protein C, protein S, and antithrombin are caused by numerous mutations. Consequently, molecular diagnostic tests are not clinically useful in evaluating patients for these disorders.

A variety of antigenic and functional assays are available to measure protein C levels [14]. Antigenic assays include ELISA, electroimmunoassay (Laurell rocket technique), and radioimmunoassay. The antigenic assays measure protein C levels (normal and des-carboxy forms), so these methods are not affected by oral anticoagulant therapy. However, antigenic assays will not measure protein C function and will therefore not detect qualitative protein C abnormalities. Functional protein C assays are preferred since they will detect both quantitative and qualitative protein C deficiency. Functional assay methods include clotting and chromogenic techniques. A common functional assay method uses Protac®, a snake venom activator of protein C. The APC generated is then measured in a PTT-based assay (clotting method) or in an amidolytic assay (chromogenic substrate method). The CAP Consensus Study recommends use of the latter chromogenic assay, primarily because therapeutic heparin levels do not affect assay results. Anticoagulant therapy and elevated factor VIII levels will affect the clot-based methods [14]. However, the Russell's viper venom-based clotting method containing heparin neutralizer eliminates the effects of both elevated factor VIII levels and therapeutic heparin.

One important aspect of interpreting protein C levels is that there is age-dependence for protein C levels; younger patients may not be correctly classified unless pediatric reference intervals for protein C levels are considered. Table 6.4 summarizes pediatric reference intervals for coagulation analytes, including protein C.

Numerous acquired variables impact protein C levels (Table 9.3), and interpretation of abnormal results should be made with caution. It is useful for the laboratory to provide a comment on factors that may result in low protein C levels, to assist clinicians in correct interpretation of the test. Ideally, evaluation of protein C deficiency should be done at a time distant from the acute thrombotic event when anticoagulation has been completed for at least 2 weeks.

9.12 Protein S Assay

As with protein C assays, antigenic and functional methods are available to measure protein S levels [16]. Total protein S antigen assays can be performed using ELISA methods, Laurell rocket technique, radioimmunoassay, etc. Functional assays can

Table 9.3 Acquired conditions associated with protein C, protein S, and antithrombin deficiency

Analyte	Causes of deficiency
Protein C	DIC
	Acute thrombosis
	Vitamin K deficiency, including oral anticoagulant therapy
	Newborn infants, children
	Liver disease
	Post-operative state
Protein S	DIC
	Acute thrombosis
	Inflammatory illness of any cause
	Vitamin K deficiency, including oral anticoagulant therapy
	Newborn infants, children
	Liver disease
	Pregnancy
	Nephrotic syndrome
Antithrombin	DIC
	Acute thrombosis
	Liver disease
	Oral contraceptives
	Nephrotic syndrome
	Pregnancy
	Heparin (therapeutic levels)

Source: Kjeldsberg et al. [15], with permission
Conditions, drugs, or diseases listed in this table may result in acquired deficiency of protein C or S, or antithrombin. These causes should be considered before evaluating patients for inherited deficiency
Abbreviation: *DIC* disseminated intravascular coagulation

be either based on PT or PTT clotting assays. The "gold standard" test for protein S has been identified as free protein S antigen levels [16]. Unlike protein C measurement, functional protein S assays are less useful because factor V Leiden (APC resistance) interferes with functional protein S assays results. For example, spuriously low protein S levels may be seen in patients with factor V Leiden when tested with functional assays for protein S.

Free protein S levels can be measured by monoclonal antibody-ELISA. Functional protein S assays are based on PT or PTT assays. Diluted patient plasma is added to protein S-deficient plasma in the presence of APC and factor Va for the PTT format assay. Positive results in the functional assay should be confirmed with another assay method, e.g., a free protein S assay.

Numerous acquired conditions may result in protein S deficiency (Table 9.3), and these must be considered in the interpretation of a low protein S test result. It is useful to comment on these acquired etiologies when reporting the test results to

assist clinician interpretation. Age-dependent reference ranges are necessary to correctly classify patients (Table 6.4). Additionally, males have higher mean plasma levels of protein S, so gender should also be taken into account when reporting protein S levels.

9.13 Antithrombin Assay

Functional and antigenic assays are available to measure antithrombin levels [17]; functional methods are preferred since many antithrombin-deficient patients have qualitatively-abnormal molecules that would be missed if antigenic assays were used. Functional assays for antithrombin measure heparin cofactor activity using a chromogenic substrate method to assay thrombin or factor Xa inhibition. Antigenic methods include Laurell immunoelectrophoresis, radial immunodiffusion, and microlatex particle immunoassay [17].

Plasma antithrombin levels can be markedly decreased by therapeutic heparin, and long-term warfarin treatment may increase antithrombin levels. Therefore, patients should be tested off of anticoagulant therapy. Other acquired conditions that can affect antithrombin results are listed in Table 9.3. Pediatric reference ranges should be used to correctly classify laboratory results of children (see Chap. 6, Table 6.4).

9.14 Laboratory Testing for Other Inherited Thrombotic Disorders

Assays exist to measure numerous other analytes that have been linked to thrombosis, for example, dysfibrinogenemias, heparin cofactor II, and fibrinolytic components (plasminogen, tissue plasminogen activator, and plasminogen activator inhibitor-1). However, the association between these parameters and thrombosis is either weak or unproven, and the CAP Consensus Conference has recommended that they not be tested for (Table 9.2).

Assay for elevated factor VIII levels is controversial. Factor VIII is an acute-phase response protein, so elevated levels would be seen during the acute event. Also, the assay for factor VIII is clot-based, so heparin therapy would affect the measurement. A summary of the CAP recommendations on thrombophilia testing is given in Table 9.2.

9.15 The Acquired Thrombotic Disorders

Table 9.4 summarizes the acquired thrombotic disorders. These include autoimmune disorders such as vasculitis and antiphospholipid antibodies, hematologic disorders such as the myeloproliferative disorders, paroxysmal nocturnal hemoglobinuria, thrombotic thrombocytopenic purpura, and heparin-associated thrombocytopenia with thrombosis, metabolic disorders such as obesity, diabetes

Table 9.4 Acquired thrombotic disorders

Autoimmune disease
Vasculitis
Antiphospholipid antibodies
Inflammatory bowel disease
Hematologic diseases
Myeloproliferative disorders (polycythemia vera, essential thrombocytosis)
Plasma cell disorders (Waldenstrom's macroglobulinemia)
Heparin – associated thrombocytopenia with thrombosis
Paroxysmal nocturnal hemoglobinuria
Metabolic disorders
Diabetes
Hyperlipidemia
Obesity
Miscellaneous disorders
Trauma
Post-surgery
Cancer
Hormone therapy
Nephrotic syndrome

and hyperlipidemia, and miscellaneous disorders such as pregnancy, nephrotic syndrome, hormone therapy, and cancer. Laboratory evaluation of thrombotic thrombocytopenic purpura and heparin-associated thrombocytopenia are discussed in Chaps. 7 and 8. D-dimer assays which are frequently elevated in cancer patients with thrombosis are discussed in Chap. 5. Antiphospholipid antibodies are frequently assayed in the coagulation laboratory and will be discussed in this chapter.

Testing for antiphospholipid antibodies (anticardiolipin antibodies, antibodies to β_2-glycoprotein-1, lupus anticoagulant) is appropriate for thrombosis patients (arterial and venous), especially if they have idiopathic clots without a family history, or if there is autoimmune disease. Testing for antiphospholipid antibodies is also appropriate for evaluating patients with recurrent miscarriage. Criteria for diagnosis of antiphospholipid antibody syndrome require demonstration of antibody persistence for 12 weeks (Table 9.5).

9.16 Anticardiolipin Antibody Assay

A standardized ELISA is recommended using high-sensitivity microtiter plates; these plates permit greater antigenic density and improve detection of antibodies [19]. The buffer system is also important. Reporting of antibody titer is critical in interpretation of an anticardiolipin antibody ELISA result. Moderate or high titer antibody levels are required for diagnosis, and the laboratory should provide clinicians with an interpretive comment that allows correct classification of patients.

Table 9.5 Revised clinical and laboratory criteria for the antiphospholipid antibody syndrome

Clinical criteria	
Vascular thrombosis –	Arterial or venous or small vessel
or	
Pregnancy morbidity –	One or more unexplained deaths of a morphologically-normal fetus at or beyond 10 weeks gestation
	or
	One or more premature births of a morphologically – normal neonate before the 34th week of gestation because of eclampsia/ preeclampsia or placental insufficiency
	or
	Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation (with exclusion of anatomic, hormonal or genetic causes)
Laboratory criteria	
Anticardiolipin antibody:	(I_gG/I_gM), in moderate or high-titer, present on at least 2 occasions 12 weeks apart, measured by standardized ELISA
or	
Lupus anticoagulant:	Present on at least 2 occasions 12 weeks apart measured by ISTH criteria (Table 9.6).
or	
Anti-β_2-glycoprotein-1 antibody:	(I_gG/I_gM), present on at least 2 occasions 12 weeks apart, with levels >99th percentile measured by a standardized ELISA

Source: Miyakis et al. [18]
Definite APS is present if at least one clinical and one laboratory criteria are present

Positive results for IgG antibodies are considered the most important, although positive results for IgM antibodies that persist may also be clinically important. The utility of IgA anticardiolipin antibody testing is uncertain.

A consensus conference on laboratory criteria used to diagnose antiphospholipid antibody syndrome also recommends ELISA testing for antibodies to β_2-glycoprotein-1. Antibody (IgG/IgM) levels >99th percentile that persist for at least 12 weeks are required to meet this diagnostic criterion. Table 9.5 summarizes the clinical and laboratory criteria for diagnosing patients with antiphospholipid antibody syndrome.

9.17 Lupus Anticoagulant (LA) Assay

The LA is an antiphospholipid antibody that affects phospholipid-based coagulation assays; it is clinically associated with thrombosis and miscarriage. The LA antibody actually recognizes a protein in a phospholipid-protein complex; the protein is usually β_2-glycoprotein-1 or prothrombin. Assays to detect the LA are coagulation-based, and therefore can be impacted by numerous variables that affect clot-based tests, including sample collection, processing, and the presence of anticoagulants.

Table 9.6 ISTH Criteria for laboratory diagnosis of the LA

Prolongation of at least one phospholipid-dependent coagulation test with platelet – poor plasma (PTT, dilute PT, DRVVT, kaolin clotting time, etc.)
Failure to correct the prolonged clotting time when patient and normal plasma are mixed
Correction of the originally-prolonged clotting time by addition of excess phospholipid
Exclusion of other inhibitors to coagulation (heparin, factor VIII antibodies)

Source: Brandt et al. [21]
Abbreviations: *ISTH* International Society of Thrombosis and Haemostasis, *LA* lupus anticoagulant

A good quality phlebotomy (non-activated sample) is necessary. Platelet-poor plasma (platelet count <10,000 μl) is critically important; if the sample platelet count is higher, many LAs will not be detected since platelet phospholipid in a frozen-thawed sample will neutralize antibody activity [18–20].

Guidelines have been published recommending laboratory criteria for diagnosis of the LA. It is suggested that more than one test system (phospholipid-dependent coagulation assay) be used to optimize identification of the LA. Test options include the PTT, dilute PT, dilute Russell's Viper venom time (DRVVT), kaolin clotting time, Taipan venom test and Textarin time. International Society of Thrombosis and Haemostasis (ISTH) guidelines for laboratory detection of the LA are summarized in Table 9.6.

If the screening test demonstrates a prolonged clotting time, a mixing study is performed with normal plasma. If the mixing study result fails to correct, then an inhibitor is suspected. When excess phospholipid or hexagonal-phase phospholipids are added to the screening test sample, the prolonged clotting time shortens or corrects, validating that the originally prolonged clotting time was due to antibodies to phospholipid. Lastly, other inhibitors to coagulation should be excluded clinically or by the laboratory (heparin, factor VIII antibodies).

"Integrated" coagulation reagents have been developed that permit testing for the LA using the ISTH criteria. For example, a DRVVT kit is available that uses reagents for screening and confirmation of the LA. Similarly, the Staclot LA® test also uses a methodology to screen and confirm the LA. Some of the integrated LA kits contain a heparin neutralizer that permits performing the test on patients receiving heparin therapy.

9.18 Utility of Laboratory Thrombosis Testing

The availability of hypercoagulability or thrombosis panels and identification of new thrombophilia disorders has led to increased test ordering of these assays. There are two reasons to test patients – to screen other family members (especially females) who may be similarly affected, and to possibly change the management of the patient being tested. The current literature is controversial on this subject, with some investigators insistent that widespread testing is useful, while others state that the information obtained is of limited usefulness. Based on the

literature (summarized in the review article by Rondina et al.), several conclusions can be drawn:

1. Testing for the inherited disorders (factor V Leiden, prothrombin gene mutation, protein C and S deficiencies, antithrombin deficiency) is of limited utility in guiding treatment for the affected patients. Large clinical trials have demonstrated that positive results for these tests do not predict recurrent thrombosis, and therefore, do not assist clinicians in determining duration of anticoagulation.
2. Testing for the above inherited disorders may be useful if the patient being tested has female siblings or children for whom a diagnosis might change management (e.g., hormonal therapy).
3. Testing for homocysteinemia is controversial. While patients who have this diagnosis made can be treated with vitamins, at this time, there is no data that shows a clear-cut clinical benefit from suppressing homocysteine levels.
4. Testing for antiphospholipid antibodies (anticardiolipin antibodies, antibodies to β_2-glycoprotein, lupus anticoagulant) is helpful in patient management, not because it changes the intensity of anticoagulation, but rather because patients should be anticoagulated for the duration that antiphospholipid antibodies persist.

Most clinicians are not aware of these limitations of thrombosis testing. Laboratory directors should educate their clinicians so that they are aware of when thrombosis testing can be helpful (family screening), what tests to order (functional assays for the natural anticoagulants), and when to order them (not acutely; not while the patient is anticoagulated), etc.

9.19 Key Points

- The most common inherited thrombotic disorders are activated protein C resistance (factor V Leiden), the prothrombin gene mutation, and homocysteinemia.
- Antithrombin deficiency, and deficiencies of proteins C and S are very uncommon. Optimal assays for these analytes include functional coagulation methods or free protein S antigen assay.
- If assays for antithrombin, protein C, and protein S are to be done, postpone testing until the patient has completed anticoagulation and the patient is clinically well.
- Be aware that inherited thrombosis testing is most useful for family screening. There is little, if any, data that such results alter management of the patient with thrombosis.
- Pediatric reference intervals are now available for inherited thrombosis analytes (antithrombin, protein C, and protein S deficiencies).
- Appropriate testing for antiphospholipid antibodies includes standardized assays for anticardiolipin antibodies, β_2-glycoprotein-1 antibodies, and the lupus anticoagulant.

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Monitoring of Anticoagulant Therapy

10

Sterling T. Bennett

10.1 Monitoring Anticoagulant and Antiplatelet Therapy

Anticoagulant and antiplatelet medications are widely used in the prevention and treatment of thromboembolism. For many years, the antithrombotic therapeutic armamentarium was limited essentially to heparin, warfarin, and aspirin, but over the past several years the number of medications has increased substantially, with a consequent demand for better understanding of the laboratory aspects of therapeutic monitoring.

Laboratory monitoring falls into two broad categories: general monitoring and specific monitoring. General monitoring is directed toward the assessment of bleeding or other untoward effects of therapy. Tests include hematocrit, hemoglobin, platelet count, occult blood, and so forth. The need for general monitoring is common to all anticoagulants and will not be discussed in detail in this chapter.

Specific monitoring is directed toward the assessment of the specific antithrombotic effects of a given medication and is the focus of this chapter. The basics of antithrombotic therapies and the laboratory tests used in their monitoring will be discussed. A summary of anticoagulant monitoring is provided in Table 10.1.

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Table 10.1 Summary of anticoagulant monitoring

Anticoagulant	Mode of action	Plasma half-life	Monitoring test(s)	Therapeutic range	Reversal	Notes
Warfarin (vitamin K antagonists)	Interference with post-translational γ-carboxylation of vitamin K-dependent clotting factors	20–60 h	INR	2.0–3.0, moderate intensity 2.5–3.5, high intensity	Vitamin K, prothrombin complex concentrates (PCC), fresh frozen plasma, recombinant factor VIIa	
Heparin, unfractionated, standard-dose IV or high-dose SC	Potentiation of AT inactivation of factors IIa and Xa	0.5–2.5 h	PTT Anti-Xa heparin assay	Method-specific 0.3–0.7 IU/mL	Protamine sulfate	The PTT therapeutic range endpoints are the PTT responses corresponding to the anti-Xa heparin therapeutic range
Heparin, unfractionated, low-dose SC	Potentiation of AT inactivation of factors IIa and Xa		None	N/A	None	
Heparin, unfractionated, high-dose IV	Potentiation of AT inactivation of factors IIa and Xa	0.5–2.5 h	ACT	Procedure-specific	Protamine sulfate	Target ACT is determined by the nature of the procedure, e.g., cardiac catheterization, cardiopulmonary bypass, ECMO, etc.
Heparin, LMW	Potentiation of AT inactivation of factors IIa and Xa	3–4 h	Anti-Xa LMW heparin assay	0.6–1.1 IU/mL, twice daily dosing 1.0–2.0 IU/mL, once daily dosing (for enoxaparin)	Partial reversal by protamine	Monitoring of LMW heparin concentration is not routinely required, but is indicated in renal impairment, morbid obesity or low body weight, prolonged therapy, or young age

Fondaparinux	Potentiation of AT inactivation of factor Xa	17–21 h	None routinely. Anti-Xa fondaparinux assay for renal impairment.	0.3–0.6 mg/L	None	Monitoring of fondaparinux concentration is not routinely required, but is indicated in renal impairment
Argatroban	Inhibition of thrombin by reversibly binding to its active catalytic site	24 min	PTT ACT (PCI and CPB procedures)	1.5–3.0 times baseline, not to exceed 100 s >300 s	None	Therapeutic targets may not be reliable for all PTT assays due to the highly variable dose-response
Bivalirudin	Inhibition of thrombin by reversibly binding to its active catalytic site and substrate-binding site	25 min	None routinely ACT for renal impairment	Procedure-specific	None	Dosage is weight-based. Monitoring is not routinely required, but patients with renal impairment should be monitored with the ACT with a therapeutic range determined by clinical protocol
Dabigatran	Inhibition of thrombin by reversibly binding to its active catalytic site	12–17 h	None	N/A	None	When laboratory assessment is needed, the ECT, ECA, or PDTT are preferred tests, but not generally available. A normal TT rules out the presence of dabigatran. A normal PTT essentially rules out therapeutic concentrations

(continued)

Table 10.1 (continued)

Anticoagulant	Mode of action	Plasma half-life	Monitoring test(s)	Therapeutic range	Reversal	Notes
Rivaroxaban	Competitive inhibition of active site of factor Xa	5–9 h	None	N/A	None	When laboratory assessment is needed, the anti-Xa assay calibrated with rivaroxaban calibrants is preferred, but not generally available. A normal PT essentially rules out therapeutic concentrations
Apixaban	Competitive inhibition of active site of factor Xa	~12 h	None	N/A	None	When laboratory assessment is needed, the anti-Xa assay calibrated with apixaban calibrants is preferred, but not generally available. A normal PT essentially rules out therapeutic concentrations

This table identifies tests for monitoring the anticoagulant effect only. Other tests required for general monitoring of anticoagulation therapy, including hemoglobin or hematocrit, platelet count, occult blood, creatinine, etc., are not specifically listed

Abbreviations: *ACT* activated clotting time, *PTT* activated partial thromboplastin time, *AT* antithrombin, *INR* International Normalized Ratio, *LMW* low molecular weight, *IV* intravenous, *SC* subcutaneous, *PCI* percutaneous coronary intervention, *CPB* cardiopulmonary bypass, *ECT* ecarin clotting time, *ECA* ecarin chromogenic assay, *PDTT* plasma-diluted thrombin time, *TT* thrombin time

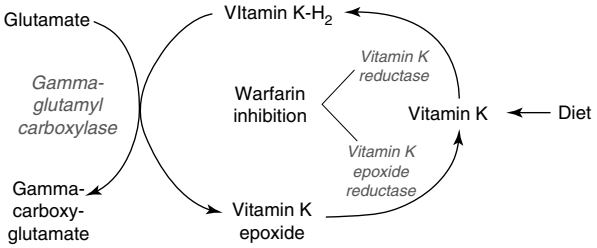

Fig. 10.1 Vitamin K cycle and warfarin inhibition of key enzymes

Table 10.2 Half-lives of vitamin-K-dependent proteins

Protein	Half-life, hours
Factor II	60
Factor VII	4–6
Factor IX	24
Factor X	48–72
Protein C	8
Protein S	30

10.2 Warfarin

Warfarin is an oral anticoagulant medication, widely used for the prevention of thromboembolic events, including deep venous thrombosis (DVT), pulmonary embolism (PE), myocardial infarction, and stroke [1, 2].

