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To cite this article: I.C.A. Munnix , R. Van Oerle , P. Verhezen , P. Kuijper , C.M. Hackeng , H.I.J. Hopman-Kerkhoff , F. Hudig , D. Van De Kerkhof , A. Leyte , M.P.M. De Maat , R.F.M. Oude Elferink , J. Ruinemans-Koerts , M. Schoorl , J. Slomp , H. Soons , A. Stroobants , E. Van Wijk & Y.M.C. Henskens (2020): Harmonizing light transmission aggregometry in the Netherlands by implementation of the SSC-ISTH guideline, Platelets, DOI: [10.1080/09537104.2020.1771549](https://doi.org/10.1080/09537104.2020.1771549)

To link to this article: <https://doi.org/10.1080/09537104.2020.1771549>



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Published online: 11 Jun 2020.



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Harmonizing light transmission aggregometry in the Netherlands by implementation of the SSC-ISTH guideline

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Abstract

Light transmission aggregometry (LTA) is considered the gold standard method for evaluation of platelet function. However, there are a lot of variation in protocols (pre-analytical procedures and agonist concentrations) and results. The aim of our study was to establish a national LTA protocol, to investigate the effect of standardization and to define national reference values for LTA. The SSC guideline was used as base for a national procedure. Almost all recommendations of the SSC were followed e.g. no adjustment of PRP, citrate concentration of 109 mM, 21 needle gauge, fasting, resting time for whole blood and PRP, centrifugation time, speed and agonists concentrations. LTA of healthy volunteers was measured in a total of 16 hospitals with 5 hospitals before and after standardization. Results of more than 120 healthy volunteers (maximum aggregation %) were collected, with participating laboratories using 4 different analyzers with different reagents. Use of low agonist concentrations showed high variation before and after standardization, with the exception of collagen. For most high agonist concentrations (ADP, collagen, ristocetin, epinephrine and arachidonic acid) variability in healthy subjects decreased after standardization. We can conclude that a standardized Dutch protocol for LTA, based on the SSC guideline, does not result in smaller variability in healthy volunteers for all agonist concentrations.

Keywords

Light transmission aggregometry, platelet aggregation, platelet disorders, platelet function, platelets, standardization

History

Received 22 October 2019

Revised 14 May 2020

Accepted 15 May 2020

Published online 10 June 2020

Introduction

Light transmission aggregometry (LTA) is considered the gold standard method to investigate patients with suspected abnormalities of primary hemostasis due to inherited or acquired defects of platelet function [1]. In this method, the platelet aggregation pattern in response to an agonist is measured in platelet-rich plasma (PRP) by turbidometry [2]. Different national and international guidelines state that LTA plays an important role in the diagnostic work-up of patients with bleeding tendency [3–9]. Novel techniques based on whole-blood analysis, e.g. impedance aggregometry and platelet

function analyzer (PFA), have been developed, but these lack sensitivity for detection of mild platelet function disorders [10,11] or are only complementary to LTA, such as flow cytometry [12]. Thus, LTA continues to be one of the most helpful tools for the evaluation of suspected platelet function disorders.

However, (pre-)analytical aspects of LTA methodology have not been adequately standardized [13]. There are many variables that affect the outcome of platelet aggregation, e.g. method of blood sampling, preparation of PRP or choice of agonists and their concentrations [14]. Recent surveys by proficiency testing organizations have also identified variations in LTA practices and suggested the need for guidelines to standardize LTA [15–17] since LTA is time-consuming, technically challenging, poorly reproducible and requires a relatively large volume of fresh blood. Standardization is also necessary to maintain this technique in small laboratories and to compare results from patients referred to other hospitals for diagnosis or treatment. During the last decade, several attempts have been made by different organizations to develop LTA guidelines, e.g. the CLSI and the Scientific and Standardization Committee of the

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International Society on Thrombosis and Hemostasis (SSC-ISTH) guidelines [18–22]. The clinical impact of using different methods for LTA is unknown, but it could lead to over and underdiagnosis of platelet disorders. In the Netherlands, the Society for Hematological Laboratories (VHL, Vereniging Hematologische Laboratoria) and the Working group Hemostasis Diagnostics of the Dutch Society for Thrombosis and Hemostasis (WHD-NVTH) collected data from 25 LTA-using laboratories in a national survey on LTA. A large variety of procedures were reported mostly on fasting, resting, smoking, caffeine use, centrifugation procedures and platelet adjustment of PRP (unpublished results). Subsequently, the VHL/WHD-NVTH developed a national LTA protocol, based on the recommendations of the Platelet Physiology Subcommittee of the SSC-ISTH [18]. With the introduction of this national LTA protocol, we aimed to achieve a higher degree of standardization in the Netherlands for LTA.

