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Recommendations for the Standardization of Light Transmission Aggregometry: A Consensus of the Working Party from the Platelet Physiology Subcommittee of SSC/ISTH

M. Cattaneo,[1] C. Cerletti,[2] P. Harrison,[3] C.P.M. Hayward,[4] D. Kenny,[5] D. Nugent,[6] P. Nurden,[7] A.K. Rao,[8] A.H. Schmaier,[9] S.P. Watson,[10] F. Lussana,[1] M.T. Pugliano,[1] and A.D. Michelson[11]

[1]Unita di Medicina III, Ospedale San Paolo, Dipartimento di Scienze della Salute, Università degli Studi di Milano, Milan, Italy; [2]Laboratori di Ricerca, Università Cattolica, 86100 Campobasso Italy; [3]School of Immunity and Infection, University of Birmingham Medical School, Birmingham; [4]Department of Medicine, McMaster University, Hamilton, Canada; Hamilton Regional Laboratory Medicine Program, Hamilton, Canada ; [5]Department of Molecular and Cellular Therapeutics, Royal College of Surgeons in Ireland, Dublin, Ireland ; [6]CHOC Children's Hospital, Hematology Research, Orange, CA, USA ; [7]Centre de Référence des Pathologies Plaquettaires, Plateforme Technologique et d'Innovation Biomédicale, Hôpital Xavier Arnozan, Pessac, France ; [8]Sol Sherry Thrombosis Research Center, Temple University School of Medicine, Philadelphia, PA, USA ; [9] Department of Medicine, Case Western Reserve University and University Hospitals Case Medical Cente, Cleveland, OH, USA ; [10]Centre for Cardiovascular Sciences, Institute of Biomedical Research, School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham, UK; [11]Center for Platelet Research Studies, Division of Hematology/Oncology, Boston Children's Hospital, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA

*The Working Party of experts was composed by: Marco Cattaneo (Chairman), Alan D. Michelson (Co-chairman), Chiara Cerletti, Paul Harrison, Cathrine P.M. Hayward, Dermot Kenny, Diane Nugent, Paquita Nurden, A.Koneti Rao, Alvin H. Schmaier, Stephen P. Watson

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Correspondence: Marco Cattaneo, Unita di Medicina III, Ospedale San Paolo; Dipartimento di Scienze della Salute, Università degli Studi di Milano. Via di Rudinì 8, 20142 Milan, Italy.

Tel.: +39 0250323095; fax: +39 0250323090.

E-mail: marco.cattaneo@unimi.it

Summary.

Light transmission aggregometry (LTA) is the most common method used to assess platelet function. However, there is no universal standard for its performance. The Platelet Physiology Subcommittee of the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis formed a working party of experts with the aim of producing a series of consensus recommendations for standardizing LTA. Due to a lack of investigations that directly compared different methodologies to perform LTA studies, there were insufficient data to develop evidence-based guidelines. Therefore, the RAND method was used, which obtains a formal consensus among experts about the appropriateness of health care interventions, particularly when scientific evidence is absent, scarce and/or heterogeneous. Using this approach, each expert scored as “appropriate”, “uncertain” or “inappropriate” a series of statements about the practice of LTA, which included pre-analytical variables, blood collection, blood processing, methodological details, choice of agonists and the evaluation and reporting of results. After presentation and public discussion at SSC meetings, the assessments were further refined to produce final consensus recommendations. Before delivering the recommendations, a formal literature review was performed using a series of defined search terms about LTA. Of the 1830 potentially relevant studies identified, only 14 publications were considered to be actually relevant for review. Based upon the additional information, 6 consensus statements were slightly modified. The final statements were presented and discussed at the SSC Meeting in Cairo (2010) and formed the basis of a consensus document, which is the subject of the present report.

Keywords: platelet aggregation, platelet disorders, platelet function, platelets, standardization.