10.2.1 Mechanism of Action

Warfarin is one of the coumarins or vitamin K antagonists. It does not have direct anticoagulant properties, but exerts its effect by inhibiting the vitamin K pathway. Clotting factors II, VII, IX, and X, and the antithrombotic proteins C and S are synthesized in the liver as inactive proteins. Vitamin K-H_2, reduced vitamin K, is an essential cofactor in the post-translational γ-carboxylation of 10–12 glutamate residues on these proteins, creating calcium-binding sites required for activity. In this process, vitamin K-H_2 is oxidized to vitamin K epoxide. In a two-step process catalyzed by the enzymes vitamin K epoxide reductase and vitamin K reductase, vitamin K-H_2 is regenerated and becomes ready to participate in another γ-carboxylation. The vitamin K cycle and its inhibition by warfarin are illustrated in Fig. 10.1. Vitamin K reductase is less sensitive to warfarin than is vitamin K epoxide reductase, enabling dietary or parental vitamin K to be reduced and overcome the effects of warfarin [1].

Warfarin decreases the availability of vitamin K-H_2 and decreases the number of calcium-binding sites on newly synthesized clotting factors. Active clotting factors are not affected by warfarin, but as they are metabolized, hypofunctional proteins replace them. Thus the onset of warfarin's effect is related to the half-life of the vitamin-K-dependent clotting factors, shown in Table 10.2. A partial effect is

achieved quickly due to the short half-life of factor VII, but the full onset of anticoagulation takes several days because of factor II's long half-life.

10.2.2 Rationale for Laboratory Monitoring

The therapeutic effectiveness of warfarin is well established, but therapeutic monitoring is required for several reasons [1]. First, warfarin has a relatively narrow therapeutic window. Under-anticoagulation greatly reduces warfarin's therapeutic efficacy, while over-anticoagulation greatly increases the risk of bleeding. Severe bleeding episodes may be fatal or lead to severe morbidity. Second, the dose-response between individuals is highly variable and may be quite variable in the same individual over time. Third, warfarin's effect may be either potentiated or inhibited by a large number of medications. For example, potentiators include acetaminophen, erythromycin, miconazole, propranolol, and cimetidine, and inhibitors include barbiturates, prednisone, carbamazepine, nafcillin, and cholestyramine, to name a few. Manufacturer's information provides over 120 examples of agents that may interfere with or potential the effect of warfarin [2]. Fourth, warfarin's effect is influenced by fluctuations in dietary vitamin K intake. Dietary vitamin K is obtained principally from green leafy vegetables, olive oil, soybean oil, cottonseed oil, and canola oil and, to a lesser extent, from butter, margarine, liver, milk, ground beef, coffee, and pears. Green tea and chewing tobacco are also rich in vitamin K. Multivitamins and herbal remedies are additional sources of vitamin K. The half-life of vitamin K is only about 1½ days, so continual intake is required, and changes in vitamin K intake affect the anticoagulant activity of warfarin within days. Fifth, coexisting diseases or illnesses may affect the absorption and metabolism of warfarin and vitamin K and the synthesis of clotting factors. Sixth, as with many medications, patient compliance is an issue with warfarin and affects the stability of the anticoagulant effect. All these factors combine to make laboratory monitoring vital for the safe management of warfarin therapy.

10.2.3 PT and INR Basics

The prothrombin time (PT) test is the assay most commonly used to monitor warfarin therapy. The PT measures the time required for the following reaction:

$$\text{Plasma} + \text{Thromboplastin} + \text{Ca}^{++} \rightarrow \text{Fibrin Clot}$$

Thromboplastins are reagent preparations rich in tissue factor and phospholipids. Thromboplastins stimulate clot formation via the extrinsic and common pathways, involving factors VII, X, V, and II, and fibrinogen. Many different thromboplastins are commercially available.

A serious limitation of the PT is that results vary considerably with different analyzers and thromboplastins and may vary with different lots of the same thromboplastin.

Consequently, PT results are inconsistent between laboratories and even within laboratories over time, making the PT inadequate for establishing therapeutic ranges and monitoring warfarin therapy, particularly when more than one laboratory is involved. An early attempt to standardize PT results was through the use of the PT ratio (PTR), namely, the PT divided by the mean normal PT. However, lab-to-lab variation was too great for the PTR to be used across laboratories for warfarin therapeutic management.

The International Normalized Ratio (INR) was developed to standardize PT values via the mathematical transformation

$$INR = \left(\frac{PT}{MNPT} \right)^{ISI} = PTR^{ISI}$$

where PT is the prothrombin time, MNPT is the mean normal PT, ISI is the International Sensitivity Index of the thromboplastin, and PTR is the prothrombin time ratio. The objective of the INR is to translate any given PT value into the PTR that would be obtained if the PT were performed by the *reference method*, which consists of PT testing using the tilt-tube method with an international reference thromboplastin preparation of the World Health Organization (WHO) [3].

Let's further examine the elements of the INR transformation. The MNPT is the geometric mean of PT determinations from a set of reference subjects (i.e., "normal" subjects) using the laboratory's analyzer and thromboplastin combination, hereafter referred to as the *working method*. Refer to Chap. 4, for additional information on the selection of reference subjects. Given n PT values, the geometric mean is calculated by the formula

$$Geometric\ \ MNPT = \left(PT_1 \cdot PT_2 \cdot PT_3 \cdot \ldots \cdot PT_n \right)^{1/n}$$

in contrast to the arithmetic mean, calculated by the formula

$$Arithmetic\ \ MNPT = \frac{PT_1 + PT_2 + PT_3 + \ldots + PT_n}{n}$$

Although substituting the arithmetic MNPT for the geometric MNPT has no practical significance under most circumstances [4], using the arithmetic mean deviates from the INR schema and may violate regulatory or accreditation standards [5].

The ISI represents the relative responsiveness or sensitivity of a working method compared to the reference method. For a given reduction in active clotting factor concentrations, the PT may be more prolonged or less prolonged, depending on the working method. Those methods that generate prolonged clotting times with relatively small reductions in clotting factor activities are considered *responsive* or *sensitive*. The sensitivity of a working method depends largely on the characteristics of the thromboplastin, but different models of analyzers with the same thromboplastin will usually exhibit some difference in sensitivity.

Warfarin therapy decreases the concentrations of active vitamin-K-dependent factors and increases the concentrations of inactive clotting factors. Inactive clotting factors are simply proteins prevented from undergoing post-translational γ-carboxylation of glutamate residues because of the decreased availability of vitamin K-H_2. They go by the unwieldy moniker of PIVKAs (Proteins Induced by

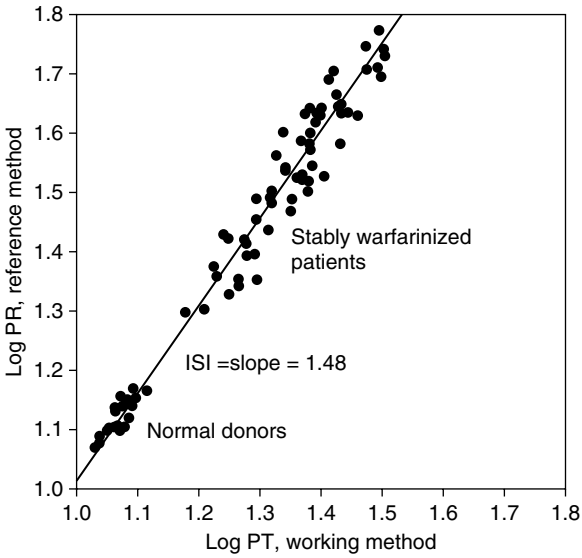

Fig. 10.2 Representation of ISI assignment. The ISI is the slope of the line from the orthogonal regression of the log-PT values from the reference method on the log-PT values from the working method. Refer to the Method Comparison section of Chap. 4, for a discussion of orthogonal regression

Vitamin K Antagonists). Thromboplastins have differential sensitivity to concentrations of active clotting factors and PIVKAs, so comparison of methods is best made when all are at a steady state, generally 6 weeks or longer after initiation of warfarin therapy.

The ISI of a working method is determined by split-sample PT testing against a reference method. According to the WHO protocol, fresh plasma samples from 20 non-warfarinized normal subjects and 60 stably warfarinized subjects are tested by both the working method and reference method. The ISI is the slope of the orthogonal regression line of the reference method log-PT on the working method log-PT, as illustrated in Fig. 10.2. Thus, a working method whose sensitivity is similar to that of the reference method will have an ISI close to 1.0, but a less sensitive working method will have a higher ISI. The College of American Pathologists recommends that laboratories use thromboplastins with an ISI $\leq$1.70 and the Clinical and Laboratory Standards Institute recommends an ISI $\leq$1.50 [6, 7], and most laboratories have adopted these recommendations [8].

In practice, most laboratories do not have the expertise or resources to determine their methods' ISI values according to the WHO protocol and rely instead on thromboplastin manufacturers to provide ISI values. Manufacturers typically assign ISI values to classes or models of analyzers, but not to specific analyzers.

10.2.4 INR and Calibration

Calibration procedures are routinely used in many areas of the clinical laboratory, particularly in chemistry, immunochemistry, and hematology assays. Calibration is fundamentally the process of determining the mathematical relationship between

Table 10.3 Comparison of a typical calibration and the INR

Component	Typical calibration	INR
Measured response	Absorbance, impedance, luminescence, etc.	Clotting time (*i.e.*, PT)
Calculated value	Concentration	INR
Standards	Calibrators	Thromboplastins
Target values	Standards traceable to definitive or reference method	Thromboplastins indexed to WHO reference thromboplastins
Calibration parameters	Slope and intercept, or coefficients of non-linear calibration equations	ISI and MNPT
Calibration "location"	Analyzer	Analyzer (MNPT) and manufacturer (ISI)
Range of accuracy	Determined in laboratory or determined by manufacturer and verified in laboratory	Difficult or impossible to determine in most labs
Calibration verification	Verified in laboratory	Difficult or impossible to do in most labs
Limitations	Matrix effects	Analyzer effects

Abbreviations: *INR* International Normalized Ratio, *PT* prothrombin time, *WHO* World Health Organization, *ISI* International Sensitivity Index, *MNPT* mean normal prothrombin time

some measurement such as absorbance or impedance and some value of interest such as an analyte concentration or cell count. Calibration equations are then used to translate measurements on patients' specimens into the values of interest.

The INR system is, in essence, a calibration of the PT where the measurement is the PT by the working method and the value of interest is the PTR that would have been obtained with the reference method. Table 10.3 compares the INR schema with a typical calibration. While there is much in common, some important differences are worth noting. In a typical calibration, calibrators with assigned target values traceable to a reference or definitive method are used on each analyzer to establish the relationship between the measured responses and the target values, yielding analyzer-specific calibration equations. Additional standards with assigned target values are then used to verify the calibration and determine or verify the range of accuracy. In the INR schema, calibrators and other standards as such do not exist. The thromboplastin reagents themselves are "calibrated" against reference thromboplastins, resulting in the assignment of a set of ISI values for classes or models of analyzers. Thus, the calibration occurs in part on each analyzer with the determination of the MNPT and in part at the manufacturer's location with the assignment of the ISI. The absence of calibrators and standards in this schema means that the INR calibration is not analyzer-specific and that laboratories cannot readily verify the accuracy of their INRs.

It should come as little surprise that a host of publications have reported inadequacies with the INR system. Interested readers may find references of these reports in other publications [1, 9]. Problems include the incorrect assignment of class- or model-specific ISI values by manufacturers and individual analyzer effects that invalidate the class- or model-based ISI values. Nevertheless, the INR system

appears to have decreased the overall lab-to-lab variation in PT values for monitoring warfarin therapy and has allowed the adoption of standardized therapeutic ranges throughout the world.

10.2.5 Plasma Calibrants

Due to the limitations of the calibration provided by the INR schema, described above, alternate approaches have emerged that use certified plasma calibrants to provide analyzer-specific calibration ("local calibration") and calibration verification [10]. Procedures for using INR calibrants are described in CLSI document H-54 [9]. Local INR calibration using plasma calibrants is appealing from both a theoretical perspective and the documented deficiencies of the INR schema, and the use of INR calibrants improves INR accuracy and decreases interlaboratory variation [11–17]. However, calibrants are not available in the United States for most coagulation test systems. In addition, several fundamental issues still need to be addressed, the most significant of which is the assignment of target INR values to calibrants. Currently, different reference thromboplastins, analytical methods, and methods of producing factor-deficient plasma may produce significantly different target INR values [13]. The ability to reliably determine the "true" INR of calibrants remains elusive. Other unresolved questions are the required precision of the calibration procedure, optimal number of calibrants, stability of calibration curves, determination of a full calibration curve versus an ISI-only calibration, and potential matrix effects of calibrants [13, 18].

10.2.6 Evaluation of Discrepant INRs

Warfarin patients generally receive INR testing over an extended period of time and often from more than one laboratory. It is not uncommon, in the author's experience, for the laboratory director to receive inquiries about INR values that appear discrepant, either between two laboratories or from one laboratory at different times. Causes of discrepant INRs are listed in Table 10.4. Some discrepancies are caused by the laboratory, some are caused by patients, and some reflect inherent limitations of the INR schema. Not all discrepancies can be resolved, but all reports of possible discrepancies should be taken seriously and evaluated to assure that correctable issues are identified and addressed.

When evaluating an INR discrepancy, the laboratory director should encourage the patient's physician to increase the thoroughness of the dietary and medication history. One vignette will serve as an illustration. A patient whose INRs had been stable for many months had a markedly elevated INR. On questioning, the patient denied any changes in diet, medications, or compliance, and repeat testing confirmed the INR elevation. Finally, the physician asked the patient to bring her medications to the office. The physician had prescribed 2 mg warfarin tablets, yet the patient's medications included warfarin tablets marked with the number 5. The patient said she had run out of her latest prescription, fished in the medicine

Table 10.4 Causes of discrepant INR values

Testing	Patient
Different citrate concentrations	Changes in diet (including herbal remedies and vitamin supplements)
Improper specimen handling	Changes in warfarin dosage
Misidentified specimen	Changes in patient compliance
Incorrect ISI assignment	Changes in medications
Incorrect MNPT determination	Variation in specimen collection time relative to warfarin ingestion
Incorrect INR equation	Biological variation in clotting factor levels
Incorrect calculations	Genetic determinants of warfarin metabolism
Incorrect entry of ISI or MNPT in computer system	
Reporting errors	
Analytical imprecision	
Analyzer problems	
Reagent problems	
Lupus anticoagulant	
Analyzer-specific effects not corrected by INR schema	

Abbreviations: *INR* International Normalized Ratio, *ISI* International Sensitivity Index, *MNPT* mean normal prothrombin time

cupboard and found some old tablets that appeared to her aged eyes to be marked with a "2" and started taking them until it was time for another appointment with her physician. Unfortunately, the number on the tablets was actually a 5, so the patient had been taking a much larger dose than necessary.

10.2.7 Therapeutic Monitoring

The recommended test for monitoring warfarin therapy is the INR. Use of any other test should be discouraged. Although several target ranges were widely used in the past, recent studies have led to the recommendation of a single target range of 2.0–3.0 for virtually all indications [19]. Guidelines for warfarin dosing, frequency of testing, and treatment of overdosage are beyond the scope of this chapter, but the interested reader is referred to an excellent review by Ageno and colleagues [1].

A few additional points about warfarin therapeutic monitoring may be useful. First, the PT assay is sensitive to changes in factor VII levels. Because factor VII has a short half-life, the PT/INR may begin to be prolonged within hours of a warfarin dose. Some non-compliant patients have admitted to taking warfarin only the day prior to testing to make it appear to the physician that the patient is compliant with the prescribed therapy. In cases where patient compliance is doubtful but the INR indicates a warfarin effect, specific factor assays showing decreased factor VII levels with normal or nearly normal factor II levels suggest that warfarin has been

taken only recently. During long-term therapy at steady state, factor II and factor VII levels should be roughly equally decreased.

