Review

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Platelet storage and functional integrity

https://doi.org/10.1515/labmed-2020-0067
Received June 16, 2020; accepted August 23, 2020; published online September 11, 2020

Abstract: Platelet transfusion is a topic of common interest for many specialists involved in patient care, from laboratory staff to clinical physicians. Various aspects make this type of transfusion different from those of other blood components. In this review, the challenges in platelet transfusion practice that are relevant for laboratory colleagues will be discussed, highlighting how the biochemical and structural characteristics of these blood elements directly affect their function and consequently the clinical outcome. More than 1,300 platelet concentrates are transfused in Germany every day, and several types are offered by their respective manufacturers. We describe the technological advances in platelet concentrate production, with a focus on how the storage conditions of platelets can be improved. Laboratory quality assessment procedures for a safe transfusion are discussed in detail. For this purpose, we will refer to the Hemotherapy Directives (*Richtlinie Hämotherapie*) of the German Medical Association.

Keywords: platelet concentrate; thrombocytopenia; transfusion.

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Isolation of human platelets

Donor selection

Platelet donor recruitment underlies the German Transfusion Act (*Transfusionsgesetz*) and follows the national guidelines for the preparation of blood and blood components [1]. Donors must be in a health condition that allows a donation without risk for themselves and the recipients. Moreover, they must be informed in detail about the risks and side effects of the donation. In addition to the general prerequisites for blood donation that we will not discuss in the present review, specific requirements have to be fulfilled for apheresis platelet donation, i.e., a platelet count of ≥150,000/μL. This value is of key importance since the blood platelet concentration after apheresis should be >100,000/μL. Drugs that impair platelet function (e.g., acetylsalicylic acid [ASA] or nonsteroidal anti-inflammatory drugs [NSAIDs]) must not have been consumed in the last seven days before donation [1].

Pooled and apheresis platelet concentrates

Platelet concentrates can be obtained either from whole blood donations with principally two different subsequent preparation procedures or directly by apheresis technologies. One option is the isolation of platelets from buffy coats (BC) derived from different, usually four to six, blood donors. To remove undesired cells (e.g., white blood cells), the centrifugation of pooled BCs is followed by a filtration step to obtain a leukodepleted concentrate [2]. At the end of the procedure, the platelets are stored in platelet additive solutions (PAS), which aim to guarantee the maintenance of the functional elements in an environment containing as little plasma as possible. Furthermore, PAS provide a buffering capacity and allow few allergic reactions or transfusion-associated complications [3]. Very promising efforts have also been made to exploit the possibility of using buffy coats as a source of hematopoietic progenitor cells for platelet production [4].

Another approach is the sequential double centrifugation of blood derived from whole blood donors. This procedure is generally very seldom used in Europe and

actually not used at all in Germany. It includes a first centrifugation step at low speed, which separates the blood in two phases. In the upper phase, platelets are enriched in the so-called platelet rich plasma (PRP). In the lower phase, erythrocytes and white blood cells are sedimented. In a second centrifugation step, the platelets harvested in the upper phase are centrifuged and subsequently reconstituted in plasma [5].

Manufacture of platelet concentrates by automated apheresis procedures is widely used when platelets are collected from individual donors. With this method, platelets are collected from a single donor through an integrated system capable of reducing the presence of leukocytes in the final apheresis product, a process called leukodepletion. The blood is withdrawn from the donor through an apheresis needle and flows through a sterile conduit until it reaches the machine in which the platelet isolation procedure takes place. Once the platelets have been isolated by differential centrifugation, blood deprived of platelets and a small amount of plasma flows back into the donor. This procedure is particularly advantageous in the case of platelet refractoriness, i.e., poor platelet increments after transfusion. This condition is usually due to antibodies present in the plasma of the donor directed against common antigens, such Human Leukocyte Antigen I (HLA I), on the surface of leukocytes and platelets [6, 7].

In Germany, the current guidelines demand for pooled units as well as for apheresis platelet concentrates a minimum number of $\geq 2 \times 10^{11}$ platelets per unit [1]. The choice between a pooled and an apheresis product is dictated from a clinical point of view by various factors. First, in many clinics, the economic factor must be considered (apheresis products are much more expensive), but the clinical needs of the patient should be the guiding criterion that influences the choice. In patients who show no signs of immunization, there are no comparative data from prospective randomized trials available [8, 9]. An undisputed indication for platelet concentrates from apheresis is the occurrence of HLA- or HPA-antibodies. Alloimmune thrombocytopenia is a pathology affecting fetuses and newborns due to maternal antibodies directed against platelet antigens. These factors can impede platelet increment and therefore require individually matched platelet concentrates [1, 10].