Introduction

Light transmission aggregometry (LTA), which was independently developed in 1962 by Born [1] and O'Brien [2] is considered the gold standard for testing platelet function, because it provides important information that is essential for the diagnostic work-up of patients with platelet function defects. LTA measures the transmission of light through a sample of platelets in suspension (platelet-rich plasma [PRP], washed platelets or gel-filtered platelets), which increases when platelets are aggregated by an agonist. LTA is a time-consuming and technically challenging technique that is affected by many pre-analytical and analytical variables, and these must be carefully controlled for by expert personnel. For this reason, LTA should be performed only in specialized laboratories.

Alternative methods to measure platelet aggregation in PRP or whole blood have been developed (*e.g.,* impedance aggregometry, 96-well plate aggregometry, single platelet counting, flow cytometry) [3-7]), several of which enable faster and more user-friendly study of the platelet response to aggregating agents. Despite these potential advantages, the majority of these techniques have not been widely adopted and, at variance with LTA, fail to provide important additional diagnostic information that can identify defect of platelet function, such as platelet shape change, the occurrence of secondary wave of aggregation or platelet deaggregation.

Although popular and widely used, the LTA technique is not standardized [8], despite the fact that guidelines have been published [9-11]. Surveys organized by proficiency testing organizations identified variations in LTA practices and the need for guidelines to standardize LTA [12-14]. In July 2006, the Platelet Physiology Subcommittee of the ISTH formed a Working Party of 11 experts with the aim of producing a series of consensus recommendations for standardizing LTA. As a first step in LTA guideline development, the

Working Party organized the largest and most detailed worldwide survey on LTA methodology [15]. The survey confirmed the very high variability in LTA practices worldwide, indicating that methodological standardization is necessary. The information gathered in the survey contributed to the development of ISTH methodological guidelines for LTA, which are in the subject of the present report by the Working Party.

Methods

RAND Method

Clinical or laboratory guidelines are traditionally based upon reviewing the evidence of the medical and scientific literature. However, it is unfortunately common that the literature does not provide definitive answers for a variety of reasons, including absence of evidence, and low quality and/or contradictory evidence. It was clear to the experts of the Working Party that not enough relevant studies had been published that compared different methodologies to perform LTA studies, which would have helped to develop evidence-based guidelines.The Working Party therefore used a formal consensus method (the RAND methodology) [16] to develop its recommendations.

The RAND method – developed by the RAND Corporation in the 1980s – is intended to obtain a formal consensus among expert groups about the appropriateness of health care interventions, particularly when scientific evidence is absent, scarce and/or heterogeneous [9]. A series of statements about LTA practice were formulated by the chairman (MC) and the co-chairman (ADM) of the Working Party. For each statement, a form was prepared, which each member of the Working Party scored for appropriateness from 1 (completely inappropriate) to 9 (fully appropriate). Ballots were blinded to the other members. The extreme scores (highest and lowest) were discarded, the median of the remaining scores was calculated and the area containing the majority of the ballots defined the classification of

each statement about LTA practice: inappropriate (scores 1-3), uncertain (4-6) and appropriate (7-9).

After the first RAND run, statements were changed according to the comments/suggestions that the reviewers wrote on their RAND forms. A second RAND run was organized to score the revised statements. Recommendations were then presented at the ISTH SSC Meeting in Vienna (2008) and discussed with the audience. Based on the comments and suggestions that were raised during that meeting, statements were revised and a third RAND run was organized among the experts of the Working Party. The revised recommendations were again presented at an ISTH SSC Meeting (Boston, 2009) and discussed with the audience. Based on the comments and suggestions that were raised during that meeting, statements were again revised and a fourth RAND run was organized among the experts of the Working Party. At this point, before final approval of the recommendations, it was decided that a formal, full literature review should be performed, in order to verify, where possible, the recommendations of the Working Party.