Second, heparin in therapeutic doses does not significantly affect the PT/INR with most working methods, since most thromboplastin reagents contain heparin neutralizers. For patients on both heparin and warfarin, the INR reflects only the warfarin effect. The situation is different for direct thrombin inhibitors (DTIs) and activated factor X (factor Xa) inhibitors, discussed later in this chapter. DTIs and factor Xa inhibitors affect clot-based assays that involve thrombin or factor X, including the PT. In patients being treated with warfarin and a DTI or factor Xa inhibitor, the INR reflects the effects of both warfarin and the other antithrombotic medication. During the conversion from a DTI or factor Xa inhibitor to warfarin, the INR therapeutic target should be increased or the dose of DTI should be decreased [20–23].

Third, patients with significantly supratherapeutic INRs are frequently treated with oral vitamin K to partially reverse the warfarin effect. Many physicians believe the reversal will occur within a few hours, but significant lowering of the INR may take 24 h or longer due to the relatively long half-life of warfarin [2].

10.3 Unfractionated Heparin

Unfractionated heparin (UFH) is an IV or subcutaneously administered anticoagulant, widely used for the treatment and prevention of thromboembolic events, including deep venous thrombosis, pulmonary embolism, stroke, myocardial infarction, unstable angina, and some cases of disseminated intravascular coagulation (DIC), and for anticoagulation during cardiopulmonary bypass, percutaneous coronary intervention, hemodialysis, and extracorporeal membrane oxygenation (ECMO) procedures.

10.3.1 Mechanism of Action

UFH is a heterogeneous group of anionic mucopolysaccharides, called glycosaminoglycans, with anticoagulant properties. The molecular weight range is 3,000–30,000 Da, averaging 15,000–18,000 Da, around 45–50 saccharides. Despite carrying the name *heparin* because it was originally extracted from liver [24], pharmaceutical preparations are usually derived from porcine intestinal mucosa or bovine lung. The term “heparin” generally refers to unfractionated heparin, so named because of its heterogeneity in size and function. In this chapter, the abbreviation UFH will be used for unfractionated heparin and LMWH for low-molecular weight heparin (discussed later).

Heparin, either UFH or LWMH, exerts its anticoagulant effect via several mechanisms, but the most important by far is the potentiation of the serine protease inhibitor antithrombin (AT), formerly called antithrombin III. By itself, AT is a slow inhibitor of thrombin. Heparin binds to AT through a specific pentasaccharide sequence, inducing a conformational change that increases the rate of binding to thrombin by 1,000-fold. This relationship is illustrated in Fig. 10.3. Heparin-AT also inhibits factor Xa, factor IXa, factor VIIa-tissue factor complex, factor XIa,

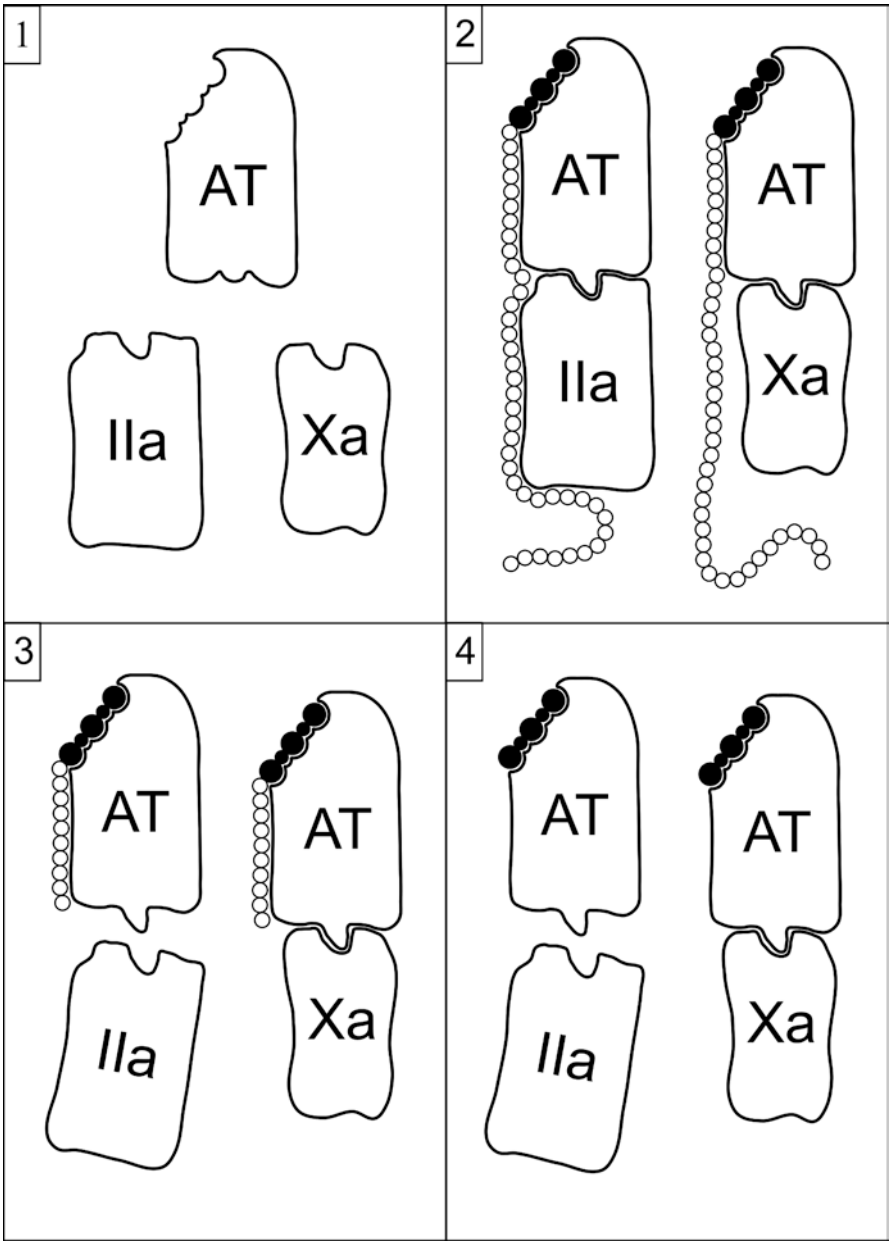

Fig. 10.3 Schematic representation of the potentiation of AT by heparin and its derivatives. *Panel 1* illustrates the lack of activity of AT in its native state against factor IIa (thrombin) or factor Xa. *Panel 2* shows the interaction and activity of UFH and AT. The binding of UFH to AT occurs through a specific high-affinity pentasaccharide sequence (*shaded*), inducing a conformational change in AT that markedly enhances its activity. The long saccharide chain of UFH is needed for anti-IIa but not anti-Xa activity. *Panel 3* shows the interaction and activity of LWMH and AT. The binding of LMWH to AT occurs via the high-affinity pentasaccharide. AT-LMWH complexes in which the LWMH molecules have fewer than 18 saccharides lose anti-IIa activity but retain anti-Xa activity. LMWH molecules of 18 or more saccharides retain anti-IIa activity. *Panel 4* shows the interaction and activity of fondaparinux and AT. Fondaparinux is a synthetic version of the high-affinity pentasaccharide. Fondaparinux-AT complexes do not have anti-IIa activity

factor XIIa, and plasma kallikrein. It indirectly inhibits thrombin-induced activation of platelets and factors V and VIII. Thrombin is tenfold more sensitive to heparin-AT inhibition than is factor Xa, and factor Xa is more sensitive than the other coagulation factors. AT binds covalently to the active serine centers of coagulation factors, then heparin dissociates and can be reutilized [25].

In pharmaceutical preparations, only one-third of UFH molecules possess the high-affinity pentasaccharide required for AT potentiation. The remaining two-thirds have minimal anticoagulant activity at therapeutic levels. Inhibition of thrombin by heparin-AT requires heparin molecules of 18 or more saccharides. Complexes with small heparin molecules inhibit factor Xa and other factors, but not thrombin.

Several other minor mechanisms contribute to the anticoagulant effect of UFH. At high concentrations, UFH molecules of 24 or more saccharides, with or without the high-affinity pentasaccharide, bind to heparin cofactor II and catalyze the inactivation of thrombin. The non-specific binding of heparin to platelets inhibits its platelet function (usually) and contributes to the hemorrhagic effects of heparin therapy. Platelet binding is more pronounced with large heparin molecules than with small heparin molecules. Heparin induces the secretion of tissue factor pathway inhibitor (TFPI) by endothelial cells, which reduces the procoagulant activity of the factor VIIa-tissue factor complex. Finally, heparin binds to von Willebrand factor (vWF), inhibiting platelet adhesion mediated by vWF [24].

10.3.2 Rationale for Laboratory Monitoring

Heparin is a highly effective anticoagulant when present in an adequate concentration. Laboratory monitoring is required for a number of reasons. First, UFH has a relatively narrow therapeutic window. When used for treatment, inadequate concentrations fail to produce the intended therapeutic response with increased risk of recurrence or extension of thromboembolism, while excessive concentrations yield an increased risk of hemorrhagic complications. When used for anticoagulation for invasive procedures, failure to achieve an adequate level of anticoagulation may lead to procedure failure, clotting in extracorporeal circuits, or embolic events. Second, the dose-response is highly unpredictable between patients and even in the same patient over time. Non-specific binding of heparin to endothelial cells, macrophages, platelets, and a variety of plasma proteins makes the dose-response highly variable. Even with weight-based loading doses and infusion rates, therapeutic levels of anticoagulation are difficult to achieve and maintain. Third, the rate of heparin resistance is high. Heparin resistance, defined as a requirement for >35,000 U UFH per 24 h to achieve a therapeutic level, occurs in up to 25 % of patients treated with UFH. Heparin resistance is classically caused by AT deficiency, particularly with AT levels <25 %, but is also associated with increases of factor VIII, fibrinogen, platelet factor 4, and heparin-binding proteins. Many of these proteins are acute-phase reactants whose concentrations rise during the acute episodes that create the need for heparin therapy.

10.3.3 Heparin-Induced Thrombocytopenia

One complication of UFH deserves special mention in the context of laboratory monitoring. Heparin-induced thrombocytopenia (HIT) is a serious, potentially life-threatening complication that requires routine monitoring of platelet counts during UFH therapy if clinicians consider the risk of HIT to be 1 % or higher [26]. HIT is caused by antibodies that recognize heparin-platelet factor 4 complexes on the surface of platelets. Antibody binding to Fc receptors activates platelets, with release of prothrombotic microparticles and activation of coagulation, leading to thrombin generation and, often, venous or arterial thrombosis [24, 26]. Thrombocytopenia results from the removal of activated platelets from circulation and is the most common clinical manifestation of HIT. In 85–90 % of cases, the platelet count falls below 150×10^9/L. In another 5–10 % of cases, the platelet count falls more than 30 % below baseline, but not below the 150×10^9/L threshold. HIT is associated with a 20–75 % risk of venous or arterial thromboembolic events including deep venous thrombosis (DVT), pulmonary embolism (PE), myocardial infarction (MI), stroke, occlusion of extremities or digits, and so forth. It should be noted that HIT is *not* the non-immune-mediated, transient, modest drop in platelet counts commonly observed with heparin therapy, although this phenomenon has also been called HIT or Type I HIT.

HIT is typically recognized by platelet count monitoring, but thrombosis precedes thrombocytopenia in up to 25 % of cases. For individuals who have not previously received heparin, HIT usually occurs 5–10 days after initiation of heparin therapy. For those who have previously received heparin, particularly within the prior 100 days, HIT may occur within 24 h ("rapid-onset HIT"). In a small number of HIT patients, the onset of thrombocytopenia begins several days after heparin has been stopped ("delayed-onset HIT"). The risk of HIT varies by patient group, gender, and type and duration of heparin exposure. This risk of HIT is 1–5 % in postoperative patients, 0.1–1 % in medical patients, and <0.1 % in obstetric patients. Women have twice the risk as men [26].

Two types of assays are used to detect the presence of HIT antibodies (i.e., anti-heparin-PF4 antibodies), namely, activation assays and antigenic assays. Activation assays are based on the ability of serum or plasma HIT antibodies to activate platelets in the presence of heparin. One or more measures of platelet activation are used, particularly serotonin release and platelet aggregation. These tests are non-standardized and vary widely in their accuracy. Variability between donor platelets and variability between heparin preparations are confounding factors, and alterations in the concentrations of platelets, heparin, and patient serum may produce different results. Nevertheless, in experienced hands the activation assays are more predictive than antigenic assays, with sensitivity as high as 98 % with specificity approaching 100 %. Antigenic assays are based on the detection of antibodies that bind to heparin-PF4 complexes or to polyvinyl sulfonate-heparin complexes. Antigenic assays are more standardized, technically easier, and have more rapid turnaround time than activation assays. Antigenic assays detect clinically insignificant antibodies more frequently than do activation assays, and antigenic assays may miss antibodies formed against proteins other than PF4, but overall the sensitivity of

antigenic assays is 94–100 %, with specificity of 82–92 %. Antigenic assay methodologies are enzyme immunoassays (EIA) and a gel centrifugation assay, although only enzyme immunoassays are currently approved in the United States.

The high sensitivity of antigenic assays yields a very high negative predictive value, such that a negative antigenic assay essentially rules out HIT in a patient with a low pretest probability. On the other hand, the moderate specificity produces a low positive predictive value (PPV) of 50 % or less, particularly in patients with a low pretest probability. The PPV may be increased by selecting patients with an intermediate to high pretest probability of HIT. The best-studied clinical prediction rule is the 4T's score, based on thrombocytopenia, timing, thrombosis, and plausible other causes.

Specificity and PPV of antigenic assays may also be increased by testing for only IgG antibodies. Polyvalent assays detect IgG, IgA, and IgM antibodies, but specimens with positive EIA results due to IgA or IgM antibodies do not generate serotonin release in activation assays. IgG-only antigenic assays have equivalent sensitivity with greater specificity than polyvalent assays [27]. In addition, the optical density (OD) values of antigenic EIA tests correlate with the results of serotonin release assays. Low-positive OD values are associated with a low probability (<5 %) of a strongly positive serotonin release assay, whereas high-positive OD values are associated with a high probability (~90 %) of a strongly positive serotonin release assay [28, 29]. Clinicians may find it helpful for the laboratory to report the OD value in addition to the interpretation of the antigenic test result.

For more information about HIT, the interested reader is referred to an excellent review by Linkins et al. [26].

10.3.4 PTT Basics

The activated partial thromboplastin time (PTT) test is the assay most commonly used to monitor UFH therapy. The PTT measures the time required for the following reaction:

$$\text{Plasma} + \text{Phospholipid} + \text{Activator} + \text{Ca}^{++} \rightarrow \text{Fibrin Clot}$$

Clot formation occurs via the intrinsic and common pathways, involving high molecular weight kininogen, prekallikrein, factors XII, XI, IX, VIII, X, V, and II, and fibrinogen. Many different phospholipid and activator reagents are available commercially [30].

The PTT is prolonged by IV UFH and has been used successfully in the management of UFH therapy for many decades. However, as with PT results for monitoring warfarin therapy, PTT results vary widely with different analyzers and reagents and are inconsistent between laboratories. PTT values may vary considerably even with different reagents or different lots of reagent from the same manufacturer. Unlike with the PT assay, the PTT has no INR-equivalent for standardizing results and establishing universal therapeutic ranges. Each laboratory must establish therapeutic range limits for its working method and must verify or adjust the limits whenever a reagent change is made. See the discussion below for determination of therapeutic ranges.

The popularity of the PTT for monitoring UFH therapy is based on several factors. First, there is a logical appeal in a test that provides a physiologic measurement of the anticoagulant effect rather than simply providing a heparin concentration. Second, the PTT is widely available with relatively short turnaround time, has good reproducibility, and is inexpensive. Third, clinicians have decades of experience with the PTT and have achieved a comfort level with its use for UFH monitoring. Fourth, until recently there have not been good alternatives. These characteristics have combined to provide a general level of clinical satisfaction with the PTT assay.

The PTT also possesses some limitations and drawbacks for monitoring UFH therapy [24, 31]. First, the PTT is a non-standard assay, as described above, so therapeutic ranges must be established in each laboratory. Different types of UFH may produce different PTT response curves. Second, the PTT is useful only for monitoring therapeutic IV doses or high-dose subcutaneous UFH therapy. The PTT is not useful for standard-dose subcutaneous therapy, due to limited PTT response, or for the high-dose IV UFH required for cardiac catheterization or other procedures, due to an excessive PTT response. Third, the PTT is affected by variables other than UFH, including increased concentrations of factor VIII and fibrinogen, decreased concentrations of AT or intrinsic and common pathway proteins, lupus anticoagulants, and thrombolytic agents. When lupus anticoagulants are present, the PTT becomes unpredictable for assessing UFH response and should not be used. The anti-Xa assay, discussed below, is the test of choice for patients with lupus anticoagulants. Fourth, specimens have relatively short stability, particularly when heparin is present. See Chap. 2, for additional discussion of specimens. Overall, the advantages of the PTT are perceived as outweighing the disadvantages for most patients, so the PTT continues to enjoy widespread use for monitoring IV UFH therapy.

10.3.5 Anti-Xa Heparin Assay Basics

The heparin anti-Xa assay, or factor Xa inhibition test, is increasingly used to supplement or replace the PTT for monitoring heparin therapy. The principle of the anti-Xa assay is the inhibition of factor Xa by AT-heparin complexes, as illustrated in the following reactions [31]:

$$\text{AT-Heparin} + \text{Xa} \rightarrow \text{AT-Heparin-Xa} + \text{Residual Xa}$$
$$\text{Residual Xa} + \text{Substrate} \rightarrow \text{Signal}$$

In these reactions, the AT-heparin complex is provided by the test plasma and factor Xa is provided as a defined quantity of reagent. The AT-heparin complexes inhibit factor Xa essentially in a 1:1 ratio. Residual factor Xa is available to react with a substrate to form a signal, usually a chromogen, whose strength is inversely proportional to the heparin concentration. The signal is compared against a standard curve to yield a quantitative factor Xa inhibitory effect in IU/mL. The standard curve is dependent on the heparin preparation used. Different curves need to be established for UFH and LMWH.

The anti-Xa assay has several favorable characteristics. First, the anti-Xa assay is simple to perform and automatable on many coagulation analyzers. Second, citrated specimens used for other coagulation tests may be used without additional special handling. Third, the test is not affected by coagulation factor concentrations, lupus anticoagulants, or other biological variables. Fourth, the anti-Xa assay is sensitive to the anticoagulant effect of LMWH, fondaparinux, and factor Xa inhibitors, discussed below, making it more versatile than the PTT for anticoagulant monitoring. Fifth, the anti-Xa assay can theoretically be standardized to provide consistency between laboratories and facilitate the adoption of common therapeutic ranges.