In vitro activation of platelets during collection

Apheresis procedures cause, albeit to varying degrees depending on the technique, a certain degree of platelet activation, which represents a phenomenon at the basis of their physiological function. Platelet activation markers used in diagnosis are represented by surface molecules, such as P-selectin (CD62P, GMP-140), a receptor for white blood cells (WBCs). Under normal conditions, CD62P is contained inside the α-granules, and the degranulation process is considered the initial stage of platelet activation, which is followed by exposure of the receptor at the membrane. Monitoring the concentration of this receptor is of some importance, as it has been implicated in platelet clearance processes by WBCs that results in the reduction in efficacy of the administered preparation [11]. Moreover, the expression of this receptor leads to the activation of WBCs, which in turn are able to activate macrophages, which are responsible for the release of pro-inflammatory cytokines [12]. In this way, the transfusion of activated platelets can lead to a moderate increase in the inflammatory response, which may be the basis of transfusion reactions. However, studies have shown that P-selectin expression at the membrane level is reversible, thus limiting platelet clearance by WBCs [13]. Lysosomal exocytosis can also be used as a read-out of platelet activation by cell separators. CD63 (GP53) is a membrane protein present on lysosomes that can be monitored to assess lysosomal activity [14]. Activation of the integrin complex glycoprotein IIb/IIIa (fibrinogen receptor) is a further marker of platelet activation. This receptor mediates both adhesion of platelets to the exposed extracellular matrix and platelet cross-linking [15]. A study comparing three different types of cell separators regarding the risk of inducing platelet activation has shown that activation of the fibrinogen receptor is a common occurrence; in this case, it was shown to be a reversible phenomenon since the parameter decreased to normal values after 2 h [16]. The same study also characterized the expression of glycoprotein IX (CD42b), a membrane protein present on platelets, which constitutes the receptor of the von Willebrand factor. A decrease in glycoprotein IX during apheresis was unaffected after a 2 h resting period of the platelet concentrate and a subsequent 2 h agitation [16].

Although many of the structural changes following platelet activation during apheresis are reversible, the initial lesion derived from the isolation process plays an essential role in the quality of the platelet concentrate and in the efficiency of the transfusion, defined as the increase in the number of platelets and the effect on bleeding after the patient has received the product [11, 17]. Therefore, a question that arises is how platelet activation can be reduced during apheresis. Reducing the harvesting time has been shown to lead to a decrease in platelet activation; this is explained by the fact that contact with the plastic

devices of the separation system is a factor that can increase the probability of platelet activation. Remarkably, reducing the apheresis time brings different benefits depending on which apheresis system is used for the collection [16]. Another matter of discussion is whether a resting period for the apheresis product after collection can decrease the activation of platelets that occurs during the procedure. Skripchenko and colleagues demonstrated that although many parameters that correlate with post-transfusion recovery could be improved by a 1–6 h rest period, no indices of platelet activation could be corrected by resting [18]. Further studies are required to confirm these results to understand whether the reduction in platelet activation can result in a better clinical outcome after platelet transfusion. Donor variability as a source of different levels of platelet activation during apheresis was also studied, but the results were inconclusive. Hagberg and coworkers were not able to identify any decisive correlation between platelet activation and demographic characteristics or the lifestyle of the subjects included in the study [16]. However, platelet activation during the production process is not an exclusive phenomenon of apheresis and activation of platelets also occurs in pooled platelet concentrates [19].

Storage of platelet concentrates

Storage length and temperature

According to current practice, platelet concentrates are usually stored in sterile oxygen-permeable bags of different plasticizers like polyvinylchloride and 2-(diethylhexyl)phthalate or polyvinylchloride at 22 °C ± 2 °C [20, 21]. Storage at temperatures outside of this range should be avoided, as this can damage the platelets. Since temperatures at approximately 22 °C favor the growth of bacteria and microorganisms, platelet concentrates can be stored for up to five days. Furthermore, room temperature increases the platelet metabolic rate and leads to a reduction in platelet functionality, a process called “platelet storage lesion”. To achieve the best clinical outcome, the shortest possible storage period should be targeted [22].