Literature Review

Search Strategy

The Medline electronic database was searched for relevant published studies between 1966 and November 2009, without any language restriction, using the following medical subject headings (MeSH) terms and free text terms: (1) “platelet aggregation” OR “platelet agglutination”; (2) lumiaggregometry” OR "light transmission aggregometry" OR "light transmittance aggregometry" OR "platelet reactivity tests" OR "platelet function tests" OR "platelet function test" OR "Born aggregation". The search was supplemented by manually reviewing the reference lists from primary or review articles and by direct consultation among members of working group to identify additional relevant studies.

Data extraction

All references were reviewed manually and data extracted using the following inclusion criteria: 1) Studies that directly compared different methodological approaches in studying LTA including case control, prospective cohort or retrospective cohort studies; (2) Studies in which one of the outcomes of interest (i.e., the methodological approach to study LTA) was a pre-specified endpoint. By this method 1830 potentially relevant studies were identified and screened for retrieval. The titles and abstracts of these were divided in 5 groups, and each group was reviewed by 2 panel members. Based on their assessments, 1803 studies were excluded, leaving 27 for detailed evaluation. After review of the full manuscripts by the panel members, 5 further studies were excluded. These 12 manuscripts, coupled with 2 extra studies identified by manual review and by consultation among members of the working group, resulted in a total of only 14 relevant studies [17-30] that were used to help refine the RAND survey and write the present recommendations (Figure 1). Disagreement among experts was resolved by consensus and, when necessary, by asking the opinion of the chairman (MC).

Final refinement of the recommendations

Based on the information that was gathered from the selected 14 publications, 6 consensus statements were slightly changed and a final, fifth RAND run among the Working Party experts was organized. The final statements were presented and discussed at the ISTH Subcommittee Meeting in Cairo (2010) and it was agreed that these could form the basis of a consensus document which is the subject of the present report by the Working Party.

Recommendations of the Working Party on Standardization of Light Transmission Aggregometry, of the SSC Subcommittee of Platelet Physiology of ISTH

The recommendations of the Working Party on standardization of LTA include 70 statements, which have been grouped into 8 sections: 1) clinical usefulness of LTA; 2) pre-

analytical variables; 3) blood collection; 4) preparation of PRP and platelet-poor plasma (PPP); 5) assessment of PRP quality; 6) methodology; 7) choice of agonists; 8) evaluation and reporting of results.

1. Clinical usefulness of LTA

- LTA is clinically useful for the study of subjects with bleeding disorders (median score 9, range 8-9) .
- LTA should not be used for the identification of subjects at risk of thrombosis, except in research (median score 7, range 5-9).

The experts agreed that this is an area that still needs further studies and standardization.

LTA should not be used for monitoring subjects on antiplatelet therapy, except in research (median score 7, range 4-7).

New, faster and more efficient point-of-care tests are available for this purpose, but LTA could be used when the new tests are not available. However, based on the negative results of 3 large randomized trials [31-33], which used the point-of-care test VerifyNow $P2Y_{12}$, and a large observational study, which used LTA to tailor antiplatelet treatment with clopidogrel [34], convincing data to recommend monitoring antiplatelet therapy by laboratory tests, including LTA are still lacking.

Based on the above 3 recommendations, the following recommendations apply only to the use of LTA for diagnosing patients with bleeding disorders, in whom the presence of a platelet function disorder is suspected.