Anti-Xa assays have some limitations and drawbacks. First, the anti-Xa assay is more expensive than the PTT. Second, it may not be financially or technically feasible to offer the anti-Xa assay in small laboratories, depending on test volume and instrumentation. Third, different UFH preparations may yield different standard curves. It is generally not feasible to establish a unique standard curve for every UFH preparation, so the potential exists for the standard curve of an anti-Xa assay to be non-representative of the anticoagulant response for some UFH preparations. The same limitation applies to LWMH preparations. Fourth, interlaboratory standardization of anti-Xa assays is more theoretical than actual. In some cases, the anti-Xa assay's interlaboratory variability is greater than the PTT's [24].

Two variations of the anti-Xa assay deserve mentioning. The first variation is a clot-based rather than chromogenic assay. Factor V, fibrinogen, activator, and calcium serve as the "substrate" and the clotting time serves as the "signal." The clotting time is proportional to the heparin concentration. Clot-based assays tend to be less precise than chromogenic assays and may be affected by more biological variables. The second variation involves an initial step of adding excess AT to the test specimens in a quantity that assures all heparin becomes complexed with AT. The theoretical appeal of this approach is that it detects "free" heparin and provides a truer estimate of heparin concentration. The alternate view is that the value of interest is the anticoagulant effect, not the heparin concentration. In AT deficiency, the anticoagulant effect may be muted physiologically due to a decrease in AT-heparin complexes, but the anti-Xa assay results may appear to show a therapeutic response due to the inclusion of "free" heparin. Currently, there is no clear superiority of either the AT-supplemented or non-supplemented anti-Xa assays [32].

10.3.6 ACT Basics

The activated clotting time (ACT) is a whole-blood clotting test commonly used to monitor high-dose UFH therapy, particularly in cardiac catheterization and cardiopulmonary bypass procedures. The ACT consists of the following reaction [31]:

$$\text{Whole Blood} + \text{Particulate Activator} \rightarrow \text{Fibrin Clot}$$

Celite and kaolin are commonly used as activators. Typically, the ACT is a point-of-care test because non-anticoagulated blood specimens cannot be transported to a central laboratory quickly enough to avoid pre-test activation of clotting.

The ACT offers several advantages for UFH monitoring. First, the ACT has a wide dose-response range so it can be used for assessing high-dose UFH therapy. Second, as a point-of-care test, the ACT is simple to perform and results are available with rapid turnaround time. The rapid turnaround facilitates its use in assessing both induction and reversal of UFH anticoagulation in near-real-time during procedures. Third, extensive clinical experience over several decades provides a high level of familiarity and comfort with its use. Fourth, suitable alternatives are not readily available. Consequently, the ACT is widely used for high-dose UFH management.

The ACT also has several important limitations and drawbacks. First, the ACT is non-standard and imprecise. The inter-analyzer variance is generally large, even with analyzers of the same make and model using the same lot of activator. Even duplicate simultaneous results from the same analyzer may vary considerably [33]. Second, because non-anticoagulated blood is used, results are affected by deficiencies in specimen collection and application technique, including delayed application of specimens. Third, the ACT reaction follows the same pathways as the PTT reaction, so the ACT is affected by the same biological variables. In addition, because whole blood is used, the ACT is also affected by variations in platelet count inasmuch as platelets provide phospholipid surfaces for reactions of the intrinsic and common pathways. By contrast, in the PTT assay phospholipid is provided as a reagent in a controlled amount. Fourth, although the dose-response of the ACT extends into the standard-dose UFH range, it is less precise and offers no advantages over the PTT for this use. Fifth, many ACT analyzers have only basic functionality and cannot be interfaced with information systems for data management.

A few issues concerning ACT testing should be considered. First, many different activator reagents are available commercially, the most common being celite, kaolin, and combinations of celite and kaolin. Different reagents generate different ACT values. One of the more important differences is the effect of aprotinin, a serine protease inhibitor commonly used in coronary artery bypass graft surgery. Celite is considerably more responsive to aprotinin than is kaolin. Controversy remains about the value of including or excluding the aprotinin effect in ACT monitoring, and depending on one's view, either celite or kaolin becomes the preferred activator [31, 34]. Second, despite the ACT's high imprecision and poor inter-analyzer concordance, it has a well-established role in the management of high-dose UFH anticoagulation. The ACT's utility in this setting is due to the fact that the goal of UFH dosing is not to hit some precise target but rather to assure that the anticoagulation level is above some threshold. The ACT's coarse estimate of clotting time is sufficient in this regard, but likely would not be if more precise dosing were required. Finally, while most ACT methods detect formation of a clot, one method (i-STAT, Abbott Laboratories, Abbott Park, Illinois) detects thrombin generation rather than clot formation. This method has been shown to correlate reasonably well (within the context of a very imprecise test) with the widely used Hemochron ACT (ITC, Edison, New Jersey) [35]. However, one potential source of major discrepancies is fibrinogen deficiency or dysfibrinogenemia because fibrinogen is measured in the clot-based ACT's pathway, but not the i-STAT ACT's pathway.

10.3.7 Therapeutic Monitoring

The tests and therapeutic ranges for UFH monitoring are listed in Table 10.1. For information about UFH administration and frequency of testing, the interested reader is referred to an excellent review by Garcia and colleagues [24].

The evidence for titration of heparin dosage to either PTT or anti-Xa targets is weak and published therapeutic ranges have not been confirmed by randomized trials, but is still standard practice [24]. Manufacturer's information states that dosage is adequate in adults when the PTT is 1.5–2 times normal; in pediatric patients, the PTT should be maintained at 60–85 s, assuming this reflects an anti-Xa level of 0.35–0.70 IU/mL [25]. The College of American Pathologists recommends establishing the PTT therapeutic range for each analyzer/reagent combination by comparing PTT results to heparin activity in patients' specimens [5], since the PTT response to *in vitro* heparin spiking overestimates the anti-Xa levels in patients on UFH therapy [36]. One note of caution is that both the PTT and anti-Xa assays show wide interlaboratory variability [37, 38] and the correlation between anti-Xa and PTT values is low. There is large uncertainty in the estimates of the PTT therapeutic range limits derived from regression analysis of PTT on anti-Xa values. Consequently, the choice of therapeutic ranges is not clear cut [5, 24, 25].

Once the PTT therapeutic range has been established, the implementation of a new PTT reagent or reagent lot does not necessarily require that a new therapeutic range be determined, provided that the heparin response does not change significantly [5]. Olson and colleagues described a method for comparing the responsiveness of PTT reagents with fresh patient specimens and monitoring the cumulative difference of means. As long as the cumulative difference remains within ±5 s, the PTT therapeutic range does not need to be reestablished [31].

Target ACT ranges are established by clinical protocols for each type of procedure.

10.4 Low-Molecular-Weight Heparin

Low-molecular-weight heparin (LMWH) is a subcutaneously administered anticoagulant, used for the treatment and prevention of thromboembolic events, including deep venous thrombosis, pulmonary embolism, stroke, myocardial infarction, and unstable angina [24, 39].

10.4.1 Mechanism of Action

LMWH is prepared from UFH by chemical or enzymatic depolymerization. The molecular weight ranges from 1,500 to 10,000 Da, averaging 4,000–5,000 Da, around 15 saccharides [24]. As does UFH, LWMH exerts its principal anticoagulant effect by the potentiation of AT via binding of a specific pentasaccharide sequence present on only one-third of LMWH molecules. A comparison of LMWH with UFH is given in Table 10.5. The differences between LMWH and UFH are virtually

Table 10.5 Comparison of unfractionated heparin, low-molecular-weight heparin, and fondaparinux

Property	UFH	LMWH	Fondaparinux
Mode of action	Activation of AT	Activation of AT	Activation of AT
Molecular weight, daltons	3,000–30,000	1,500–10,000	1,728
Molecules containing high-affinity pentasaccharide, %	33	33	100
Administration	IV or SC	SC	SC
Plasma half-life, h	0.5–2.5	3–4	17–21
Binding to plasma proteins, endothelial cells, platelets, and macrophages	Much	Little	None
Dose-response	Unpredictable	Predictable	Predictable
Dosage	Titrated to lab value	Fixed dose or weight-based	Fixed dose or weight-based
Anti-Xa/anti-IIa ratio	1.2	2–4	No anti-IIa activity
PTT at therapeutic levels	Prolonged	Not prolonged or only slightly prolonged	Not prolonged or only slightly prolonged
Thrombocytopenia 50,000–100,000/μL, incidence, %	1.0–1.5	1.0–1.5	0
Thrombocytopenia <50,000/μL, incidence, %	0.2	0.1	0
HIT (immune-mediated thrombocytopenia), %	0.1–5	<0.1–1	None
Bleeding complications, %	3–5	Same or lower	Same or higher
Protamine reversal	Effective for IV, less effective for SC	Less effective	Not effective

Abbreviations: *AT* antithrombin, *UFH* unfractionated heparin, *LMWH* low-molecular-weight heparin, *IV* intravenous, *SC* subcutaneous, *PTT* activated partial thromboplastin time

all attributable to the different binding properties of the shorter and longer saccharide chains. Shorter chains show less non-specific binding to plasma proteins, endothelial cells, macrophages, and platelets, resulting in a much more predictable dose-response; longer plasma half-life, allowing once- or twice-daily dosing; and reduced anti-IIa activity, of uncertain significance. In addition, reduced binding to platelets is believed to be responsible for a lower incidence of heparin-induced thrombocytopenia compared to UFH.

10.4.2 Rationale for Laboratory Monitoring

Routine laboratory monitoring of the anticoagulant effect of LMWH is not required for most patients because of the highly predictable dose-response. Monitoring may be indicated in some special situations, including renal

insufficiency, morbid obesity, low body weight, newborns, young children, and patients receiving prolonged therapy [24, 39]. As with UFH, HIT occurs with LMWH therapy, although at a much lower frequency, so routine monitoring of platelet counts may be required only for surgical patients or for medical patients who received UFH prior to LMWH [26].

10.4.3 Therapeutic Monitoring

When monitoring is required, the test of choice is the anti-Xa assay. Because each brand of LMWH has unique properties, the anti-Xa LMWH assay's standard curve should be established with LMWH preparations used in the facilities served by the laboratory. A standard curve established with one brand of LMWH should not be considered valid for other brands. The therapeutic targets of anti-Xa activity measured 4 h after administration of enoxaparin, the best-studied LMWH preparation, are shown in Table 10.1.

10.5 Fondaparinux

Fondaparinux is a subcutaneously administered synthetic analog of the AT-binding pentasaccharide found in active molecules of UFH and LMWH, approved for use in the treatment of acute venous thromboembolism (VTE) and prevention of recurrent VTE [24, 40].

10.5.1 Mechanism of Action

Fondaparinux exerts its anticoagulant effect by the potentiation of AT. A comparison of fondaparinux with LMWH and UFH is shown in Table 10.5. Significant properties of fondaparinux include an absence of anti-IIa activity because it is too small to bridge AT to thrombin; no detectable binding to plasma proteins or cells, providing a highly predictive dose-response with excellent bioavailability following SC injection; and a longer half-life than either UFH or LMWH, 17–21 h, allowing once-daily dosing. In addition, fondaparinux has only rarely been associated with heparin-induced thrombocytopenia, cannot be reversed by protamine sulfate, and is not effective in patients with moderate or severe AT deficiency. Fondaparinux is excreted unchanged in the urine, so renal insufficiency is a contraindication to its use.

10.5.2 Rationale for Laboratory Monitoring

Routine laboratory monitoring of the anticoagulant effect of fondaparinux is not required for most patients because of the highly predictable dose-response. It seems

prudent to consider monitoring in extremes of body weight, young age, and prolonged use.

10.5.3 Therapeutic Monitoring

When monitoring is desirable, the test of choice is the anti-Xa assay whose standard curve is established using fondaparinux. The therapeutic target is shown in Table 10.1. It should be noted that the anti-Xa fondaparinux assay does not have clinical validation at this point in time.

10.6 Bivalirudin

Bivalirudin is an IV-administered direct thrombin inhibitor (DTI) approved for anticoagulation of patients with unstable angina undergoing percutaneous transluminal coronary angioplasty (PTCA) or with HIT, or at risk of HIT, undergoing percutaneous coronary interventions [41]. Bivalirudin is intended for use with aspirin.

10.6.1 Mechanism of Action

Bivalirudin is a derivative of hirudin. Hirudin is a naturally occurring polypeptide of 65 amino acids with thrombin-inhibiting activity, produced in trace amounts by the leech *Hirudo medicinalis*. Bivalirudin is a synthetic combination of 8- and 12-peptide fragments of hirudin that bind to thrombin's active catalytic site and substrate-binding site, respectively. Thrombin slowly cleaves bivalirudin with recovery of thrombin's active-site function. Bivalirudin has a predictable dose-response and a short half-life of only 25 min. Its efficacy is unaltered by AT deficiency. Only 20 % of bivalirudin is excreted in the urine. Bivalirudin has not been shown to react with heparin-induced antibodies. No antidotes are available, but the short half-life and reversible binding limit bleeding complications.

10.6.2 Therapeutic Monitoring

Because of the predictable dose-response and short half-life, routine laboratory monitoring of bivalirudin is not required.

10.7 Argatroban

Argatroban is an IV-administered DTI approved for prophylaxis or treatment of thrombosis in adult patients with HIT and as an anticoagulant in adults with or at risk of HIT undergoing percutaneous coronary intervention [20].

10.7.1 Mechanism of Action

Argatroban, a synthetic derivative of L-arginine, is a competitive inhibitor of thrombin that binds reversibly to thrombin's active catalytic site. It inhibits both clot-bound and free thrombin. It has a predictable dose-response and its efficacy is unaltered by AT deficiency. No antidotes are available and bleeding complications may be hard to treat, but the short plasma half-life of 24 min somewhat mitigates this disadvantage. Argatroban is metabolized primarily in the liver, so its use should be limited in hepatic impairment. It has no renal excretion and can be used in patients with severe renal impairment. Argatroban does not react with heparin-induced antibodies [20, 24].

10.7.2 Rationale for Laboratory Monitoring

Argatroban has a relatively narrow therapeutic window. Overdosage is of particular concern because of the absence of an antidote.

10.7.3 Therapeutic Monitoring

For the treatment or prevention of thromboembolism, the PTT is used for routine laboratory monitoring with a manufacturer-recommended therapeutic range of 1.5–3.0 times the patient's baseline PTT, but <100 s [20]. Although this therapeutic range was determined in clinical trials, its transferability to all laboratories seems doubtful given the wide inter-laboratory variation of the PTT. See the PTT section above for other considerations of the PTT for therapeutic monitoring. For anticoagulation in percutaneous coronary interventions, the ACT is used for dosage titration, with a therapeutic range of 300–450 s.

10.8 Dabigatran

Dabigatran is an oral DTI approved to reduce the risk of stroke and systemic embolism in patients with non-valvular atrial fibrillation, for the treatment of VTE in patients who have been treated with a parenteral anticoagulant for 5–10 days, and to reduce the risk of recurrence of VTE in previously treated patients [1, 21].

10.8.1 Mechanism of Action

The pharmaceutical preparation, dabigatran etexilate (Pradaxa), is a prodrug that is hydrolyzed to dabigatran. Up to 40 % of dabigatran is then conjugated to four different acyl glucuronides with similar pharmacologic activity. Dabigatran and active metabolites are competitive inhibitors of free and clot-bound thrombin. The time to

peak effect is 1.5 h after ingestion, and the half-life is 12–17 h. Excretion of absorbed dabigatran (3–7 %) is primarily renal (>80 %). Dabigatran is dialyzable.

Compared to warfarin, dabigatran has a more rapid onset of action, more predictable anticoagulant effect, lower potential for dietary interactions, and lower potential for drug interactions. The principal disadvantages are irreversibility and lack of proven strategies for managing overdosage, bleeding complications, or emergent surgery or other invasive procedures. Another disadvantage is that dabigatran is taken twice daily, whereas warfarin is taken once daily.

10.8.2 Ecarin Clotting Time Basics

Simple, widely used coagulation tests have limitations for measuring dabigatran's effect. The PT/INR and PTT are relatively insensitive and the relationship between the PTT and dabigatran concentration is nonlinear. The usual form of the thrombin time (TT), also called the thrombin clotting time (TCT), has a linear response to dabigatran concentration, but it is overly sensitive. The ecarin clotting time (ECT) has a linear dose-response throughout the range of concentrations expected during prophylaxis or therapy and is thought to be the most reliable test for dabigatran assessment [1, 42]. Ecarin is an enzyme derived from the venom of the Indian saw-scaled viper, *Echis carinatus*. Ecarin activates prothrombin through proteolytic cleavage, producing meizothrombin. Meizothrombin's activity is inhibited by DTIs, but not by UFH, LMWH, fondaparinux, warfarin, Xa inhibitors, or lupus anticoagulants. A variation of the ECT is the ecarin chromogenic assay (ECA) in which patient specimen is mixed with ecarin and excess prothrombin, meizothrombin is formed in inverse proportion to the concentration of inhibitor (eg, dabigatran), and a chromogenic substrate is formed in proportion to the amount of meizothrombin generated. Limited availability is a major drawback of the ECT or ECA. In the United States, ECT and ECA reagents are currently labeled for research use only (RUO).

10.8.3 Plasma-Diluted Thrombin Time Basics

The TT measures the time required for the following reaction:

$$\text{Plasma} + \text{Thrombin} \rightarrow \text{Fibrin Clot}$$

The TT is very sensitive to UFH and exquisitely sensitive to DTIs, including dabigatran. The TT shows a linear response to dabigatran, but the rate of prolongation is too rapid for practical use as a measurement of dabigatran concentration [43, 44]. A modification of the TT, the plasma-diluted thrombin time (PDTT), preserves the TT's linear response to dabigatran concentration, but at a much slower rate that allows for more precise quantification of dabigatran [45]. In the PDTT, the TT is performed on a sample consisting of one volume of citrated plasma diluted with three volumes of pooled normal plasma, and the result is compared to a

dose-response curve constructed using dabigatran plasma calibrant reference materials. As with ECT and ECA reagents, dabigatran plasma calibrants are currently labeled for research use only in the United States.