The continuous request for platelet concentrates in the clinic, combined with the fact that their half-life is limited, has driven biomedical research to continuous efforts to lengthen the time of storage. To improve the storage quality of platelets, many techniques have been adopted, but continuous shaking at room temperature turned out to be the best option. Nevertheless, cold storage has been evaluated as an alternative method to reduce both disadvantages, but it remains controversial whether the benefits outweigh the undesired effects. A study showed that a temperature below 15 °C causes an alteration of platelet morphology with a consequent decrease in survival [23, 24]. The process of refrigeration below certain temperatures is a triggering factor for apoptosis due to an irreversible remodeling of the platelet to a spheroidal shape and a decrease in the mitochondrial membrane potential mediated by the arachidonic acid complex [25]. Cold stored platelets are currently not available for clinical treatment in Germany. They might offer some advantages since their capability to aggregate and adhere is potentially higher than that of platelets kept at room temperature [26]. On the other hand, the possible suitability of cold stored platelets for acutely bleeding patients (e.g. polytrauma, cardiac surgery) is not the same as the suitability of these preparations for the care of patients with hyporegenerative thrombocytopenia who require platelets that ideally remain in circulation for several days.

A decrease in pH, which is responsible for lowering platelet viability, was also shown to occur after storage at room temperature; conversely, a slowdown of platelet metabolism as a result of cold storage is able to decrease the rate of glycolysis and the production of lactic acid and consequently maintain the pH at physiological levels [27].

Furthermore, cold storage leads to lower levels of P-selectin expression and better adhesion properties to surface-coated von Willebrand factor and fibrinogen [28]. However, other studies affirm the opposite, demonstrating an increase in platelet activation indices, such as an enhanced expression of P-selectin on the surface following refrigeration, but registering normal P-selectin values after transfusion [29]. With regard to the length of cooling, we distinguish short-term and long-term refrigeration, which span from a few hours to days, respectively. Characteristic changes following refrigeration are actin rearrangement, glycan clustering and lipid raft aggregation, which have an impact on the life span of transfused platelets [30–32].

An alternative to long-term cooling is the cryopreservation of platelets at temperatures between –65 and –80 °C. Dimethylsulfoxide (DMSO) and trehalose are the most commonly used cryoprotective agents. High concentrations of DMSO of approximately 5–6% are required to guarantee efficient cryopreservation. However, washing of platelets after thawing to remove excess toxic DMSO can decrease both platelet number and activity [33]. A major advantage of the use of trehalose is that the organization of the membrane structure of frozen platelets is unperturbed after thawing, and in this way, lateral phase separation as a consequence of long-term refrigeration is prevented [34].

Khuri and colleagues conducted a randomized clinical trial in which cardiac surgery patients were transfused with cryopreserved or liquid-stored platelets, respectively. The primary endpoint of the study was the assessment of the clinical effects and efficiency of transfusions of cryopreserved platelets for a maximum of two years in comparison with those of transfusions of conventionally stored platelets. The authors conclude that frozen platelet concentrates have, at least in some respect, superior efficiency in controlling acute bleeding. They observed that cryopreserved platelets favor the prevention of blood loss and limit the need for further transfusions after cardiothoracic surgery, which reflects greater coagulation efficacy than liquid-stored platelets. This can be in part explained by the fact that frozen platelets are already activated to some extent and thus more prone to adhere to the injured endothelium than non-activated platelets [35]. Some questions still remain to be answered to further confirm the safety of these products; it is a matter of debate whether the higher procoagulant effect of cryopreserved platelets can increase thrombotic risk in the long term. Notably, a multicentric randomized study showed the safety and effectiveness of cryopreserved platelets in the treatment of bleeding patients with thrombocytopenia [36]. Further investigations need to be carried out regarding the use of DMSO as a cryopreserving agent, since it could also result in high neuronal and renal toxicity. Nevertheless, different platelet storage techniques might be adopted according to the pathology and the indications for transfusion.

Microbial contamination

The risk of the microbial contamination of platelet products has been drastically reduced due to preventive diagnostic measures and the sensitivity of laboratory tests currently in use. However, because of the storage conditions adopted for platelet concentrates (oxygen-permeable bags, storage at room temperature and nonstop shaking), these preparations are particularly prone to bacterial growth. Since platelet transfusion is mostly administered to severely ill patients, the higher risk of bacterial contamination requires even more accurate diagnostic measures.