2. Pre-Analytical Variables

- Blood samples for LTA should be collected after a short rest period for the subject (median score 7, range 2-8), to attenuate the effect of exercise-induced adrenaline release on platelet aggregation.
- Blood samples for LTA should be collected from subjects who refrain from smoking for at least 30 minutes (median score 7, range 5-8), to attenuate the effect of exercise-induced adrenaline release on platelet aggregation.
- Blood samples for LTA should be collected from subjects who abstain from caffeine for at least 2 hours (median score 7, range 3-8).
- A record of all drugs that the subject has taken in the week prior to testing should be collected (median score 7, range 7-9).
- Treatment with drugs known to reversibly inhibit platelet function (e.g. NSAIDs) should be stopped at least 3 days before sampling (median score 8, range 7-9).
- Treatment with drugs known to irreversibly inhibit platelet function (e.g. aspirin, thienopyridines) should be stopped at least 10 days before sampling (median score 8, range 8-9).
- When treatment with drugs that inhibit platelet function cannot be stopped before sampling, drug-induced effects on platelet function should be considered when interpreting the LTA results (median score 9, range 8-9).
- **It is uncertain** whether blood samples for LTA should be collected from fasting subjects (median score 5, range 3-8). Although variations in glycemia and lipidemia might slightly affect the results of LTA studies, it is uncertain whether or not these effects interfere significantly with the diagnosis of platelet function disorders.

The effects of light meals on the results of LTA studies are probably negligible. However, patients should not be studied after having meals associated with high

fat content, to avoid the formation of chylomicrons in plasma, which will interfere with light transmission.

- **It is uncertain** whether treatment with any drug should be stopped before sampling (median score 4, range 1-8).

It is impossible for some patients to stop all medications before being sampled for LTA studies.

3. Blood Collection

Blood samples for LTA studies should be collected using the following precautions to minimize platelet activation during the procedure.

- Blood samples for LTA should be drawn with minimal or no venostasis (median score 8, range 7-9).

It is also suggested that, if a tourniquet needs to be used, it should immediately be released as soon blood collection begins.

- Blood samples for LTA should be drawn using a needle of at least 21 gauge (median score 8, range 7-9).
- Blood samples for LTA should be drawn into plastic (polypropylene) or siliconized glass tubes (median score 9, range 8-9).
- Blood samples for LTA should be drawn into a buffered anticoagulant to help keep the pH stable during processing and testing (median score 7, range 3-8).
- Blood samples for LTA should be drawn into 109 mM sodium citrate, buffered anticoagulant (median score 8, range 5-9).
- Blood samples for LTA should be drawn into 129 mM sodium citrate, buffered anticoagulant (median score 7, range 4-9).

Although 5 experts declared their preference for one of the two suggested concentrations of sodium citrate, the final recommendation was that either concentration of sodium citrate is acceptable, as long as its use is consistent in each center.

- The first 3-4 mL of blood drawn should be discarded or used for tests other than LTA (median score 8, range 5-9).
- When difficulties are encountered in obtaining sufficient blood for LTA, underfilled tubes may only be used to exclude severe platelet function disorders, such as Glanzmann thrombasthenia or Bernard-Soulier syndrome (median score 7, range 5-8).

4. Preparation of PRP and PPP

- Blood samples should be allowed to "rest" at room temperature for 15 min before centrifugation (median score 8, range 6-8).
- PRP should be prepared by centrifuging blood samples at 200 x g for 10 min (median score 8, range 5-9).

This recommendation was recently validated by Femia et al, who demonstrated that centrifugation of blood samples at 200xg (or 250xg) for 10 min appears to be the best condition for preparing PRP for LTA studies, both in terms of the degree of contamination of PRP by other blood cells and of platelet reactivity [35]. Similar results in terms of platelet reactivity were reported by Merolla et al [36].

- PRP should be prepared by centrifuging blood samples at ambient temperature (approximately 21°C) (median score 8, range 8-9)
- PRP should be prepared by centrifugation without using a brake (median score 9, range 8-9)
- PRP should be prepared by blood sedimentation for samples with very large platelets (median score 8, range 5-9)

- **It is uncertain** whether it is advisable to keep the tubes at a 45° angle when preparing PRP by sedimentation of blood samples with very large platelets (median score 6, range 4-8).
- PPP should be prepared by centrifuging whole blood, or the tubes of blood from which PRP was removed, at ambient temperature at 1500 x g for 15 minutes (median score 8, range 8-9)