10.8.4 Therapeutic Monitoring

The anticoagulant effect of dabigatran does not ordinarily need to be assessed, and the use of routine laboratory monitoring has not been studied [1, 21]. Laboratory assessment may be useful in patients with reduced renal function or in those who develop bleeding or thrombosis, need an invasive procedure while receiving dabigatran, may have taken an overdose, or appear coagulopathic with an uncertain medication history [1, 44]. When available, the ECT, ECA, or PDTT is the test of choice. Otherwise, a normal TT excludes the presence of dabigatran, and a normal PTT essentially excludes a therapeutic or supratherapeutic concentration of dabigatran, but does not rule out any dabigatran effect [44], although it should be noted that the dabigatran sensitivity of PTT reagents varies widely [46]. The INR schema is not suitable for assessing dabigatran. Other approaches to the use of commonly available tests have been reported [47].

10.9 Rivaroxaban

Rivaroxaban (Xarelto) is an oral factor Xa inhibitor approved to reduce the risk of stroke and systemic embolism in patients with nonvalvular atrial fibrillation, for the treatment of VTE, to reduce the risk of recurrent VTE, and for the prophylaxis of VTE in patients undergoing knee or hip replacement surgery [23].

10.9.1 Mechanism of Action

Rivaroxaban selectively and competitively blocks the active site of factor Xa, both free and prothrombinase-complex-bound. The time to peak effect is 2–4 h after ingestion, and the half-life is 5–9 h. Excretion of absorbed rivaroxaban (60–100 %) is primarily renal (>66 %). Active metabolites have not been identified [1].

Compared to warfarin, rivaroxaban has a more rapid onset of action, more predictable anticoagulant effect, lower potential for dietary interactions, and lower potential for drug interactions. Rivaroxaban's efficacy and bleeding risk profile are at least as favorable as those of warfarin and LMWH. The principal disadvantages of rivaroxaban are acute irreversibility and lack of proven strategies for managing overdosage, bleeding complications, or emergent surgery or other invasive procedures. Compared to dabigatran, rivaroxaban has once-daily dosing (in most indications) and may be used in mild to moderate renal impairment.

10.9.2 Therapeutic Monitoring

Dose titration to a laboratory value is not required and has not been studied [1, 23]. Laboratory assessment may be useful in patients who develop bleeding or thrombosis or need an invasive procedure while receiving rivaroxaban, may have taken an overdose, or appear coagulopathic with an uncertain medication history. Rivaroxaban inhibits endogenous factor Xa, causing a dose-dependent prolongation of the PT and anti-Xa assays and, to a lesser extent, the PTT. The PT may be used to assess rivaroxaban effect. A normal PT excludes a therapeutic peak concentration of rivaroxaban, but does not exclude the presence of subtherapeutic concentrations or trough therapeutic concentrations [48]. PT reagents show substantial variability in rivaroxaban sensitivity [46], so it is recommended that laboratories evaluate their response curves using commercially available rivaroxaban calibrants [44]. The INR should not be used. The anti-Xa assay, calibrated for rivaroxaban, is preferred over the PT due to its stronger correlation with rivaroxaban effect [44]. It should be noted that rivaroxaban calibrants are currently labeled for research use only in the United States.

10.10 Apixaban

Apixaban (Eliquis), like rivaroxaban, is an oral factor Xa inhibitor that selectively blocks the active site of both free and prothrombinase-complex-bound factor Xa. The time to peak blood concentration is 3–4 h after ingestion, and the half-life is ~12 h. Apixaban is taken twice daily. Excretion of apixaban is renal (~27 %) and fecal. Active metabolites have not been identified [1, 22]. Apixaban is similar to rivaroxaban for test considerations and effects [44].

10.10.1 Other Anticoagulants

Many other new anticoagulant drugs are in development or clinical trials. For an overview, the interested reader is referred to an excellent article by Weitz and colleagues [49].

10.10.2 Antiplatelet Agents

Platelets are an integral component of the hemostatic system and are involved in both physiological and pathological thrombosis. Antiplatelet therapies are effective for the prevention of platelet-rich thrombus formation in the arterial system by blocking one or more pathways involved in platelet activation and aggregation and are commonly used in the prevention of myocardial infarction and stroke in patients with or at risk for coronary or peripheral artery disease and in the prevention of thrombotic events in patients treated with percutaneous coronary intervention [50, 51]. The most

common antiplatelet therapies are aspirin, the thienopyridines (clopidogrel, prasugrel, and ticlopidine), the glycoprotein IIb/IIIa antagonists (abciximab, tirofiban, and eptifibatide), dipyridamole, and cilostazol.

10.10.3 Mechanisms of Action

For a description of the pathways and mechanisms involved in the antiplatelet effects of these agents, interested readers are referred to an excellent review by Eikelboom and colleagues [50].

10.10.4 Basics of Platelet Function Testing

Light transmission aggregometry is the historical standard for platelet function testing, but involves a number of factors that make it impractical for widespread, rapid testing. Growth in antiplatelet therapeutics has fueled a commensurate surge in methods for platelet function testing. Basic descriptions of various platelet function test methods are provided in Chap. 3. Comparisons of methods and antiplatelet response profiles are shown in Table 10.6, modified from Gurbel and colleagues [52].

10.10.5 Rationale for Laboratory Testing

Despite theoretical platelet inhibition on the basis of known mechanisms, *in vitro* testing of platelet function demonstrates high platelet reactivity in up to one-third of patients receiving antiplatelet agents. Estimates of the prevalence of high on-treatment platelet reactivity are affected by differences in patient characteristics, comorbidities, concomitant therapies, test methods, and cutoffs used to define high reactivity [50]. Risk of thrombotic events may be 1.5–5 times higher in patients with high on-treatment platelet reactivity than in those with demonstrable platelet inhibition. Similarly, those with higher than usual platelet inhibition are at increased risk of bleeding complications [51]. Platelet function testing has been proposed for the following uses:

- Individualized, lab-directed therapy. The identification of poor responders and subsequent changes in dosage or choice of agents is particularly appealing. However, large, controlled trials have failed to demonstrate improvement in outcomes with individualized anti-platelet therapy directed by laboratory testing [50]. In contrast, a meta-analysis of nine randomized trials showed benefit to individualized therapy, particularly in high-risk patients [51]. Concerns have been raised about limitations of the large studies and about the small size and heterogeneity of the other randomized trials, so lab-directed antiplatelet therapy remains controversial, although published evidence-based guidelines recommend against the routine use of platelet function tests for therapeutic management [51].

Table 10.6 Platelet function tests for assessing antiplatelet therapy

Test	Description	Advantages	Drawbacks	Prognostic value	Aspirin responsive	Thieno-pyridine responsive	GP IIb/IIIa inhibitor responsive
Bleeding time	Measures time to cessation of bleeding following skin incision	In vivo; physiological	Affected by platelet count and non-platelet factors; variable; scarring	No	No	No	No
Platelet aggregometry	Measures changes in light transmittance through platelet-rich plasma after addition of platelet agonists	Historical "gold standard"	Technically difficult; time consuming; expensive	Yes	Yes	Yes	Yes
PFA-100 (Sysmex)	Measures time to closure of small aperture under high shear after addition of platelet agonists to whole blood	Simple; rapid; extensively studied	Affected by platelet count and non-platelet factors; unfamiliar terminology (closure time)	Yes	Yes	No	No
VerifyNow (Accumetrics)	Measures light transmittance through whole blood added to well containing platelet agonist-coated beads	Simple; rapid; extensively studied; FDA-approved for antiplatelet monitoring	Expensive cartridges; unfamiliar units (aspirin reactive units, P2Y12 reactive units)	Maybe	Yes	Yes	Yes
Plateletworks (Helena)	Measures platelet count in whole blood drawn in tube containing platelet agonists and compares with baseline platelet count	Simple; rapid; uses CBC analyzer	Not well studied	No	Yes	Yes	Yes

(continued)

Table 10.6 (continued)

Test	Description	Advantages	Drawbacks	Prognostic value	Aspirin responsive	Thieno-pyridine responsive	GP IIb/IIIa inhibitor responsive
Thromboelastograph PlateletMapping (Haemoscope)	Measures clot strength after addition of platelet agonists to whole blood weakly clotted by reptilase and factor XIII	Simple; provides additional information on clot formation and clot lysis	Not well studied	Yes	Yes	Yes	Yes
VASP Phosphorylation (BioCytex)	Measures presence of phosphorylated vasodilator-stimulated phosphoprotein (VASP) in platelets by flow cytometry after addition of platelet agonists	Directly related to inhibition of clopidogrel's target receptor (P2Y12)	Requires flow cytometer; time consuming; expensive; research use only in USA	Yes	No	Yes	No
AspirinWorks (Corgenix)	Measures urinary 11-dehydrothromboxane B_2	Simple specimen collection	Reference lab test; expensive; long turnaround	Yes	Yes	No	No

Modified from Gurbel et al. [52]

- Prognosis. Evidence supports the clinical utility of platelet function testing as a prognostic marker in patients treated with percutaneous coronary intervention. High on-treatment platelet reactivity in this patient population is predictive of the risk of major adverse clinical events, but is less clear in medically managed acute coronary syndromes or stable coronary artery disease [51]. Although the evidence base for clinical management of high on-treatment reactivity is currently weak, physicians may find it useful to integrate prognostic information with other clinical data and patient factors to direct the course of care.
- Bleeding risk. The link between high on-treatment platelet reactivity is well established, but the association between low on-treatment platelet reactivity is less clear. Some evidence is emerging that supports the concept of a "therapeutic window" for clopidogrel, wherein low platelet reactivity indicates a bleeding risk and high platelet reactivity indicates a thrombotic risk [51].
- Preoperative risk assessment. When a patient on clopidogrel requires surgery, standard practice is to discontinue treatment at least 5 days preoperatively. However, high variability in clopidogrel response and high variability in platelet function recovery times suggest a role for platelet function testing to identify perioperative bleeding risk. Observational studies demonstrated a correlation between platelet function and bleeding in patients undergoing cardiac surgery. Measurement of the antiplatelet effect of clopidogrel before surgery has been used to identify patients who may safely proceed to surgery before the standard waiting period [51].

10.10.6 Therapeutic Monitoring

A report from the American College of Cardiology's Working Group on On-Treatment Platelet Reactivity summarized published guidelines regarding the use of platelet function testing in the management of antiplatelet therapy [51]. In short:

- Routine clinical use of platelet function testing to manage antiplatelet therapy is not recommended, but may be considered in selected cases, particularly in patients at high risk for poor clinical outcomes.
- For patients with a low therapeutic response to clopidogrel, it may be reasonable to change to another antiplatelet agent.
- For patients on antiplatelet therapy, it is reasonable to make decisions about surgical delay based on platelet function test results rather than arbitrary use of a specified period of delay.

Because virtually every aspect of antiplatelet therapeutic monitoring is subject to some degree of controversy, it is important for the laboratory director to have an ongoing dialog with clinicians to determine the test methods and indications appropriate to the institution's patient population.

10.11 Effects of Anticoagulants on Hemostasis Tests

Anticoagulant drugs are commonly used in both inpatients and outpatients. Different anticoagulants affect coagulation tests to varying degrees. One of the challenges for both clinicians and laboratories is in assessing whether abnormal coagulation test results are due to the presence of anticoagulants, either as therapeutic agents or specimen contaminants, or abnormalities in the hemostatic system. Table 10.7 summarizes the effects on coagulation tests of the anticoagulants discussed in this chapter [1, 20–25, 39–41, 44, 46, 53–57].

10.12 Common Pitfalls

Therapeutic monitoring of anticoagulation requires laboratories to produce reliable test results and, for some assays, therapeutic ranges. Some of the more common problems include:

- The PT thromboplastin manufacturer does not provide the correct ISI for the analyzer.
- The laboratory does not correctly determine the MNPT for the INR calculation.
- The laboratory does not assess the accuracy of its INRs by comparison with prior results, other laboratories, or plasma calibrants.
- The INR is calculated incorrectly.
- The PTT therapeutic range for UFH therapy is not determined initially by the laboratory or is not verified with each reagent lot change.
- Incorrect materials are used to establish the standard curves for anti-Xa assays; for example, LMWH heparin is monitored using an anti-Xa assay set up with UFH.
- The poor interchangeability of ACT results between analyzers is not recognized.
- The influence of anticoagulants on common coagulation tests is not recognized. This is particularly true for the newer oral anticoagulants that don't require routine laboratory testing.
- The anti-Xa assay is used to assess the anticoagulation effect of oral factor Xa inhibitors for which it has not been calibrated.

10.13 Summary and Key Points

Laboratory monitoring of anticoagulation therapy is critical for the efficacious and safe use of anticoagulants. Some anticoagulants require monitoring of their specific anticoagulant effects, but many do not. All anticoagulant therapy requires some level of general monitoring for complications such as occult bleeding. This chapter has focused on specific monitoring. Some key points are:

- Warfarin therapy is widely used and monitored with the INR, a mathematical transformation of the PT involving a manufacturer-assigned ISI and laboratory-

Table 10.7 Effects of anticoagulant medications on coagulation tests[a,b]

Anticoagulant	PT[a]	PTT	TT	Fib	DD	Anti-Xa	ACT	DRVVT	VWF act	APC Res	Plsm act	PC act	PC Ag	PS act	AT act	F.II	F.VIII	F.IX	F.X	PLT Agg
Warfarin	↑↑	0–↑	0–↑	0	0	0	0–↑	↑↑	0	0[c]	0	↓	↓	↓	0	↓↓	0	↓↓	↓↓	0
Heparin, unfractionated, standard dose IV or high-dose SC	0–↑	↑↑	↑↑	0	0	↑↑	↑↑	↑↑	0	0[c]	0	↑	0	↑	0	↓	↓	↓	↓	0
Dabigatran	0–↑	↑↑	↑↑	0–↓	0	0	↑↑	↑↑	0	↑	0	0–↑[d]	0	0–↑[d]	0–↑[e]	↓	↓↓	↓↓	0	0
Abixaban	↑↑	↑	0	0	0	↑↑	↑↑	↑↑	0	?	0	0–↑[d]	0	0–↑[d]	0	0	↓	↓	↓	0
Rivaroxaban	↑↑	↑	0	0	0	↑↑	↑↑	↑↑	0	?	0	0–↑[d]	0	0–↑[d]	0	0	↓	↓	↓	0
Argatroban	↑	↑↑	↑↑	↓↓	0	0	↑↑	↑↑	0	?	0	↑	0	↑↑	0	↓↓	↓↓	↓↓	↓	0
Bivalirudin	0–↑	↑↑	↑↑	↓	0	0	↑↑	↑↑	0	?	0	↑	0	↑↑	0	↓	↓↓	↓↓	0	0
Fondaparinux	0	0–↑	↑	0	0	↑↑	↑↑	0	0	?	0	0	0	↑	0	0	0	0	0	0
Heparin, unfractionated, low-dose SC	0	0–↑	↑	0	0	0–↑	0–↑	0	0	?	0	0–↑	0	0–↑	0	0	0	0	0	0
Heparin, unfractionated, high-dose IV	↑	↑↑	↑↑	0	0	↑↑	↑↑	↑↑	0	↑↑	0	↑	0	↑	0	↓	↓	↓	↓	0
Heparin, LWM, therapeutic	0	0–↑	↑	0	0	↑↑	0–↑	↑	0	↑	0	↑	0	↑	0	↓	↓	↓	↓	0
Heparin, LWM, prophylactic	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Abbreviations and symbols: *ACT* activated clotting time, clotting, *Anti-Xa* anti-Xa heparin, LMW heparin, or fondaparinux assay, chromogenic, *APC Res* activated protein C resistance, clotting, *AT act* antithrombin activity (functional), chromogenic, *DD* D-dimer, EIA or immunoturbidimetric, *DRVVT* dilute Russell viper venom time, *F.II* factor II (prothrombin), clotting (PT-based), *F.VIII* factor VIII, clotting (PTT-based), *F.IX* factor IX, clotting (PTT-based), *F.X* factor X, clotting (PT-based), *Fib* fibrinogen, clotting, *PC act* protein C activity (functional), clotting, *PC Ag* protein C antigenic, EIA, *Plsm act* plasminogen activity (functional), clotting, *PLT Agg* platelet aggregation, *PT* prothrombin time, clotting, *PTT* activated partial thromboplastin time, clotting, *TT* thrombin time, clotting, *VWF act* von Willebrand factor activity (functional), platelet agglutination, ? unknown, 0 unaffected or not significantly affected, ↑ mild to moderate increase or prolongation, ↑↑ moderate to marked increase or prolongation, ↓ mild to moderate decrease, ↓↓ moderate to marked decrease

[a]References are noted in the text

[b]Effects are dose-dependent and reagent-dependent

[c]No effect if method uses dilution with factor V-deficient plasma; clotting times are prolonged if method uses undiluted specimen

[d]False elevation in PTT-based assays; no effect in chromogenic assays

[e]False elevation in thrombin-based assays; no effect in factor Xa-based assays

determined MNPT or involving a calibration procedure in the laboratory. The purpose of the INR is to transform PT values into the PT ratio that would have been obtained had the specimen been tested by a reference method. Use of the INR has facilitated the evolution of global therapeutic ranges, but the INR schema does not, as yet, assure full comparability of results between laboratories.

- UFH therapy is widely used, although it is being replaced by other agents in several clinical settings. A major limitation is a highly variable dose-response between patients due largely to non-specific binding of large heparin molecules to plasma proteins, endothelial cells, monocytes, and platelets. The PTT is the principal test used for therapeutic monitoring, but it also exhibits a highly variable response to heparin. It is incumbent on each laboratory to establish therapeutic range limits and to verify or re-establish the limits whenever a reagent lot change is made or a new reagent is implemented. The PTT is not useful for monitoring LMWH or high-dose UFH therapy.
- The ACT is used for monitoring high-dose UFH anticoagulation, generally for cardiac catheterization, cardiopulmonary bypass, or related procedures. The ACT has a proven clinical record, but suffers from poor precision and poor comparability between analyzers. Therapeutic ranges are generally determined from published clinical protocols.
- LMWH and fondaparinux exert their anticoagulant effects through the activation of AT, as does UFH, but with far more highly predictable dose-responses. Routine monitoring is not required except in special circumstances including body weight extremes, young age, renal dysfunction, and prolonged therapy.
- The anti-Xa assay is increasingly used for monitoring UFH, LMWH, and fondaparinux therapy. A specific standard curve must be established for each type of anticoagulant. PTT therapeutic ranges are defined by anti-Xa therapeutic range limits, but there is significant variation in anti-Xa measurements between reagents and in the relationship between PTT and anti-Xa values.
- Direct thrombin inhibitors, including argatroban, bivalirudin, and dabigatran, selectively inhibit thrombin and do not require AT activity. Argatroban requires therapeutic monitoring with the PTT with limits defined in terms of PTT ratios. Laboratories do not have the means of assessing whether these ratios are suitable for their PTT methods. Dabigatran does not routinely require laboratory assessment, but when needed, the ECT, ECA, or PDTT are the most reliable tests. However, reagents or calibrants are labeled for research use only (RUO) in the United States.
- Oral factor Xa inhibitors, including rivaroxaban and apixaban, do not require dose titration to a laboratory value. When testing is needed, a normal PT essentially excludes the presence of therapeutic concentrations, but does not exclude the possibility of subtherapeutic concentrations. The anti-Xa assay is more reliable, but must be calibrated to the individual agents. At this time, calibrants are labeled for research use only in the United States.
- Therapeutic monitoring of antiplatelet therapy is appealing due to relatively high rates of non-response; however, lab-directly therapy has not been demonstrated to be clinically efficacious. Nevertheless, platelet function testing may be appropriate and help guide therapy in selected situations.