This study had three aims:

- (1) To investigate whether the use of a standardized LTA protocol could reduce variation (per agonist concentration) in general by analyzing maximum aggregation in healthy volunteers.
- (2) To investigate whether the use of a standardized protocol for LTA could reduce variation in five hospitals that participated both before and after standardization by analyzing maximum aggregation in healthy volunteers.
- (3) To calculate national reference values per agonist concentration by using all healthy volunteers results after standardization.

Materials and Methods

Participating Laboratories

A total of 10 laboratories (together including 129 healthy subjects) participated in the survey in 2013 (before standardization) and 11 laboratories (134 healthy subjects) in 2016 (after standardization). Five laboratories participated before and after standardization. Details of the participants are summarized in Table I.

Table I. The number of Dutch laboratories that participated in this survey before and after introduction of a standardized LTA protocol, including the instruments used for aggregometry.

Laboratory number	Before standardization (n = 10)	After standardization (n = 11)	Instrument
1	Participated	-	A; Chronolog
2	Participated	Participated	B; PAP8
3	Participated	-	A; Chronolog
4	Participated	-	C; APACT
5	Participated	-	A; Chronolog
6A*	Participated	Participated	C; APACT
6B*	Participated	Participated	C; APACT
6C*	-	Participated	A; Chronolog
7	Participated	Participated	C; APACT
8	Participated	Participated	A; Chronolog
9	Participated	Participated	A; Chronolog
10	Participated	-	C; APACT
11	-	Participated	A; Chronolog
12	-	Participated	A; Chronolog
13	-	Participated	C; APACT
14	-	Participated	A; Chronolog
15	-	Participated	A; Chronolog
16	-	Participated	D; AggRAM

*laboratory number 6 participated with different protocols/sub-laboratories (A,B,C); gray colored lines participated in both years.

Healthy Subjects

Healthy volunteers were included anonymously (no registration of age or gender) with the only exclusion criteria of taking drugs known to interfere with platelet function. Information was recorded on fasting, diet instructions and a light meal prior to sample collection. Citrated blood was drawn from healthy volunteers according to the local venepuncture procedure, CCKL/ISO-15189 guidelines and the WMA Declaration of Helsinki.

Instruments

Most laboratories used one type of instrument for LTA, either Chrono-Log Corporation (Haverston, PA, USA), APACT, (LABiTec, Ahrensburg, Germany), PAP-8 (Biodata Corporation, Horsham, PA, USA) or Aggram (Helena BioSciences, Europe).

The instrument type per participant is shown in Table I.

Before standardization in 2013, each participating laboratory used their local protocol for LTA which will not be specified here. Overall, 10 different procedures for LTA were used in the 10 laboratories in the Netherlands before standardization.

The diversity in reagent concentrations in 2013 (before standardization) and the fixed concentrations used after standardization in 2016 is summarized in Table II.

In 2016 the national VHL protocol, based on the SSC-ISTH protocol according to Cattaneo et al., was approved [18]. The protocol describes pre-analytical variables regarding blood collection and sample preparation, centrifuge settings, aggregometry devices and settings, which are summarized in Supplementary Tables S1 and S2.

Used reagent brands were not recorded in 2013 and the reagents used in 2016 are summarized in Supplementary Table S3.

Statistics

Data were anonymized before import into a database and analyzed using GraphPad Prism 7.0 (GraphPad Software). Differences in LTA results of healthy subjects (not normally distributed) between 2013 and 2016 were tested on significance with a non-parametric Mann–Whitney U test. Reference intervals (95% interval, normally distributed) were determined by parametric analyses using EP-evaluator (Data Innovations LLC).