A study by Schrezenmeier and colleagues demonstrated by routine bacterial culture screening that the overall frequency of bacterial growth in platelet concentrates was between 0.06 and 0.09% [37]. The risk of viral transmission is considerably lower [38] (compare Figure 1). The risk of infection for a single patient increases with the number of platelet concentrates received, which is different depending on the specific disease. In everyday clinical practice, several measures can be adopted to limit the number and severity of bacterial contaminations and consequently of adverse reactions and sepsis. Donor selection is the first measure. Donors are asked before each donation about their general health condition and if they had manifested symptoms that could indicate the presence of a bacterial infection in the period prior to the donation. However, the questionnaires rely on the ability of donors to recognize symptoms potentially related to an infection and, in any case, are unable to identify asymptomatic bacteremia. For this reason, an analysis of the patient's blood (e.g., inflammation indices, white blood cell count) is mandatory, together with laboratory tests aiming at identifying eventual bacterial contamination. Culture-based microbiological tests and rapid detection methods represent the standard bacterial screening methods in use. Classic culture methods consist of the inoculation of culture media with 4–10 mL of the platelet concentrate within 24 h of withdrawal. A growth phase follows, and in the case of negative results, the platelet product can be approved for transfusion and eventually recalled if bacterial growth is found later after this period. BacT/ALERT from

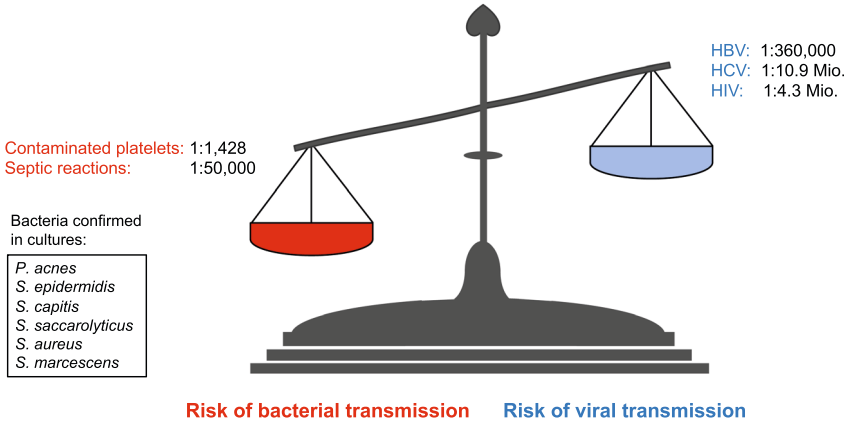

Figure 1: Risk of bacterial and viral transmission [37, 38].

BioMérieux is a widely used screening system used for platelet concentrates [39]. These methods, however, focus on aerobic bacterial species, and anaerobic and facultative strains might remain undetected. Furthermore, slow-growing species, together with the low sensitivity of bacterial culture, may put at risk the reliable identification of bacterial contaminations [40, 41]. Rapid detection methods rely on the identification of the bacterial genome in the blood of the donor. With the advent of real-time quantitative PCR (RT-qPCR), it is possible to quickly and reliably identify the presence of bacterial DNA in a few hours. Although this technique is highly sensitive and specific, there is a risk of false positive results due to the use of polymerases extracted from bacteria, free bacterial DNA/RNA or possible contamination of genomic DNA from the donor. Therefore, continuous verification of any contamination of the singular reaction components (primers, polymerase, water and buffer) is mandatory [42]. The big advantage of this method is the possibility of amplifying the genetic material of many bacterial strains at the same time. The bacterial genes that are targets of amplification by PCR should possibly have a low mutation rate, eliminating the risk over time of false negative results; genomic regions present within the 16S and 23S bacterial ribosomal subunits (housekeeping genes) are a standard choice that fulfills this requirement [43]. The RNA polymerase β-subunit is also a suitable target for this purpose [44]. The highest risk for bacterial contamination is at the end of the shelf life. To reduce the risk of a fatal transfusion reaction due to contaminated products, in 2008 the Permanent Working Group on Blood of the Federal Ministry of Health (*Arbeitskreis Blut des Bundesministeriums für Gesundheit*) decided to limit the storage period for platelet concentrates to four days (96 h), calculated from midnight on the collection day. However, if a validated and accredited procedure for the detection of microbial contamination is applied, the maximum storage duration can be prolonged to 5×24 h.

A second line of preventive measures for potential transfusion-related septicemia must be taken by the transfusing physician at the time of platelet concentrate administration. These consist of visual control of the product to detect contamination before transfusion, correct transfusion practice to avoid secondary contamination, and close monitoring of the patient to recognize the first signs of a septic reaction and take appropriate measures in time [45].