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Pharmacogenetic Testing for Anticoagulant and Antiplatelet Therapies

11

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11.1 Introduction

There is growing interest in the use pharmacogenetic testing to guide drug therapy when safety or efficacy may be significantly impacted by patient genotype. In the coagulation arena, the anticoagulant drug warfarin and the antiplatelet drug clopidogrel are examples of medications that have unpredictable bioavailability and available pharmacogenetic tests. These drugs are widely used in cardiovascular medicine to treat and prevent thromboembolic events. Testing for warfarin sensitivity involves evaluation of genetic variants in VKORC1 and CYP2C9 while testing to predict clopidogrel response involves evaluation of CYP2C19 variants. This chapter discusses currently available information regarding the role of pharmacogenetic testing to guide the use of these drugs.

11.2 Pharmacogenetic Testing for Warfarin

Warfarin is an oral vitamin K antagonist anticoagulant widely used for the prevention and treatment of thromboembolic events. Warfarin exhibits wide interindividual variability in response (due to influences from race, body weight, gender, diet, comorbidities, concomitant medications, and genetic factors) and has a narrow therapeutic window with potential hemorrhagic or thrombotic complications if

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incorrect doses are administered, necessitating laboratory monitoring with the International Normalized Ratio (INR). Warfarin's effect on the vitamin K-dependent coagulation factors is mediated by inhibition of the vitamin K epoxide reductase (VKORC1) enzyme complex [1]. VKORC1 reduces oxidized vitamin K and regenerates the vitamin K needed to create functional forms of coagulation factors II, VII, IX, and X through post-translational modification [1]. Variants in the VKORC1 gene are known to affect warfarin response [1]. Several liver cytochrome P450 (CYP) enzymes play a role in warfarin metabolism, including CYP2C9, CYP2C19, CYP2C8, CYP2C18, CYP1A2, and CYP3A4 [1]. The CYP2C9 enzyme is particularly important in the metabolism of S-warfarin, which is the most potent anticoagulant form [1–3]. Point mutations in the CYP2C9 gene result in reduced function enzyme variants and decreased warfarin clearance.

There are two common single nucleotide polymorphisms (SNPs) in the VKORC1 gene promoter, −1639G>A and −1173C>T, that exist in linkage disequilibrium, meaning that they are tightly linked and almost always found together in the same haplotype [3–5]. These variants result in decreased expression of the VKORC1 protein, causing increased sensitivity to warfarin as compared to the wild-type allele [2, 4]. The sensitive haplotype is present in up to 40 % of Caucasians and greater than 80 % of Asians, but is less common in African Americans. Because they are linked, laboratory tests usually identify only the −1639G>A mutation [3].

The wild-type CYP2C9 allele has full function and is designated as *1. Individuals with the *1/*1 genotype are classified as extensive metabolizers [6]. Reduced function alleles CYP2C9*2 and CYP2C9*3, found in approximately 11 and 7 % of Caucasians, respectively, are the most common CYP2C9 variants affecting warfarin metabolism [1, 7], although lower frequency reduced function alleles such as *5, *6, and *11 also occur. The *2 allele reduces warfarin metabolism by approximately 30 % while the *3 allele reduces metabolism by 80–90 %, resulting in reduced daily warfarin requirements [4]. Individuals who are heterozygous or homozygous for these alleles are classified as poor metabolizers [6].

Together, the CYP2C9 and VKORC1 genotypes account for a large proportion of the variation in warfarin response (up to 45 % in Caucasians and up to 30 % in African Americans) [2]. Variant genotypes have been associated with adverse events, such as bleeding, in some studies [1, 4]. The combination of genotype and clinical information may account for 50–70 % of variation in response [4]. The Coumadin label (common formulation of warfarin) currently provides information about expected maintenance doses based on genotyping and taking into account effects of CYP2C9*2 and *3 alleles in combination with the VKORC1 −1639G>A variant (see Table 11.1) [7]. The recommended warfarin dose decreases as the number of reduced function alleles increases. It should also be noted that subjects with genetic variants may require a prolonged amount of time to reach steady state due to the increased drug half-life.

Several algorithms are available that utilize both genotype and clinical information to predict warfarin maintenance dose requirements [1, 8, 9]. One such algorithm is available at www.WarfarinDosing.org. This algorithm takes into account numerous clinical factors, including certain concomitant medications, and allows

Table 11.1 Expected modification of daily warfarin maintenance doses based on genotyping results

CYP2C9						
VKORC1	*1/*1	*1/*2	*1/*3	*2/*2	*2/*3	*3/*3
GG	Typical	Typical	Moderate ↓	Moderate ↓	Moderate ↓	Significant ↓
AG	Typical	Moderate ↓	Moderate ↓	Moderate ↓	Significant ↓	Significant ↓
AA	Moderate ↓	Moderate ↓	Significant ↓	Significant ↓	Significant ↓	Significant ↓

Adapted from information presented in the Coumadin package insert [7]
Typical dose = 5–7 mg/day, moderately decreased = 3–4 mg/day, significantly decreased = 0.5–2 mg/day

the information to be saved and modified based on additional information from INR monitoring. In a study of this algorithm, the pharmacogenetic equation used explained a little over 50 % of the variability in warfarin dose in both derivation (n = 1015) and validation (n = 292) cohorts [8]. Performance of this algorithm was superior to equations that accounted only for clinical factors, which explained approximately 20 % of dose variability [8]. Current algorithms are limited by a lack of detailed guidance regarding dosing intervals and laboratory monitoring.

Small prospective studies have compared the use of pharmacogenetics-guided warfarin dosing with traditional management. These studies have shown mixed results, with three showing no difference in the time in therapeutic range (TTR) and one showing increased TTR in the pharmacogenetic-guided group [1, 10–12]. These small studies did not show differences in thrombotic events, major bleeding, or survival [10]. In 2013, results from three randomized controlled trials were reported in the New England Journal of Medicine. Two of these trials compared genotype-guided dose determination with clinical algorithms and showed no significant differences in the primary outcome of TTR or other endpoints [13, 14]. The third trial compared a pharmacogenetic algorithm with the local standard of care [15]. In this study, genotype-guided dosing was associated with a higher percentage of TTR than standard dosing during warfarin initiation.

When estimating warfarin dosing, it is critically important to consider potential interactions with other medications, including drugs with possible synergistic anticoagulant effects, impact on vitamin K status, or effects on warfarin metabolism. Caution must be used not only with prescription medications, but also over-the-counter and herbal preparations. Numerous medications are metabolized by the liver cytochrome P450 system and may either inhibit or induce these enzymes, with potential impacts on warfarin metabolism. Common drugs that may influence required warfarin does include amiodarone, statins, antibiotics, and antifungals.

There are several FDA-cleared warfarin genotyping assays utilizing a variety of methodologies [5]. These tests identify the CYP2C9*2 and *3 alleles and the VKORC1 promoter mutation −1639G > A, but do not detect other warfarin sensitivity variants or mutations causing warfarin resistance [5]. The wild-type allele (*1) is not directly detected, but is inferred when other variants are not identified, a situation that can lead to genotyping errors in the presence of untested polymorphisms [5, 6]. In an analysis of 5 years of proficiency testing (PT) data from the College of

American Pathologists (CAP), participating laboratories demonstrated greater than 98 % accuracy in identifying samples with the wild type CYP2C9 diplotype (*1/*1, 10 surveys), as well as samples containing variant alleles with diplotypes of *1/*2 (4 surveys), *1/*3 (4 surveys), and *2/*3 (2 surveys) [6]. Similarly, accuracy in identifying wild-type (G/G) versus variant (GA and AA) genotypes of VKORC1 exceeded 96 % in ten total surveys [6]. In the CAP PT analysis, laboratories were also asked to provide expected phenotype. Genotype-phenotype concordance ranged from 89 to 96 % for CYP2C9, with the lowest values seen for prediction of the *1/*2 and *1/*3 phenotypes, and from 89 to 94 % for VKORC1, with the lowest value for prediction of the AA phenotype [6]. Laboratories providing genotyping results should provide guidance on expected phenotype, but are generally limited in their ability to provide comprehensive information due to the need for detailed clinical data. Although current tests can yield results in a few hours, testing availability is usually limited to reference laboratories or larger hospital-based laboratories, which may limit utility to impact initial therapy [16]. Point-of-care assays for warfarin genotyping have been studied, but are not widely used [17]. Problems with reimbursement of warfarin pharmacogenetic testing are an ongoing issue for clinical services and laboratories. This testing is not currently reimbursed by Medicare outside of clinical trial settings [5].

Despite the theoretical advantages of warfarin dose adjustment based on genotyping, the testing is not currently considered the standard of care. Current practice guidelines from the American College of Chest Physicians and American College of Medical Genetics do not recommend routine use of pharmacogenetic testing to guide warfarin dosing at the initiation of therapy due to a lack of evidence supporting this approach and probable lack of cost-effectiveness [3, 10]. There are specific groups where pharmacogenetic testing may be useful including medication-compliant subjects requiring unusually low or high doses to maintain a therapeutic INR, or subjects who may be prescribed prophylactic warfarin in the future [3]. Pharmacogenetic-guided warfarin dosing, if utilized, does not eliminate the need for close clinical follow-up and laboratory monitoring via the INR.

11.3 Pharmacogenetic Testing for Clopidogrel

Clopidogrel is an oral thienopyridine antiplatelet medication that irreversibly blocks the platelet surface $P2Y_{12}$ ADP receptor, inhibiting platelet aggregation and reducing the risk of adverse cardiovascular outcomes in patients with cardiovascular disease [18]. Clopidogrel is administered as an inactive prodrug that undergoes a 2-step conversion to an active thiol metabolite by the liver cytochrome P450 (CYP) system [19]. Several CYP enzymes, including CYP2C19, CYP3A, and CYP2B6, and CYP1A2, are involved in the metabolism of clopidogrel, but CYP2C19 is considered the principle enzyme responsible for its activation [18–20]. Genetic polymorphisms in CYP2C19 influence generation of the active metabolite. In March 2010, after several previous labeling changes, the Food and Drug Administration (FDA) added a boxed warning to the package insert of clopidogrel informing prescribing physicians about potential risks for adverse cardiovascular events in clopidogrel

Table 11.2 Summary of CYP2C19 variants and effect on clopidogrel metabolism

CYP2C19 variant	Description	Clopidogrel metabolism status	Possible therapeutic approach[a]
*1	Wild-type	*1/*1 = extensive metabolizer	Standard clopidogrel dosing
*2 or *3	Loss of function	1 allele = intermediate metabolizer 2 alleles = poor metabolizer	Consider alternative antiplatelet therapy if not contraindicated
*17	Increased function	*1/*17 or *17*17 = ultra-rapid metabolizer[b]	Standard clopidogrel dosing

[a]Possible therapeutic approaches are based on recommendations for acute coronary syndrome patients treated with percutaneous coronary intervention and based on findings from large meta-analyses [25]

[b]The *17 allele also interacts with loss of function alleles if present, resulting in intermediate metabolizer status [25]

poor metabolizers who generate lesser amounts of the active metabolite [19]. The warning states that testing is available to identify a patient's CYP2C19 genotype and advises consideration of alternative treatment or treatment strategies in subjects who are poor metabolizers. Alternative treatment strategies could include administering higher doses, replacing clopidogrel with other agents such as prasugrel or ticagrelor, or addition of a third antiplatelet agent to dual antiplatelet regimens, although evidence-based alternatives have not been established in clinical trials [19]. Prasugrel is a more potent $P2Y_{12}$ inhibitor that is converted to its active metabolite in a single step and has very few reports of poor responders [19, 21, 22]. However, prasugrel is associated with increased rates of bleeding as compared to clopidogrel [19]. Ticagrelor is a reversible non-thienopyridine $P2Y_{12}$ inhibitor that does not require activation [19, 23].

The CYP2C19*1 allele is the wild-type allele (full function) and several alleles associated with reduced or absent function have been described (*2, *3, *4, *5, *6, *7, and *8, others). CYP2C19*2 and *3 are non-functional and represent the most common reduced function alleles in whites and Asians [19]. The *2 allele (24 % of whites, 30 % of African Americans, and 50 % of Asians have at least one copy) encodes a cryptic splice site that results in complete loss of enzyme activity, while the *3 allele results in introduction of a stop codon [20, 24]. Using the CYP2C19 genotype, individuals can be phenotypically classified as ultra-rapid, extensive, intermediate, or poor metabolizers (see Table 11.2) [6, 25]. Extensive metabolizers have no loss-of-function (LOF) alleles and generate expected amounts of active drug, intermediate metabolizers carry 1 LOF allele, and poor metabolizers carry 2 LOF alleles (2 to 14 % of the population) [25, 26]. Several studies have revealed an increased risk of major adverse cardiac events in subjects with reduced function alleles, particularly poor metabolizers who have significantly lower exposure to the active metabolite and less inhibition of platelet aggregation [19, 26–28].

Clinically available CYP2C19 genotyping tests generally characterize the most common LOF alleles CYP2C19*2 and *3 by targeted real-time polymerase chain reaction (PCR). Some tests also evaluate less common LOF alleles and may look for *17, which is associated with ultra-rapid metabolism, increased exposure to the

active metabolite, increased platelet inhibition, and increased risk of bleeding (see Table 11.2) [26]. The *17 variant (3–21 % of the population) results from a promoter mutation that results in increased CYP2C19 gene transcription and this hyper-functional variant interacts with LOF alleles, if present [20, 25]. As in the warfarin genotyping tests, the presence of the wild-type allele (*1) is often inferred when other variants are not identified, a situation that can result in systematic genotyping errors when there are variants present that are not identified by the testing panel [6]. When interpreting results, it is important to know which CYP2C19 variants can and cannot be identified by the test.

Pharmacogenetic results should include interpretive comments that provide information on the expected phenotype. This is fairly straightforward for extensive metabolizer (*1/*1) and poor metabolizer genotypes (2 reduced function alleles), and more difficult to predict for the heterozygous condition with 1 wild-type allele and 1 reduced function or increased function allele [6]. Due to reports of higher rates of adverse cardiac events in *1/*2 CYP2C19 heterozygotes, it is important to classify these subjects as intermediate rather than extensive metabolizers [6, 25]. Interpretations may also provide therapeutic recommendations, although definitive recommendations based on clinical trials evidence are not currently available. The recent Clinical Pharmacogenetics Implementation Consortium Guidelines summarize therapeutic recommendations based on CYP2C19 status for acute coronary syndrome patients managed with percutaneous coronary intervention (PCI), based on the findings of large meta-analyses (see Table 11.2) [25]. The guidelines do not recommend widespread adoption of the therapeutic recommendations when clopidogrel is used for other indications.

Genotyping assays have traditionally been performed only by specialized laboratories and results may not be available for several days, hampering their ability to influence therapy in the acute setting. A point-of care assay for CYP2C19 genotyping of the *2, *3, and *17 alleles (Spartan RX CYP2C19 Assay, Spartan Bioscience Inc., Ottawa, Ontario, Canada) has recently been FDA-cleared for In Vitro Diagnostic use. The device was used in a proof-of-concept trial (RAPID GENE) in PCI patients where carriers of the *2 allele were treated with prasugrel rather than clopidogrel and additional trials are ongoing [29]. Cost may be an issue for CYP2C19 genotyping since the testing is not reliably reimbursed [19].

The College of American Pathologists offers a proficiency testing (PT) program for pharmacogenetics that includes CYP2C19 genotyping. An analysis of PT data from 2007 to 2012 demonstrated good genotype accuracy for identification of the *1, *2, *3, and *8 alleles [6]. For instance, accuracy for the *1/*1 (9 challenges), *1/*2 (6 challenges), and *2/*2 (1 challenge), and *2/*3 (1 challenge) genotypes were approximately 98, 97, 88, and 96 %, respectively. Accuracy for certain uncommon or rarely tested genotypes showed poor accuracy, but the findings were based on very limited data.

It should be noted that even in patients with two fully functional CYP2C19 alleles (*1/*1, extensive metabolizer), there may be other factors that contribute to residual platelet reactivity and on-clopidogrel treatment failure. These include compliance, differences in intestinal absorption (affected by ABCB1 genotype),

interactions with other drugs metabolized by the same pathways, CYP enzymes other than CYP2C19 or paraoxonase 1 (PON1) that impact generation of the active metabolite, and increased platelet reactivity [4, 18, 19, 22, 30–32]. For instance, the clopidogrel package insert warns against concomitant use with drugs that inhibit CYP2C19, such as omeprazole [18]. Overall, CYP2C19 polymorphisms appear to account for only a small percentage (~12 %) of the reported variability, and most of the variability in clopidogrel response is yet to be explained [19, 24, 26].

In addition to the focus on testing for CYP2C19 genotype, there is a large body of literature evaluating tests of clopidogrel-induced platelet inhibition, most commonly platelet function tests. These tests include light transmission aggregometry, impedance aggregometry, PFA-100®, VerifyNow® $P2Y_{12}$, Plateletworks®, VASP phosphorylation, and others [30]. A 2007 meta-analysis reported a prevalence of 21 % of subjects undergoing percutaneous coronary intervention as having inadequate clopidogrel response, as measured by a variety of laboratory tests of platelet inhibition [33]. Importantly, persistent platelet reactivity while on clopidogrel therapy has been associated with adverse clinical outcomes in a number of studies [18, 22, 30, 33]. However, standardization of these tests is lacking and the tests tend to show poor agreement in the classification of clopidogrel response [30].