Results

Overall Variation in Maximum Aggregation of Healthy Volunteers Before and After Introduction of a Standardized LTA Protocol

Figure 1a-i demonstrates observations of the variation in maximum platelet aggregation with various concentrations of ADP,

Table II. Agonists concentrations used before (2013) and after (2016) standardization.

Agonist	Before standardization	After standardization (recommended by the SSC-ISTH guideline)
ADP low	0.5–2.5 μ M	2 μ M
ADP intermediate	4–5 μ M	5 μ M
ADP high	10–20 μ M	10 μ M
Collagen low	0.2–2 μ g/ml	1–2 μ g/ml
Collagen high	4–10 μ g/ml	5–10 μ g/ml
Ristocetin low	0.25–0.6 mg/ml	0.3–0.7 mg/ml
Ristocetin high	1.0–1.5 mg/ml	1.2–2.0 mg/ml
Epinephrine low	5 μ M	5 μ M
Arachidonic acid	0.5–1.6 mM	1 mM

collagen, ristocetin, epinephrine and arachidonic acid in healthy volunteers before and after standardization using different analyzers. ADP concentrations of 0.5 to 2.5 μM (Figure 1a) show in general high coefficients of variation (CV) in maximum aggregation ranging from 5% to 107% when measured on all instruments (Figure 1a). The use of a standardized protocol (2 μM ADP) does not reduce the CV. Lowest variation is observed before standardization when using the slightly higher ADP concentration of 2.5 μM . Figure 1b, c shows that increasing ADP concentrations (5 or 10 μM) reduces the CV without any difference before or after standardization. Intermediate or high ADP concentrations show little variability between various analyzers in terms of maximum aggregation ranges.

More or less the same effect is observed with collagen. Before standardization in 2013, low collagen concentrations (0.2–2 $\mu\text{g}/\text{mL}$) show more variation in maximum aggregation results of healthy volunteers in comparison to high collagen concentrations (4–10 $\mu\text{g}/\text{mL}$) on both Chronolog and APACT instruments (Figure 1d, e). After standardization in 2016, low collagen concentrations (1–2 $\mu\text{g}/\text{mL}$) show less variability in maximum aggregation on all instruments, compared to the LTA results of 2013 (Figure 1d). A fixed, high collagen concentration of 5 $\mu\text{g}/\text{mL}$ displays roughly the same variation before and after standardization (Figure 1e). As expected, low concentrations of ristocetin result in very low maximal aggregation responses with a few outliers (Figure 1f). The standardization procedure does not affect these results. Higher ristocetin concentrations show CV ranges of 3% to 19% before and 3–22% after standardization (Figure 1g). For epinephrine 5 μM the CV before standardization is lower (Figure 1h). Finally, LTA results of arachidonic acid are relatively stable between different instruments before and after standardization (Figure 1i).

Observational Changes in LTA Results for five Individual Hospital Laboratories Who Participated Both Before and After Standardization

Figure 2a illustrates the consequence of LTA standardization with intermediate doses of ADP for five individual hospital laboratories who participated in both surveys. The variation in maximum aggregation of hospital laboratory 2 and 6 shows no obvious change between 2013 and 2016. Standardization clearly increases variation in maximum aggregation with intermediate concentrations of ADP in hospital 7. In contrast to this, variability is reduced in both hospital 8 and 9 after standardization in 2016. Figure 2b demonstrates the results of LTA standardization with low concentrations of collagen for the same five hospitals. Standardization evidently reduces variation in maximum aggregation with low collagen doses in 4 out of 5 hospital laboratories.

Determination of General Reference Ranges

Reference intervals (95% confidence intervals, normally distributed) for maximum aggregation expressed as a percentage, for each agonist, were estimated using the LTA data from all healthy controls (Table III). For individual agonist responses, the total numbers of samples tested ranged from 42 to 107.

Maximum aggregation response to low concentrations (2 μM) of ADP in healthy volunteers shows a very broad range of results (Table III). However, less variability was seen with intermediate (5 μM) and high (10 μM) concentrations of ADP. This also accounts for low and high doses of collagen and ristocetin, epinephrine and arachidonic acid (Table III).