Another possible option to reduce the risk of bacterial contamination is photochemical inactivation, which is a standard procedure in some European countries (e.g. Switzerland). This method is based on the inactivation of bacteria by photoactive compounds in combination with ultraviolet light and can be performed directly before transfusion without further platelet manipulation. Three systems of pathogen inactivation are CE certified. Currently, only the INTERCEPT™ Blood System is available for clinical use in Germany, whereas the Mirasol® system is available in other European countries. The THERAFLEX system is under investigation in clinical studies. INTERCEPT™ Blood System employs amotosalen-HCl (S-59) combined with ultraviolet A (UVA) and is able to inactivate a broad spectrum of pathogens (viruses, bacteria, protozoa) and leukocytes [46]. Another option is the Mirasol® pathogen reduction technology (PRT) combined treatment with riboflavin (vitamin B12) and UV light. This method creates an irreversible modification of DNA and hampers DNA repair in the targeted organism or cell. Similar to INTERCEPT™, the Mirasol® technology can inhibit the replication of different viruses, bacteria and parasites together with leukocytes. This technology increases the platelet activation processes and metabolism, which in turn augments glucose consumption and lactic acid production [47, 48]. A prolongation of platelet shelf life is demonstrated to be possible if a mixture of plasma and PAS is used as storage solution [49]. THERAFLEX is based on the application of shortwave, ultraviolet light, which leads to a loss of the replication ability of pathogens and nucleated cells. Since no additional pathogen-inactivating substances are added, it is assumed that the risk of undesirable toxic reactions is limited. THERAFLEX is effective for the inactivation of leukocytes through the inhibition of proliferation and protein synthesis. In addition, antigen presentation and the release of cytokines are inhibited during storage [50]. All three methods inhibit leukocyte proliferation and thus effectively prevent graft versus host disease (GvHD). All three methods might accelerate the appearance of platelet storage lesions, like modifications of platelet shape, aggregation and changes in the intracellular platelet activation pathways [51], but the clinical impact of this finding is still unclear. After applying a validated and accredited procedure for pathogen inactivation, the maximum storage duration is 5×24 h.

Platelet additive solutions and their effect on platelets

PAS were developed in the late 1980s to improve platelet concentrate storage conditions and decrease the risk of adverse transfusion reactions. The great advantage deriving from the use of PAS is that they allow the

storage of platelets in a minimal amount of plasma with a great reduction in immunologic (e.g., isoagglutinins in plasma), allergic and overload responses due to the concomitant plasma transfusion. PAS contain electrolytes that improve platelet storage and are optimized so that the platelet shelf life and consequently their effectiveness can be maximized. Common elements present in PAS are sodium chloride, trisodium citrate, sodium acetate and/or sodium phosphate, D-mannitol, potassium chloride, and magnesium chloride/sulfate [52]. Citrate is added to avoid platelet clotting, which takes place below a value of 8 mmol/L [53]. Acetate is used because of its buffering capacity; after it is oxidized, it subtracts H^+ ions from the environment and contributes to the increase in pH. This effect is able to counterbalance the production of lactate derived from platelet metabolism [54]. The advantages of adding phosphate are its ability to counteract the pH decrease and to promote glycolysis [54]. Better control of pH can also be facilitated by the addition of potassium and magnesium. A study showed that platelet storage for seven days in PAS supplemented with potassium and magnesium was able to maintain pH levels of 7.15 ± 0.1 compared to 6.94 ± 0.05 for PAS without magnesium and potassium. Moreover, supplementation with these electrolytes has been shown to decrease the expression of activation markers such as P-selectin from 50 ± 8 to 23 ± 6% [3].