Similar to genotyping, alternative dosing strategies for patients with suboptimal platelet inhibition have not been established in clinical trials. For instance, in the GRAVITAS trial, patients receiving clopidogrel therapy were tested for high on-treatment platelet reactivity using the VerifyNow $P2Y_{12}$ test. Patients with high on-treatment reactivity were then randomized to either high- or standard-dose clopidogrel for 6 months. The trial analysis showed no clinical benefit with the higher dose regimen [34]. Similar results were seen in the ARCTIC trial with no significant improvements in clinical outcomes with platelet function monitoring and adjustment of clopidogrel therapy [35]. The TRIGGER-PCI trial was designed to evaluate the utility of switching clopidogrel poor responders to prasugrel, but the study was terminated due to low event rates.

In summary, although detection of reduced function CYP2C19 alleles or residual on-treatment platelet reactivity in clopidogrel-treated patients has been associated with adverse cardiovascular outcomes, routine testing is not currently recommended since evidence-based treatment strategies (directed either by pharmacogenetic or platelet testing) from clinical trials are lacking. Recommendations from the American College of Cardiology, American College of Chest Physicians, and American Heart Association do not support genetic or platelet function testing for clopidogrel as the current standard of care [19, 22].

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Coagulation Testing and Transfusion Medicine

12

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Transfusion Services are presented with many challenging coagulation problems. Most cases gain attention when out of the ordinary requests for blood products are received. The Transfusion Medicine Physician is often brought into a clinical scenario that has already become an emergency and quick response is imperative [1–8]. Decisions often have to be made empirically, or with the help of a very limited coagulation test menu. This chapter will focus on maximizing information from a short coagulation test menu, reviewing the tools available to correct coagulopathies, and applying these tests to emergency situations.

12.1 Essential Coagulation Assays

The basic coagulation assays available in most hospitals include a platelet count, prothrombin time (PT), partial thromboplastin time (PTT), fibrinogen, mixing study, D-Dimer, and heparin assay. These tests have already been discussed in detail, so what follows is a brief discussion on some misconceptions about certain assays and

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helpful hints regarding result interpretation in an emergency. Ultimately, the coagulation assays used to direct transfusion therapy are not as important as selecting, and implementing an appropriate algorithm to guide clinician practice [9].

12.1.1 Prothrombin Time

The PT is used to monitor the extrinsic and common soluble coagulation pathways. It is also used to monitor warfarin therapy via the international normalized ratio (INR) – a value calculated employing the PT, the mean normal PT and the international sensitivity index (see Chap. 10). The INR was developed to standardize PT results between laboratories using reagents with differing sensitivities to the anticoagulant effects of warfarin. The INR can be used to guide transfusion of fresh frozen plasma in bleeding patients receiving warfarin therapy. The INR has not been evaluated as a tool for guiding transfusion in other clinical situations (e.g., massive transfusion with multiple coagulation factor deficiencies).

A common misconception among clinicians is that heparin cannot cause a prolongation of the PT. Most PT reagents now contain substances (e.g. heparinase, polybrene) that will neutralize up to 1.0–2.0 U/mL of heparin in a patient sample resulting in a PT result unaffected by heparin. However, greater plasma heparin concentrations (e.g. heparin overdose, or status-post hemodialysis or cardiopulmonary bypass) will cause a prolongation of the PT (as well as the PTT) resulting in confusion as to the cause of clinical bleeding. An example will be presented later.

12.1.2 Partial Thromboplastin Time

The PTT is used to measure the intrinsic and common coagulation pathways. It is commonly used to monitor heparin therapy. The PTT is not as sensitive to coumadin therapy as the PT, but overdoses of warfarin can cause a prolongation of the PTT since the intrinsic or common pathways include factors II, IX, and X.

12.1.3 Fibrinogen Concentration

Fibrinogen levels are extremely valuable for diagnostic purposes and in determining therapy. However, many different methodologies for measuring fibrinogen levels are available and all have their pros and cons. Clinical laboratories generally require a rapid turn-around-time and rely on functional (rather than antigenic) fibrinogen assays. The Clauss assay, which derives the fibrinogen concentration from the clotting time of diluted plasma after addition of excess thrombin, is the most commonly used method. However, fibrinogen levels can also be calculated from the total change in optical signal observed during a PT reaction. Antigenic assays are mainly used in a reference laboratory setting. The type of assay used in a hospital is not as important as understanding how the assay performs in certain clinical situations.

Table 12.1 Effect of decreasing fibrinogen concentration on the PT and PTT

Fibrinogen deficient curve			
% Activity	Fibrinogen, mg/dL	PT (s) (normal 10.7–15.0 s)	PTT (s) (normal 25–40 s)
100	288	11	28
80	230.4	11.4	29.8
70	201.6	11.6	29.8
40	115.2	12.6	31.9
33	95.04	13.8	34.5
30	86.4	13.1	32.6
20	57.6	14.2	34.3
10	28.8	16.6	40
5	14.4	23.4	44.5
0	0	54.1	150

Abbreviations: *PT* prothrombin time, *PTT* partial thromboplastin time

For example, understanding how different heparin concentrations affect fibrinogen results is critical.

Fibrinogen levels of 80–100 mg/dL are required to achieve hemostasis in a bleeding patient. Routine fibrinogen determinations in emergency situations may seem unnecessary because the PT and PTT might be expected to become prolonged with significant fibrinogen deficiency. Unfortunately, this is not necessarily true. An example of a fibrinogen deficient response curve for PT and PTT is represented in Table 12.1. The curve is created by maintaining normal levels of all clotting factors except fibrinogen. Fibrinogen levels are then varied and the PT and PTT are measured. This curve demonstrates that fibrinogen levels can drop to approximately 58 mg/dL, while the PT and PTT remain in the normal range. It is not until fibrinogen levels drop to approximately 29 mg/dL that the PT prolongs to 1.6 s above the upper limit of normal, but the PTT is still at the upper limit of normal. A clinical condition that results in destruction of fibrinogen may not be detected if the PT and PTT are the only assays relied on to detect a low fibrinogen.

12.1.4 Prothrombin Time and Partial Thromboplastin Time Mixing Studies

Mixing studies are used to determine if a prolongation in a PT or PTT is due to a deficiency of clotting factors, heparin or inhibitors. The test is performed by mixing patient plasma, with pooled normal plasma in a 1:1 ratio. After mixing, the PT and PTT are repeated and the times should shorten close to, or within the normal range, if the prolonged results are due to a deficiency of clotting factors. In emergency situations, mixing studies can be useful in identifying the presence of heparin as a cause of unexpected bleeding, since a lack of correction suggests the presence of heparin or an inhibitor. However, correction of the PTT does not rule out the presence of heparin in the sample (Fig. 12.1). Therefore, heparin assays should replace mixing studies for this purpose.

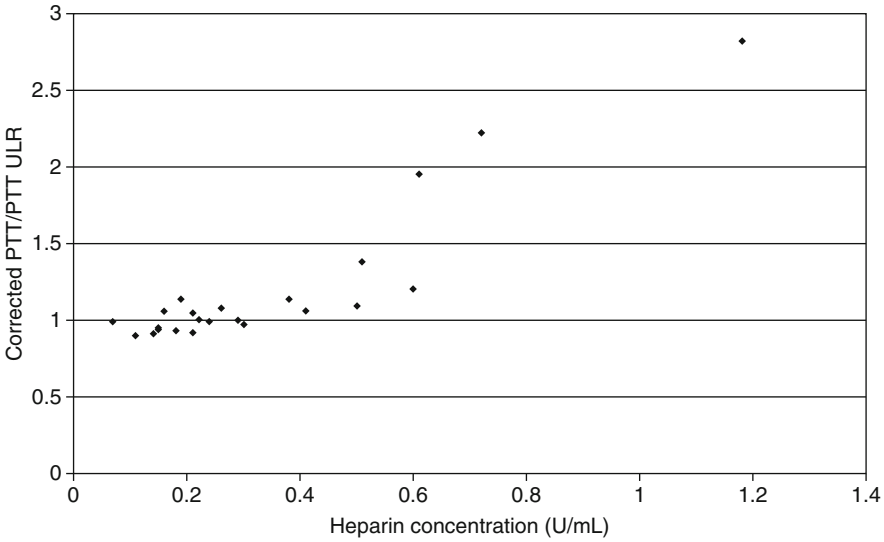

Fig. 12.1 Plot of the ratio of the corrected PTT (1:1 dilution of patient sample with pooled normal plasma) to the uncorrected PTT versus the concentration of heparin in specimens collected from patients receiving unfractionated heparin therapy. Results for corrected samples were not consistently above the upper limit of the reference interval until the heparin concentration reached 0.4 U/mL. *Abbreviations*: *ULR* upper limit of reference interval, *U* units

12.1.5 D-dimer Assay

D-dimer assays are very specific for the diagnosis of disseminated intravascular coagulation (DIC) [10]. The diagnosis of severe, acute DIC must be made quickly to find, and treat, the underlying cause. Early identification of DIC will also direct appropriate transfusion therapy as will be discussed later. Minor elevations in D-dimer are seen following routine surgical procedures, and in trauma patients, and should not be considered diagnostic for DIC [10–12].

12.1.6 Platelet Counts

Platelet counts are essential for identifying bleeding that may be secondary to thrombocytopenia and for directing transfusion therapy in a bleeding patient. This is an extremely valuable, but underutilized assay.

12.1.7 Thromboelastography

The search for the "Holy Grail" of coagulation assays has been ongoing for decades. The perfect assay would assess platelet number, platelet function and the soluble coagulation system with a single, rapid, inexpensive test. The thromboelastograph

(TEG) is the prototype example of an attempt at such an assay. TEG has been discussed previously in this book, but in short, is a whole blood clotting assay that gives a real time graph of clot formation and stability. Standard TEG analysis was expected to be the ultimate coagulation assay because of the ability to assess all components necessary for clot formation. Subsequent studies demonstrated that the standard TEG assay was not able to differentiate between the effects of viable platelets versus cryodisrupted platelets that were added to the test system. Therefore, standard TEG analysis appears to be sensitive to the amount of phospholipid in the test system, but unable to differentiate between functional and non-functional platelets [13]. New TEG assays have been developed that use platelet agonists as initiators of clot formation in addition to the original phospholipid reagent [14]. This modification was made to better assess platelet function. These new assays have significantly increased the cost of TEG without convincing evidence of clinical benefit (see Chap. 7).

12.2 Transfusion Medicine Tools: Blood Components

12.2.1 Platelets

Platelet concentrates should be transfused in a systematic fashion. This requires consideration of three questions:

1. Is thrombocytopenia causing life-threatening hemorrhaging?
2. What is the cause of the thrombocytopenia?
3. Did the patient's platelet count increase after platelet transfusion?

The primary sites of life-threatening hemorrhage due to thrombocytopenia include the brain and the lungs. If thrombocytopenia is the suspected cause of life-threatening hemorrhage, platelets must be transfused immediately.

Bleeding patients who are not in jeopardy of death or significant morbidity should not be transfused until the cause of the low platelet count is evaluated. Understanding the etiology of the thrombocytopenia is critical because transfusion of platelets in certain disease states can actually cause morbidity and even death. Two clinical conditions where platelet transfusions should be avoided are thrombotic thrombocytopenic purpura (TTP) and heparin-induced thrombocytopenia. Platelet transfusions administered to patients with these conditions can cause an increase in platelet thrombi in tissues resulting in end organ damage.

After platelets are transfused, it is important to check a platelet count to ascertain the success of the transfusion. Many clinical conditions can lead to the immediate destruction of platelets including autoimmune thrombocytopenic purpura, sepsis, fever, splenomegaly. The administration of medications such as amphotericin and anti-thymocyte globulin can also result in platelet destruction. Patients with a prior history of pregnancy and/or transfusion are at risk for developing HLA antibodies and may need HLA matched platelets to have an adequate response to platelet transfusion.

The best way to monitor platelet transfusion success is by determining a pre-transfusion platelet count and checking the count again 1 h after completing the platelet transfusion. If time does not permit waiting for an hour (e.g. the need for an invasive procedure), a post-transfusion platelet count can be checked after 10 min. The transfusion of an adult dose of platelets (one apheresis platelet unit or six pooled, random donor platelet units derived from whole blood) should increase the platelet count by 30,000–60,000/μL. Factors determining the magnitude of the response include the number of platelets in the product, the size of the patient, and whether the patient has any clinical conditions leading to refractoriness to platelet transfusions. Calculations such as the corrected count increment or recovery rate can best quantify the success of a platelet transfusion; however, these tools are usually not necessary in routine practice.

The goal of platelet transfusion is to provide adequate circulating platelet numbers to prevent or minimize bleeding. Patients with platelet counts below 5,000/μL are generally considered to be at risk for life-threatening, spontaneous hemorrhage in the central nervous system. Prophylactic platelet transfusions for patients undergoing chemotherapy are usually given when counts are between 10,000 and 20,000/μL at the discretion of the clinician. Some clinician's prefer to limit patient exposure to blood products and will tolerate minor mucosal bleeding and petechiae to avoid the risks of transfusion. Others transfuse platelets at counts closer to 20,000/μL to limit bleeding as much as possible.

Most patients are not at risk for severe bleeding when platelet counts are above 20,000 per μL, unless they are scheduled for an invasive procedure or have received anti-platelet medications. Most invasive procedures, or surgery, can be safely performed when counts are above 50–75,000 per/μL, but many surgeons aren't comfortable until counts are above 100,000/μL. Striving for platelet counts significantly above 100,000/μL is neither a prudent, nor necessary use of a precious resource. Additional platelet transfusions during an invasive procedure may be necessary, and should be guided by platelet counts obtained during surgery.

The following case history demonstrates why a systematic approach to platelet transfusion is essential to optimize patient outcome. A 60 year old male was scheduled for a total hip replacement. He had a long history of aplastic anemia that required red cell transfusions. The patient's platelet count usually ran around 30,000 per μL. He was transfused two units of apheresis platelets and taken to surgery without checking a post-transfusion platelet count. The patient required more than 70 blood product units during the procedure and almost exanguinated. Later investigation revealed the patient did not respond to the pre-operative platelet transfusions because of preformed HLA antibodies that immediately destroyed the transfused platelets.

A systematic approach to platelet transfusion could have prevented this near disaster. The patient did not have life-threatening bleeding prior to surgery, but performing a total hip replacement with a platelet count of 30,000/μL put him at risk. Prior to platelet transfusion, the cause of the patient's thrombocytopenia should have been investigated to determine if there was a contraindication to platelet transfusion. In this case the cause was aplastic anemia and platelet transfusion was indicated. Finally, post-transfusion platelet count should have been checked to see if

surgery could have been performed safely. Unfortunately, this was not done and the surgeon was not aware that the platelet transfusions had not increased the pre-operative platelet count. If the post-transfusion count had been performed, surgery would have been canceled and re-scheduled for a day when HLA matched platelets would have been available to raise the patient's platelet count to a level adequate for a safe procedure.

12.2.2 Fresh Frozen Plasma (FFP)

Plasma is frozen soon after collection to preserve levels of the labile clotting factors V and VIII. FFP not only contains all factors in the soluble coagulation system, but also natural inhibitors of the soluble coagulation system (e.g. anti-thrombin), and proteins of the fibrinolytic system (e.g. plasminogen). FFP provides all factors necessary for the delicate balance between hemostasis and thrombosis. However, FFP is used mainly in hemorrhaging patients to replace deficiencies of multiple coagulation factors.

Hospitals are required to have laboratory guidelines for the use of FFP. The most commonly used cutoff to indicate the need for FFP transfusion is a PT and/or PTT that exceeds 1.5 times the mid-range of normal [3]. The problem with this universally applied guideline is that it does not result in a standard that can be compared between different hospitals and coagulation assay methodologies. Note that an INR of 1.5 is not necessarily equivalent to a PT of 1.5 times the mid-range of normal.

An alternative to the above calculation involves using a dilution curve to provide a guideline for FFP use (Table 12.2). A dilution curve is performed by diluting

Table 12.2 Sensitivity of the PT and PTT to dilution of pooled normal plasma with normal saline

Dilution sensitivity (upper limit of normal in seconds: PT = 15.5 s, PTT = 37 s)		
Prothrombin activity curve		
% Activity	PT (s)	PTT (s)
100	13.6	29
90	14.5	30
80	15.2	31
70	15.9	33
60	17.2	36
50	19.2	40
40	22.4	48
30	27.7	64
20	40.3	99
15	52.8	>150
10	102.8	>150
5	>150	>150
1	>150	>150

Abbreviations: *PT* prothrombin time, *PTT* partial thromboplastin time

pooled, normal plasma with a known amount of crystalloid solution (e.g. normal saline) and performing PT and PTT assays on the dilutions. For example, the 50 % PT and PTT times were run on a sample of 50 % normal saline and 50 % normal plasma. The dilution curve is an in-vitro simulation of what happens when a bleeding patient is resuscitated with normal saline prior to FFP availability. The PT and PTT at the 50–40 % level are used as the guideline for FFP transfusion in the case of dilutional coagulopathy. The advantage to this approach is that it assures that the PT and PTT that are used as a guideline in a given institution fall at the same place on a dilution curve. This is not always the case when using the 1.5 times midrange of normal guideline, where the calculation may lead to a 10–20 % difference between where the PT and PTT fall on the dilution curve. While the dilution curve gives more consistency between the PT and PTT guideline values, it does not solve the problem of standardization between hospitals. For example, the 50 % cutoff will not result in the same number of seconds for PT and PTT assays in different hospitals.

Many clinicians attempt to use the INR as a guideline for FFP use. While it is true the INR will increase with a worsening coagulopathy, it has not been thoroughly evaluated as a method to direct FFP therapy. The exception would be in the setting of rapid warfarin reversal.