Discussion

Nowadays, LTA is still the most common method used in clinical laboratories to assess platelet function and is considered the gold

standard method for detecting thrombocytopeny [23–26]. However, (pre-)analytical aspects of this method have not yet been standardized worldwide [15–17] despite various efforts of experts involved in the development of national or international guidelines [18–22]. Novel techniques are developed, e.g. whole-blood flow cytometry [12,27]. Furthermore, promising results are accomplished with platelet aggregation studies under flow conditions [28]. Advantages of these new methods are the use of whole blood and low sample preparation, but they also require trained technicians and specialized equipment [29–34]. Likewise, standardization of the traditional LTA method is still essential here.

Despite the recently ISTH-SSC Platelet Physiology Subcommittee guideline for LTA [18], a Dutch survey of LTA protocols from 25 different hospitals in the Netherlands resulted in 25 different procedures (unpublished results). Adherence to a new guideline requires many changes for the individual hospitals, which are time and money consuming and the actual improvement for patient care remains unknown. This discrepancy in LTA procedures is not new, but also evident from other surveys [15–17]. Results of a worldwide survey [21] showed that common practices were identified in sample collection, processing and analysis and although some were generally considered acceptable, others were not ideal. The agonist concentrations used for LTA varied, and many laboratories used ADP, collagen, epinephrine and ristocetin, at more than one concentration, in addition to arachidonic acid. The parameters commonly used to assess LTA responses were maximum amplitude or percentage aggregation, which were considered particularly important, in addition to the presence of a ‘secondary wave,’ deaggregation, shape change and a measure of the lag phase. Furthermore, many laboratories did not have appropriate determined reference intervals or used non-adjusted PRP [21]. We examined the introduction of a national LTA protocol in the Netherlands and the consequences of standardization. Most and very important variables [35] such as used citrate concentration (109 mM) or the window between blood drawing and analysis were completely standardized by the participants in 2016 (after standardization). Our data show that standardization was not effective for the low ADP concentration. This can be explained by the fact that ADP is a batch-dependent agonist and probably highly variable in the low concentration range. The question arises whether we should still use these low concentrations of ADP. It is unknown what the low ADP concentration adds to the analyses of bleeding tendency when no discrimination can be made between healthy volunteers and patients with a bleeding tendency. ADP requires structural and extensive quality control of lots and batches [18]. Unpublished results from a small, multicentre, agonist brand study in the Netherlands on the agonists collagen and ADP showed that brand and lot differences were the most prominent for the variable aggregation patterns with low dose ADP and for deviations in the type of collagen used (non-equine collagen brands). In our study, brands were mostly coupled with instrument brands and we could not see a clear pattern in differences. Althaus et al. [36] performed an interesting study which was partly comparable with our study by questioning 15 laboratories to measure maximal aggregation in 3 PRP samples of healthy volunteers using a fixed panel of agonists that were provided by shipping to the hospitals. In this study, the highest CV was observed using the lowest ADP concentration and the CV decreased with increasing ADP levels. They contributed this effect to a lower stability of ADP in shipping.

In contrast to ADP, low doses of collagen showed improvement in variation of LTA results after standardization. Presumably, this is because not every laboratory used the same collagen preparation before standardization in 2013. Differences between bovine and equine collagen preparations in LTA have been described earlier and could be a source of variance [37]. Likewise, arachidonic acid displayed less variation in maximum aggregation results after standardization. This cannot be