Cardigan and colleagues analyzed different platelet additive solutions and their effect on cytokine levels and complement activation. The authors showed that parameters related to platelet activation (e.g., TGF-β, CD62P) and cytokine release markers (e.g., IL-8) can change depending on PAS composition and that modification of the quality and concentration of the different components of PAS can improve both the storage and safety profiles of platelet concentrates [52]. The impact of additive solutions affects the conservation and physical-chemical characteristics of the platelets; additionally, depending on the substances contained in the different solutions, there may be different clinical implications concerning the tolerability and efficacy of the product administered. The retrospective study by Tobian and coworkers demonstrated that the use of PAS significantly reduced the appearance of allergic transfusion reactions [55]. Another study by Cohn and colleagues focused on allergic (ARs) and febrile nonhemolytic transfusion reactions (FNHTRs) in patients who received platelets in PAS versus patients treated with platelets stored in plasma. The overall incidence of transfusion reactions was lower in the PAS group (0.55%) than in the plasma-treated group (1.37%). The relative risk (RR) for PAS- versus plasma-stored platelets was 0.4, with RRs of 0.350 and 0.336 for ARs and FNHTRs, respectively [56]. Transfusion-related acute lung injury (TRALI) is a life-threatening complication occurring within 6 h after transfusion. It is triggered in most cases by an immune reaction of human leucocyte antigen (HLA) antibodies present in the plasma of the donor, which react with antigens of the recipient and cause acute respiratory distress with hypoxemia and pulmonary edema [57]. Although less than 20 ml of residual plasma may be enough to trigger an immunological reaction, the addition of PAS was shown to be able to reduce the risk of TRALI events [58]. The use of PAS as storage medium can increase the risk of transfusion-transmitted bacterial infections, as demonstrated by Kreuger in a recent study. An RR of 4.36 was achieved for patients who received a transfusion of PAS-stored platelets versus those who received plasma-stored concentrates. However, several bacterial strains can be detected more easily in PAS, which offers favorable growth conditions, and this has to be taken into account when considering the higher RR for PAS over plasma as a source of bacterial contamination [59].

Quality assessment of platelet concentrates

Platelet concentrates must fulfill defined release criteria established by the quality control processes. These processes evaluate different parameters, including biochemical, physical and functional properties of the platelets. For every parameter, the time and frequency of testing are considered [1].

Swirling is a simple parameter to evaluate the morphology of platelets and can be assessed by the naked eye. It must be performed after the manufacturing of the product and prior to dispensing it. Bertolini and coworkers identified a decrease in swirling in platelet concentrates as a signal of disc-to-sphere transition in platelet morphology, which is considered a typical sign of storage lesion [60]. Since platelet morphology is considered to be one of the most important physical parameters indicating the ability of platelets to circulate in the blood and given the simplicity of the swirling assessment, it is good practice for physicians to evaluate platelet morphology directly before transfusion. Nevertheless, since platelet preparation has changed considerably (e.g. platelet additive solutions, gas-permeable bags), an absence of swirling is rarely seen and should, if it occurs, be handled with caution, as it could be a sign of bacterial contamination or malpractice.

The number of platelets present in a concentrate is another parameter assessed by quality control. According

to the Hemotherapy Directives (*Richtlinie Hämotherapie*) of the German Medical Association in agreement with the Paul Ehrlich Institute, a minimum of 2×10^{11} platelets per concentrate is mandatory. This number has to be estimated directly after production (maximum two days after withdrawal) and at the end of the shelf life [1]. After production, it is also necessary to determine the number of WBCs in the product. Less than 1×10^{6} WBCs in each unit is allowed. WBCs have a negative impact on platelet storage conditions, increasing glucose consumption and lactate production with a consequent drop in pH levels, which is why removal of leukocytes has been introduced. Moreover, a high leukocyte content has been linked to a higher incidence of adverse events such as refractoriness to transfusion, graft versus host disease (GvHD), alloimmunization to leukocyte antigens (HLA) and the risk of transmission of cytomegalovirus (CMV) infections [61]. pH decreases during storage depending on the storage conditions in use and must be held between 6.5 and 7.6. It has been demonstrated that a pH of <6 is detrimental for platelets and significantly impairs their viability [62]. Sterility testing of platelet concentrates has to be performed 24 h before expiry of the shelf life at the earliest and 72 h after this at the latest. In case of bacterial growth, the physician must be informed about the result of the antibiogram if he has already received the product.

Conclusions

Specialized and patient-centered medical approaches as well as the growing offer of diagnostic and therapeutic procedures increase the demand for supportive platelet transfusions. Production of platelet concentrates supply is undertaken in highly regulated specialized blood establishments. Clinical supply is challenged by a short shelf life and a limited number of blood donations. Continuous improvement of platelet preparations is necessary for prolonged storability and to keep up with technical developments, especially regarding the prevention of bacterial and viral transmission. Optimization of transfusion policies should be based on the cross-talk between the requests of clinical medical staff and the laboratory. New developments in the production processes of platelet concentrates must therefore meet the daily clinical demands, consider maximum transfusion efficacy and guarantee patient safety.

Research funding: None declared.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: All authors received salary within in the last 36 months from the German Red Cross Blood Service Baden-Württemberg – Hessen. The German Red Cross Blood Service Baden-Württemberg – Hessen is a non-profit organization that produces and distributes platelet concentrates. PW: Membership in Advisory Boards for Sanofi.

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