5. Assessment of PRP quality

- Grossly haemolyzed samples should be discarded (median score 9, range 7-9)
- If the sample tested is lipaemic, the final report should indicate this (median score 8, range 2-8)
- It is necessary to check the platelet count of the PRP sample tested (median score 9, range 8-9)
- The results of LTA studies could be inaccurate when the platelet count in the PRP samples is lower than 150 x 10^9/L (median score 8, range 5-9)
- Caution should be taken when interpreting abnormal results in samples with low platelet counts (median score 9, range 7-9)
- PRP with low platelet counts may be tested to exclude severe platelet function disorders (Glanzmann thrombasthenia, Bernard-Soulier syndrome, type 2B and platelet-type von Willebrand disease) (median score 8, range 5-9)
- The platelet count of PRP samples should NOT be adjusted to a standardized value with autologous PPP (median score 8, range 3-9)

Recent studies demonstrated that platelet counts in PRP within the range that is observed in PRP samples from subjects with normal platelet count in whole blood do

not affect the results of LTA studies [19-22]. Therefore, the common practice of adjusting the platelet count in PRP with autologous PPP is not recommended, because it is unnecessary and may impair the platelet responsiveness to agonists [21]. It remains uncertain what is the best practice to follow when the platelet count in PRP exceeds about $600x10^9$/L. More recently, a study showed that both methods - native and adjusted platelet count - are valid to assess a bleeding disorder [37]. Abnormalities of platelet aggregation were more frequent using adjusted platelet count both in controls and patients [37].

6. Methodology

- LTA studies must include a known normal subject, run in parallel with the subject(s) under study (median score 8, range 7-9)
- After centrifugation, PRP samples should be allowed to sit at room temperature for 15 min before testing (median score 7, range 7-9)
- PRP should be used to set 0% light transmission in the aggregometer (median score 9, range 8-9)
- Autologous PPP should be used to set 100% light transmission in the aggregometer (median score 9, range 8-9)
- LTA studies should be performed at 37°C (median score 9, range 8-9)
- During LTA testing, PRP samples should be constantly stirred at 1,000 rpm using a disposable stirrer, unless otherwise specified by the manufacturer of the aggregometer (median score 8, range 8-9)
- Before adding an agonist, baseline tracings for LTA should be observed for oscillations and stability for at least 1 minute (median score 8, range 7-9)
- The volume of agonist added for LTA should be consistent, and never more than 10% of the total sample volume (median score 8, range 7-9)

- Platelet aggregation should be monitored for a minimum of 3 minutes after adding an agonist (median score 8, range 7-9)
- Platelet aggregation should be monitored for a minimum of 5 minutes after adding an agonist that does not cause maximal aggregation by 3 minutes with most control samples (median score 8, range 8-9)
- Platelet aggregation should be monitored for a minimum of 10 minutes after adding an agonist that does not cause maximal aggregation by 5 minutes with most control samples (median score 8, range 5-9)
- LTA studies should be completed within a maximum of 4 hours after blood sampling (median score 8, range 7-9)

7. Choice of Agonists

Platelet agonists should be properly stored and checked for stability. The following platelet agonists, at the indicated concentrations, should be used for diagnostic LTA studies:

- **ADP:** 2 μM (median score 7, range 4-9)
  - higher concentrations of ADP should be used if abnormal results with 2 μM (median score 8, range 8-9)
- **Epinephrine:** 5 μM (median score 8, range 5-9)
  - higher concentrations of epinephrine should be used if abnormal results with 5 μM (median score 8, range 6-9).
- **Collagen:** use a low concentration of collagen, which is sufficient to cause the aggregation of normal platelets (e.g., 2 μg/mL Horm collagen) (median score 8, range 5-9)

- higher concentrations of collagen should be used if abnormal results with 2 µg/mL (median score 8, range 6-9).

Reference to Horm collagen (the most widely used collagen preparation for LTA studies) is given as an example and is not intended to bind laboratories to the use of this commercial preparation.