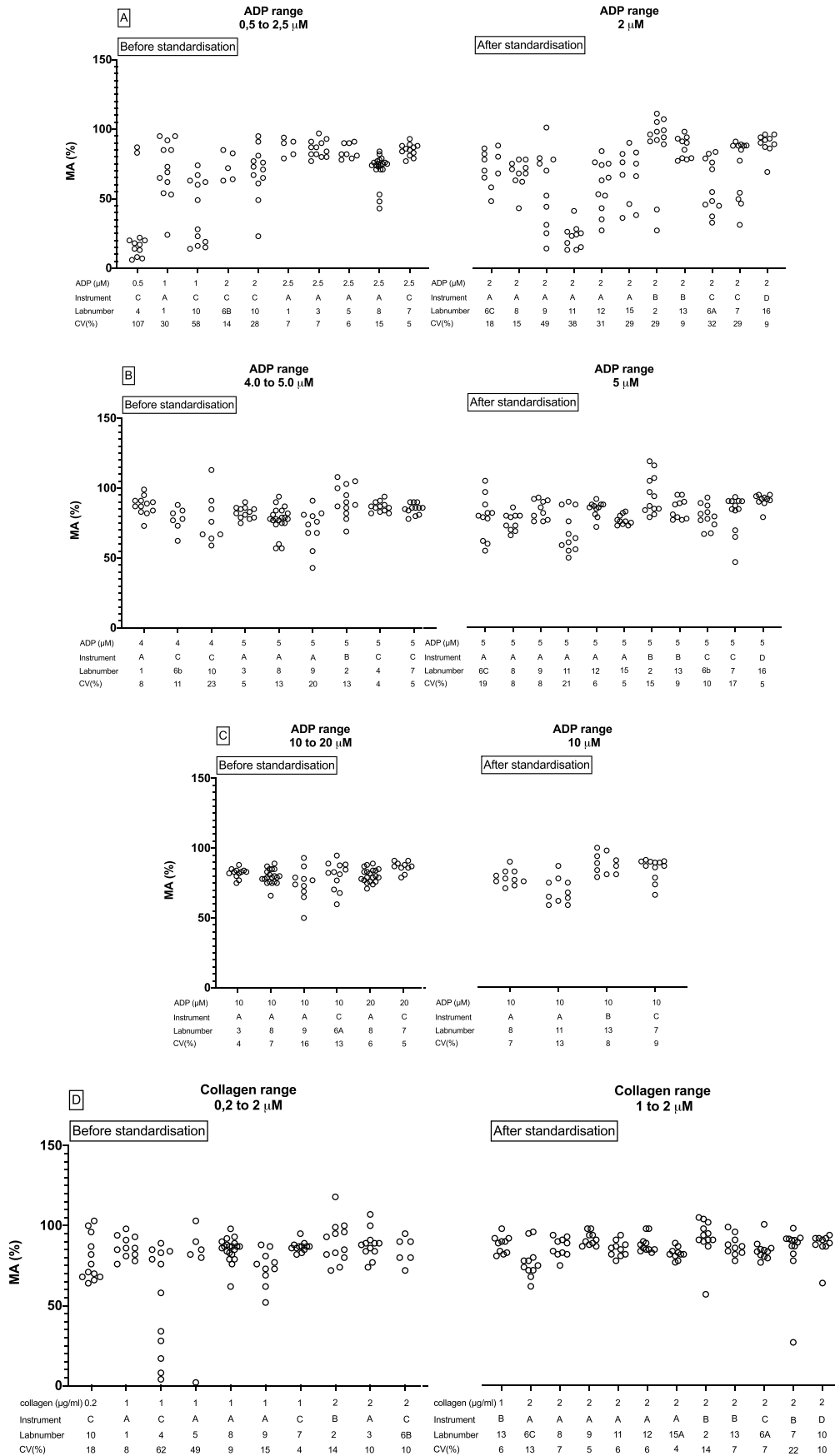