12.2.3 Cryoprecipitate

Historically, cryoprecipitate has been used to treat hemophilia A and von Willebrand disease, but now is used almost exclusively as a source of fibrinogen. The content, and indications for use of cryoprecipitate are not understood by many clinicians. Cryoprecipitate is produced from FFP by allowing FFP to thaw in a 1–6 °C environment. When kept at this temperature, the FFP melts, but 10–20 mL of precipitate will form. Refrigerated centrifugation separates the precipitate from the liquid. The precipitate can then be isolated, and when warmed, will go into solution. The result is a liquid, which contains fibrinogen, factor VIII, von Willebrand factor and factor XIII. Myths surrounding the use of cryoprecipitate include that it is a concentrated form of FFP and that it contains more fibrinogen than FFP. In fact, cryoprecipitate contains only the factors listed above, and only about 50 % of the fibrinogen contained in the original unit of FFP. Cryoprecipitate does not contain as much fibrinogen as FFP, but there is more fibrinogen per unit volume compared with FFP (i.e., it is a concentrated source of fibrinogen). Each bag of cryoprecipitate contains, on average, about 200 mg of fibrinogen. As noted earlier in this chapter, fibrinogen levels of 80–100 mg/dL are required to achieve hemostasis in a bleeding patient. To raise the fibrinogen level in an adult by 100 mg/dL requires between 10 and 30 bags of cryoprecipitate depending on the size of the patient (about two bags per 10 kg of body weight). The most common error in cryoprecipitate transfusion is delivery of a dose insufficient to significantly increase fibrinogen levels in an adult (e.g. four bags). Patient fibrinogen levels should always be re-evaluated after cryoprecipitate infusion.

12.3 Clinical Scenarios and Case Studies

12.3.1 Dilutional Coagulopathy

Massive transfusion is defined as replacement of a patient's blood volume in a 24-h period [1]. Some prefer the definition of transfusion of ten units of packed red blood cells in an adult [2], or even four packed red blood cells in a 1-h period [1]. Most massively transfused patients are initially resuscitated with crystalloids followed by packed red blood cells. Red cells contain negligible amounts of the soluble coagulation system and no viable platelets, while crystalloids contain nothing involved in hemostasis. The result is dilution of the soluble coagulation system and platelets. Each will be discussed separately, but must be evaluated together in a bleeding patient.

12.3.1.1 Dilution of the Soluble Coagulation System

Therapeutic plasma exchange (TPE) represents the best example of pure dilution of the soluble coagulation system. In TPE, whole blood is removed from the patient and directed into an automated cell separator. The various components of the blood are separated by differential centrifugation and the desired blood fraction (e.g., plasma) is removed. TPE is used in many clinical conditions where a pathologic substance in plasma (often an auto-antibody) produces a disease state. The rate of removal of the pathologic substance is dependent upon the number of plasma volumes exchanged [15] (Table 12.3).

Unfortunately, TPE does not exclusively remove the pathologic substance, and beneficial contents of plasma, such as clotting factors, are also removed. FFP should be the ideal replacement fluid because it contains clotting factors and immunoglobulins, but it carries risks of transfusion transmitted disease and adverse reactions, such as transfusion related acute lung injury. Therefore, FFP is only used as a replacement fluid for patients at risk of bleeding, or who need a specific factor contained in FFP such as the metalloprotease required for patients with TTP.

Colloids, or a combination of colloid and crystalloids, are the usual replacement fluid selected for TPE. These solutions contain no clotting factors, and patients must be monitored closely to avoid spontaneous hemorrhage and to ensure the safety of invasive procedures if they become necessary in the course of patient management. Routine laboratory testing prior to TPE should include a platelet count, PT, PTT and

Table 12.3 Effect of the number of plasma volumes exchanged on the removal of pathologic substances in plasma

Plasma volumes exchanged	Fraction removed
0.5	.39
1.0	.43
1.5	.78
2.0	.86

fibrinogen. These assays should be in the normal range before a TPE procedure is initiated. Clinicians rarely appreciate the severity of the transient coagulopathy produced by a TPE procedure. They must be reminded that all clotting factors are depleted by the procedure, and this can result in a coagulopathy more severe than that seen in hemophilia patients lacking a single clotting factor. TPE with colloid replacement is usually performed on an every other day basis to allow for recovery of clotting factors through endogenous production prior to the next procedure. However, recovery rates vary between patients, and coagulation testing prior to each procedure is prudent.

The following is a poignant case illustrating the severity of a pure, dilutional coagulopathy caused by TPE. A 77 year-old male was admitted for TPE to treat myasthenia gravis. The platelet count, PT, PTT and fibrinogen were all within the normal range prior to the procedure. The patient underwent a 1.3 plasma volume exchange utilizing a left femoral vein, dual-lumen catheter, and using a 5 % albumin solution as the replacement fluid. The procedure would have predicted approximately 70 % removal of the original clotting factors, but should not have resulted in spontaneous hemorrhage.

Three to four hours after completion of the procedure, the apheresis team was called by an intensive care doctor wondering why the patient was found hypotensive with a 4–5 unit hematoma in the right groin. A hematoma in the right groin was perplexing since the catheter used for the procedure was in the left groin, and venous lines rarely bleed after TPE. The following coagulation assay results were obtained:

PT (11–15.5 s)	29 s	Mix 16 s
PTT (25–37 s)	72 s	Mix 34 s
FIB (150–350 mg/dL)	70 mg/dL	
D-dimer	Normal	

Where FIB equals fibrinogen

The PT, PTT and fibrinogen were about what would be expected following a TPE procedure. The PT and PTT were prolonged but the mixing study corrected almost to normal, and the platelet count was above 100,000/μL. The fibrinogen was decreased as expected, and a normal D-dimer ruled-out DIC. Comparing the PT and PTT after the procedure with the dilution curve (Table 12.2) shows the results landing on the curve between the 30 % and 20 % level. This means that 70–80 % of the original clotting factors were removed. The predicted removal rate was around 70 %, but further investigation showed that the patient had received some additional crystalloid fluid when he was found hypotensive, explaining the slightly more severe coagulopathy than predicted. The patient was given eight units of FFP. The coagulopathy was corrected and the bleeding stopped.

The mystery surrounding this case involved why bleeding occurred in the right groin when the line was placed in the left femoral vein. Further investigation revealed that the physician inserting the line first attempted to place it in the right femoral vein, but aborted the attempt when he cannulated the femoral artery by mistake. He had pulled the line out, and held pressure until the bleeding had stopped,

but failed to inform anyone of the mishap. A platelet plug had stopped the initial bleeding in the artery, but once the TPE was initiated, clotting factor levels were dropped to levels that would not allow adequate fibrin formation to cement the platelet plug in place.

All cases of massive transfusion result in a similar coagulopathy when crystalloid resuscitation is used prior to FFP availability. Blood banks must have protocols in place that allow for thawing of FFP early in the course of massive transfusion. Often, FFP must be given empirically, since time may be insufficient for the laboratory to turn around basic coagulation results. When possible, thawed FFP should be available before the patient receives a complete blood volume of red cells and crystalloid.

12.3.1.2 Dilutional Thrombocytopenia

Dilutional thrombocytopenia occurs later in the course of transfusion of resuscitated patients than what would be predicted by "wash-out" mathematical calculations [1]. This probably reflects mobilization of endogenous platelet reserves including marginated platelets, platelets contained in the spleen and lungs, and platelets released early from the bone marrow. Platelet transfusions are usually given to massively transfused patients after initial transfusion of red cells and plasma, and are usually effective in maintaining platelet counts sufficient for primary hemostasis. As already noted, the effectiveness of platelet transfusions should be monitored with frequent platelet counts, a fast and inexpensive assay.

12.3.1.3 Inadvertent Heparinization

Heparin can be very problematic for the Transfusion Service, particularly when it is present in a sample due to contamination from a line draw, or, more importantly, due to unintentional heparinization of the patient resulting in unexpected bleeding. However, the presence of heparin in a sample must be suspected for the heparin assay to be ordered. Routine coagulation assay patterns can help identify samples containing heparin. The first clue to the presence of small concentrations of heparin will be a greatly prolonged PTT with a PT that is normal or close to normal. The discrepancy between the two assays is due to the heparinase or heparin-binding agent contained in the PT reagent that neutralizes the effects of the heparin. If heparin concentrations are very high, both the PT and PTT will be prolonged, usually greater than the time where laboratories stop reporting results (e.g. >150 s). Mixing studies were historically used to provide evidence for the presence of heparin, but now, once heparin is suspected, a quantitative heparin assay should be performed.

Once the presence of heparin is confirmed, the investigation must continue to determine if it is sample contamination, or if the patient had been given heparin. This may be as easy as asking the phlebotomist if the sample was obtained with a peripheral stick, or was collected from a line. The next step should be to contact the nurse to see if the patient has been placed on heparin and if the patient shows any signs of bleeding. The investigation must continue even if the floor denies overt heparin administration because of ubiquitous heparin availability in

hospitals. Patients may receive heparin for dialysis, invasive diagnostic procedures or may be status post-cardiopulmonary bypass. Patients may have large concentrations of heparin in central lines, which can be inadvertently flushed into the patient. Heparin comes in many different concentrations that can lead to an inadvertent overdose.

The following case illustrates the difficulties heparin can cause, both in diagnosis and in delaying treatment. A 2-day old full term male was in the well-baby nursery of a small community hospital when the nurse noticed an increased respiratory rate. The pediatrician was concerned that the baby was developing pneumonia and gave the child a bolus of antibiotics while arranging transportation to a children's hospital. Upon arrival at the children's hospital the child had confluent ecchymoses and signs consistent with meningitis. A lumbar puncture was performed which resulted in severe bleeding, spinal cord compression and lower extremity paralysis. An ultrasound demonstrated intra-ventricular hemorrhage as well. Fearing DIC, clinicians ordered FFP, cryoprecipitate and platelets, which led to an investigation by the blood bank physician. Laboratory results on the baby showed the following:

- PT > 150 s, mixing study > 150 s
- PTT > 150 s, mixing study > 150 s
- Fibrinogen = 0 mg/dL
- D-dimer = normal
- Platelet count = 170,000/μL

The blood bank physician covering the pediatric hospital was perplexed by the results. The PT, PTT and fibrinogen were consistent with DIC, but the platelet count, D-dimer and mixing studies were more consistent with heparinization. The physician had the assays repeated at an adjoining adult hospital where he was more familiar with the coagulation assay characteristics and the following results were obtained:

- PT > 150 s, mixing study > 150 s
- PTT > 150 s, mixing study > 150 s
- Fibrinogen = 142 mg/dL
- D-dimer = normal
- Platelet count = 165,000/μL

These results were consistent with systemic heparinization of the baby given the severe bleeding history. The nurse was contacted with an explanation the laboratory results and to recommend protamine administration. The nurse did not believe the interpretation of the results, so a heparin assay was performed which was 5.2 U/mL (recommended heparin therapeutic range equals 0.3–0.7 U/mL). The attending physician was convinced of the heparin overdose and protamine was given to the child correcting the coagulopathy.

This case makes some salient points. The first is the importance of understanding the strengths and weaknesses of coagulation assays in the hospital where you practice. The assays obtained at the children's hospital were confusing because of the fibrinogen level of zero. The blood bank physician later discovered that fibrinogen levels at the children's hospital were calculated from the rate of clot formation on the PT assay. Heparin had prevented the PT from clotting; therefore a zero fibrinogen level was calculated. The fibrinogen assay at the adult hospital was resistant to the effects of heparin. The physician knew heparin would falsely reduce the true fibrinogen level by 10–20 %, but that fibrinogen would be measured. The second point is that a heparin assay result is often required to convince clinicians that the bleeding problem is related to heparin. The final question to answer was where did the heparin come from? In this case, as in most mysterious cases of inadvertent heparin administration, the transfusion medicine physician must perform the investigation. In this case, the investigation revealed that the community hospital had decided to heplock the patient's I.V. after an antibiotic bolus was given to the child. Unfortunately, instead of 10 U/mL of heparin, the nurse was handed a concentration of 10,000 U/mL, and the child was inadvertently, systemically anticoagulated.

Heparin can complicate patient care at anytime. Know how coagulation assays in your hospital respond to heparin, and have a low threshold for performing heparin assays when unexpected bleeding occurs.

12.3.1.4 Disseminated Intravascular Coagulation

DIC is caused by a stimulus that leads to abnormal and constant thrombin generation [16]. Many etiologies for DIC exist, but the most severe, acute cases of DIC are obstetrically related. Causes of DIC in pregnant women include amniotic fluid embolus, infection and retained products of conception. While clinicians search for the underlying cause of the DIC, the blood bank must provide product support before and after the underlying cause is treated.

In addition to thrombin generation, DIC also results in activation of the fibrinolytic system and abnormal amounts of plasmin are generated [16]. Thrombin converts fibrinogen to fibrin, and plasmin not only digests cross-linked fibrin clots, but also destroys fibrinogen before in is converted to fibrin. Therefore, DIC results in a rapid, and disproportionate reduction in fibrinogen levels relative to other clotting factors. Treatment must include rapid replacement of fibrinogen, and cryoprecipitate is the ideal blood product for this purpose, as demonstrated below.

A 27 year-old female, with a pregnancy estimated at 29 weeks gestation, was admitted with premature rupture of membranes. Fetal heart tracings deteriorated and the patient was taken to the operating room for a successful cesarean delivery without immediate complications. A post-operative check on the patient was performed 1.5 h after delivery. She was pale, tachycardic, hypotensive, and oozing from the incision site with approximately one liter of blood found in the bed. She was taken emergently back to the operating room for a hysterectomy. Laboratory

Table 12.4 Selected laboratory values from a patient diagnosed with DIC

Time[a]	HCT	PLT	PT	PTT	Fibrinogen	D-dimer
(hours)	39–47 %	140–440,000/μL	13–16 s	25–36 s	150–350 mg/dL	<.5 mg/dL
Admit	39	183				
1.5	17	53	>150	>150	<7	>256
4.5	18	19	21	48	152	64
6	29	134	17	31		

Abbreviations: *HCT* hematocrit, *PLT* platelet count, *PT* prothrombin time, *PTT* partial thromboplastin time, *s* seconds, *DIC* disseminated intravascular coagulation
[a]Time in hours after admission to the hospital

results at admission and hours after the original cesarean section are shown in Table 12.4.

The laboratory findings 1.5 h after delivery, when the patient was found in shock, show the hallmarks of DIC. Most notably, no detectable fibrinogen and extremely high D-dimer levels. Thirty units of cryoprecipitate were given to rapidly correct the fibrinogen level. As previously noted, cryoprecipitate provides the most concentrated form of fibrinogen and can be rapidly thawed, pooled and infused into the patient. In addition to cryoprecipitate, the patient received nine units of packed red blood cells, two apheresis packs of platelets and seven units of FFP. The laboratory results drawn 4.5-h after the hysterectomy show dramatic improvement. By the 6-h mark, the PT and PTT had returned to normal and the patient was stable and survived without permanent sequelae.

DIC is one of the most feared clinical conditions facing Transfusion Medicine Services. Severe, acute DIC is most commonly related to obstetrical cases and has a high mortality rate. Successful treatment must be directed at the underlying cause and the blood bank must focus on rapid replacement of fibrinogen. This is best accomplished with the use of adequate amounts of cryoprecipitate. Plasma and platelets will also be required to provide the factors not contained in cryoprecipitate.

12.4 Emerging Issues in Transfusion Medicine and Anticoagulation

12.4.1 Reversing New Oral Anticoagulants (NOACs)

The "perfect" oral anticoagulant would be effective treating all thrombotic disorders, while rarely being associated with unwanted bleeding. It would be dosed once a day, be inexpensive, and not require laboratory monitoring. Another important characteristic is the ability to easily reverse the anticoagulant in case of a needed emergency invasive procedure or overdose resulting in life-threatening hemorrhage [17]. The NOACs include the Xa inhibitors rivaroxaban and apixaban. Dabigatran

is a direct thrombin inhibitor. Time will tell whether one these NOACs become the "perfect" anticoagulant, but currently they all fall far short since none of them currently have a proven protocol for rapid reversal [18, 19].

The half-life of rivaroxaban is 5–9 h, but increases in patients with renal failure and the elderly. Apixaban's half-life is 8–15 h, and is less affected by renal failure [18]. Both drugs half-lives are relatively short, but still would require days for reversal if completely dependent on metabolism. A specific antidote for Xa inhibitors is under investigation [20]. It is a recombinant protein that is a modified form of Xa, but does not function in the soluble coagulation system. It does retain the binding site for Xa inhibitors and acts as a decoy. Reversal of Xa inhibitors with the decoy protein is dose dependent and has been shown to reverse bleeding in animal models. When approved it will be marketed under the name "Andexanet alfa." A four factor prothrombin complex concentrate (PCC) has recently been licensed in the United States. There is some evidence that four factor PCC may be able to reverse Xa inhibitors [21]. However, the only studies available were done in healthy men given Xa inhibitors with the PCC completely reversing the prothrombin time. No studies are available in bleeding patients.

Dabigatran has a half live of 12–17 h, which is again too long to wait for normal metabolism before taking a bleeding patient to surgery. No antidote for this direct thrombin inhibitor is available. However, an Fab antibody fragment (aDabi-Fab) has been shown to tightly bind to dabigatran reversing the anticoagulant properties of the drug [22]. The aDabi-Fab mimics the binding site of thrombin for dabigatran, but has no effect on the soluble coagulation system. Only animal studies exist and much more research is necessary before it is ready for human use. A four factor PCC was not effective in reversing the anticoagulant effect of dabigatran in the study cited above [21]. One ex-vivo study exists evaluating the use of an activated four factor PCC (FEIBA) in reversal of dabigatran [23]. While coagulation assays were corrected ex-vivo using FEIBA, the thrombotic risk of this product should be weighed heavily against the bleeding risk in a given patient. Only 35 % of dabigatran is protein bound [18] and approximately 2/3 of the drug can be removed after 2 h of hemodialysis [19]. While dialysis could be considered, it is often difficult to find the time to place a central line, and complete the procedure, prior to the need for surgical intervention in an emergency.

The NOACs may in time prove to be the "perfect" anticoagulants when compared to warfarin. This will certainly not be the case until specific antidotes are licensed for human use. Until that time, bleeding patients on NOACs requiring surgery will remain a challenge for the blood bank.

12.5 Summary and Key Points

Transfusion services commonly encounter cases of catastrophic bleeding. The key to successful management of the cases is rapid diagnosis of the cause, using routine coagulation assays. Once the coagulopathy is understood, the appropriate component therapy can be provided. Many algorithms directing blood product transfusion

exist. The key to success is thorough understanding of the strengths and weaknesses of the assays used to support the algorithm.

- Have an early warning system in place for catastrophic bleeding and treat empirically with blood products if necessary
- Develop a limited coagulation test menu, and algorithm, to guide blood product administration during massive transfusion
- Understand the strengths and weaknesses of the coagulation assays in your hospital
- Understand how heparin affects coagulations assays, and have a low threshold for ordering a heparin assay
- Use a systematic approach for platelet transfusions
- Treat severe, acute DIC aggressively with blood products. Early focus should be on improving low fibrinogen levels using cryoprecipitate
- Become familiar with the NOACs and NOAC anticoagulant reversal strategies as they evolve

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