- **Thrombin Receptor (PAR1) Activating Peptide (PAR1-AP):** 10 µM (median score 7, range 3-9)
  - higher concentrations of PAR1-AP should be used if abnormal results with 10 µM (median score 8, range 5-9)
- The **thromboxane A_2 mimetic U46619:** 1 µM (median score 7, range 5-9)
  - higher concentrations of U46619 should be used if abnormal results with 1 µM (median score 8, range 4-9)
- **Arachidonic acid:** 1 mM (median score 8, range 6-9)
  - higher concentrations of arachidonic acid should be used if abnormal results with 1 mM (median score 7, range 4-9)
- The agglutinating agent **Ristocetin:** 1.2 mg/mL (median score 8, range 7-9)
  - If platelet agglutination induced by Ristocetin 1.2 mg/mL is normal, testing should be repeated using Ristocetin 0.5-0.7 mg/mL (median score 8, range 7-9)
  - If platelet agglutination induced by Ristocetin 1.2 mg/mL is absent, testing should be repeated using Ristocetin 2 mg/mL (median score 7, range 3-9)

8. Evaluation and Reporting of Results

- The platelet aggregation tracing should be evaluated based on:
  - presence of shape change (median score 8, range 8-9)
  - length of the lag phase (median score 8, range 5-9)
  - slope of aggregation (median score 7, range 3-9)
  - maximal amplitude or % aggregation (median score 9, range 8-9)
  - amplitude or % aggregation at the end of the monitoring period (median score 8, range 5-9)
  - deaggregation (median score 8, range 8-9)
  - visual examination of the aggregation tracings (median score 9, range 8-9)
  - the presence of a "secondary wave" induced by epinephrine (median score 7, range 5-9)
- Studies completed more than 4 hours after blood collection should be reported with a comment on this (median score 8, range 3-9)
- Clinical laboratories must establish appropriate reference intervals and validate test performance with each lot of reagents (median score 8, range 7-9) [18].

Addendum

M. Cattaneo: concept and design of the study, grading of recommendations, analysis of the literature search, analysis and interpretation of the data, writing the manuscript, final approval of the version to be published.

C. Cerletti: grading of recommendations, analysis of the literature search, analysis and interpretation of the data, revising the manuscript, final approval of the version to be published.

P. Harrison: grading of recommendations, analysis of the literature search, analysis and interpretation of the data, revising the manuscript, final approval of the version to be published.

C.P.M. Hayward: grading of recommendations, analysis of the literature search, analysis and interpretation of the data, revising the manuscript, final approval of the version to be published.

D. Kenny: grading of recommendations, analysis of the literature search, analysis and interpretation of the data, revising the manuscript, final approval of the version to be published.

D. Nugent: grading of recommendations, analysis of the literature search, analysis and interpretation of the data, revising the manuscript, final approval of the version to be published.

P. Nurden: grading of recommendations, analysis of the literature search, analysis and interpretation of the data, revising the manuscript, final approval of the version to be published.

A.K. Rao: grading of recommendations, analysis of the literature search, analysis and interpretation of the data, revising the manuscript, final approval of the version to be published.

A.H. Schmaier: grading of recommendations, analysis of the literature search, analysis and interpretation of the data, revising the manuscript, final approval of the version to be published.

S.P. Watson: grading of recommendations, analysis of the literature search, analysis and interpretation of the data, revising the manuscript, final approval of the version to be published.

F. Lussana: literature search, analysis of the literature search, analysis and interpretation of the data, revising the manuscript, final approval of the version to be published.

M.T. Pugliano: literature search, analysis of the literature search, analysis and interpretation of the data, revising the manuscript, final approval of the version to be published.

A.D. Michelson: concept and design of the study, grading of recommendations, analysis of the literature search, analysis and interpretation of the data, revising the manuscript, final approval of the version to be published.

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Legends to Figures

Figure 1 – Literature search: progression of study selection