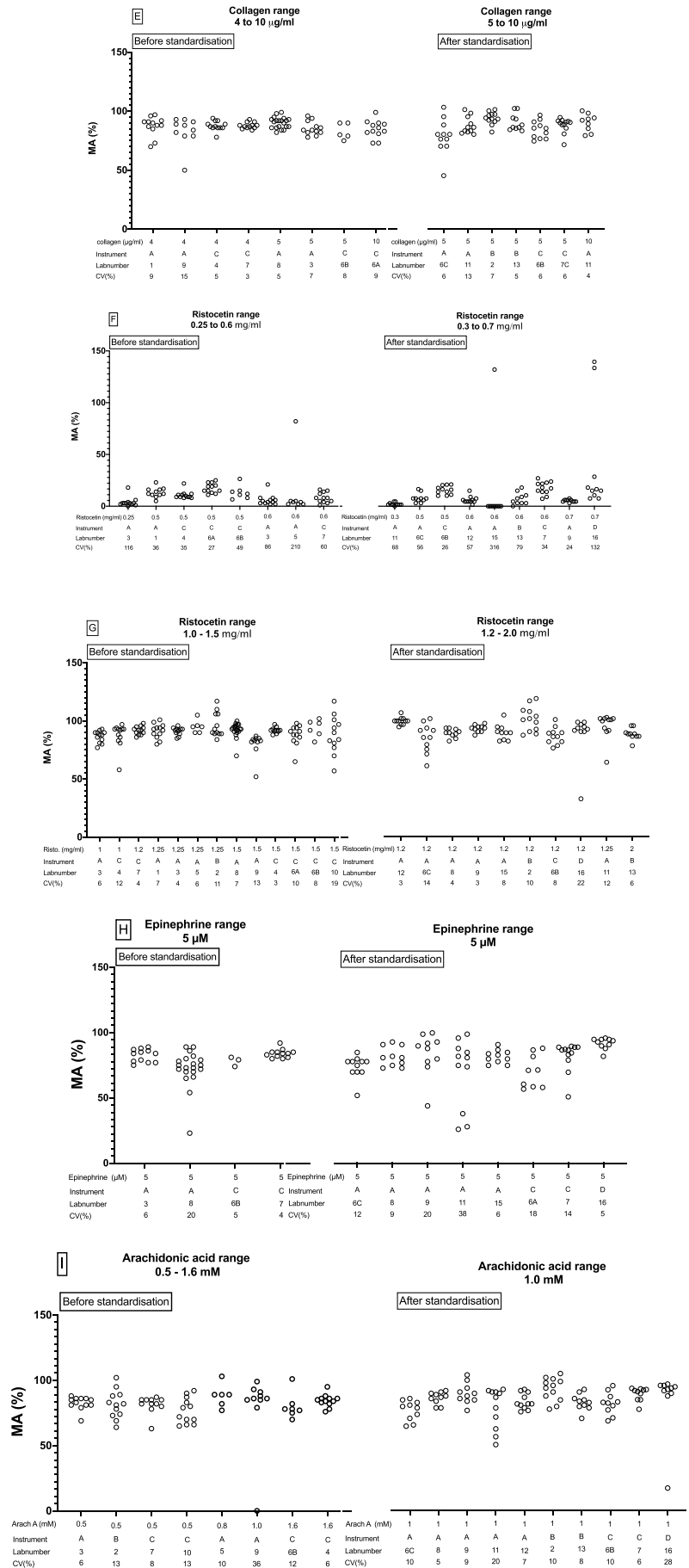


Figure 1. Changes in LTA results of healthy volunteers on different analyzers before and after standardization. Graphs show the instrument type (a: Chronolog, b: PAP8, c: APACT, d: AggRAM), laboratory number and the coefficient of variation (CV, %) in maximum aggregation after stimulation of PRP from healthy subjects with: a) 0.5–2.5 μM ADP low range, b) 4–5 μM ADP intermediate range, c) 10–20 μM ADP high range, d) 0.2–2 $\mu\text{g/ml}$ collagen low range, e) 4–10 $\mu\text{g/ml}$ collagen high range, f) 0.25–0.6 mg/ml ristocetin low range, g) 1.0–1.5 mg/ml ristocetin high range, h) 5 μM epinephrine, i) 0.5–1.6 mM arachidonic acid. Results are expressed as % maximum aggregation.

Figure 1. continued



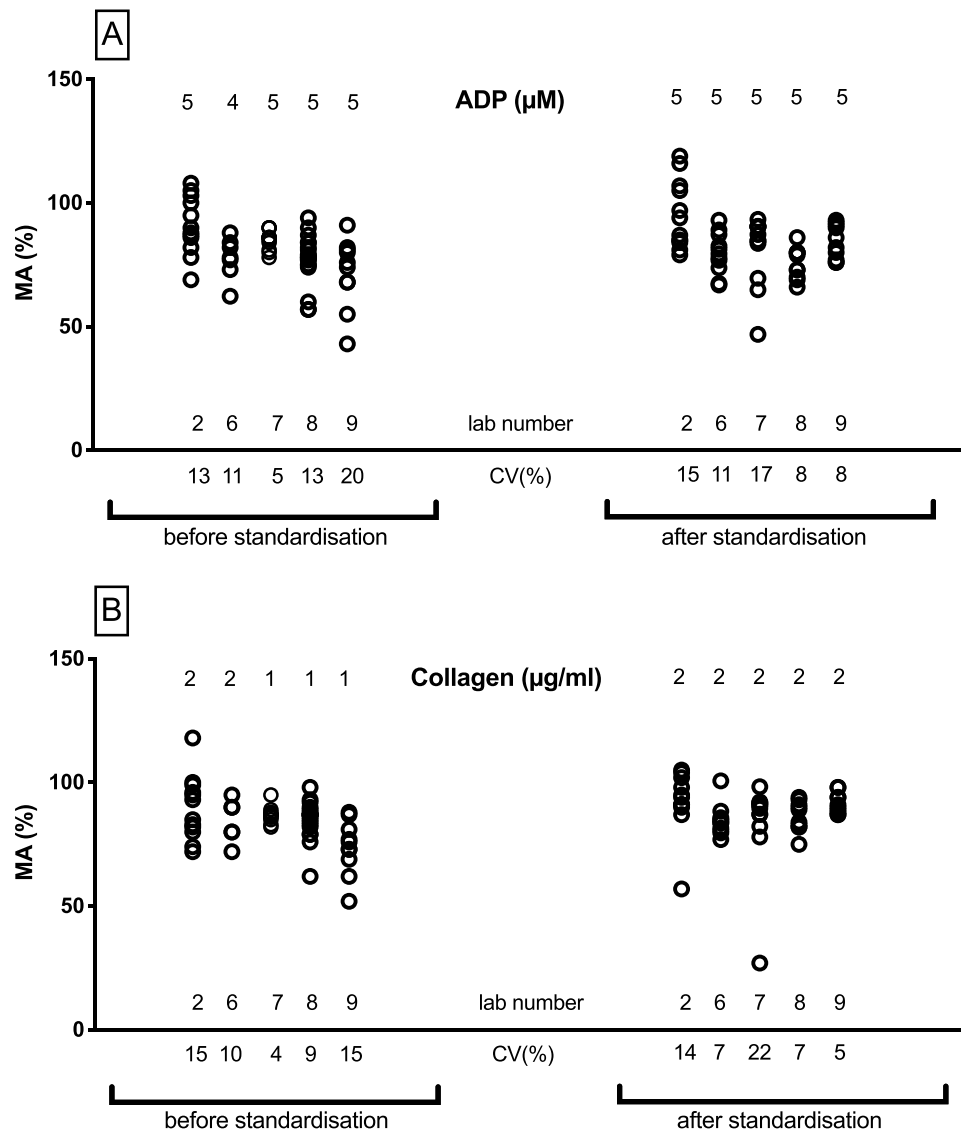


Figure 2. **The consequence of standardization for five individual hospitals who participated before and after standardization.** Diagrams show variation in maximum aggregation after stimulation of PRP from healthy subjects with a) 4–5 μM ADP or b) 1–2 $\mu\text{g/ml}$ collagen. Results are expressed as % maximum aggregation.

Table III. LTA reference intervals (95% confidence interval, normally distributed) for % maximum aggregation per agonist concentration after standardization.

Agonist	Reference interval for % maximum aggregation
2 μM ADP	17–117 (n = 106)
5 μM ADP	59–105 (n = 107)
10 μM ADP	60–101 (n = 42)
2 $\mu\text{g/ml}$ collagen	69–105 (n = 106)
5 $\mu\text{g/ml}$ collagen	75–103 (n = 56)
Low ristocetin (<0.7 mg/ml)	0–50 (n = 71)
High ristocetin (>1.2 mg/ml)	71–111 (n = 95)
5 μM epinephrine	49–111 (n = 73)
1 mM arachidonic acid	62–108 (n = 94)

explained. In case of epinephrine no significant effect of standardization was observed.

The benefits of standardization for an individual laboratory were present but variable. Using a fixed concentration of ADP had mostly no effect and brought no reduction in variation in the

majority of the hospitals. In general, there was less variation in LTA results with a fixed concentration of collagen after standardization. In conclusion, variation was not unambiguously reduced after standardization, assuming that there was protocol adherence.

A possible explanation for the fact that in general there is a low reduction in variation after standardization, might be the effect of introducing non-adjusted PRP, which was a major change for laboratories after standardization. Diluting PRP with platelet poor plasma to adjust platelet count introduces artifacts as suggested by Cattaneo et al. and should be avoided, because it artefactually inhibits platelet aggregation [18,38]. In addition, it may also lead to more variation, because of different platelet counts in PRP [39,40]. However, working with non-adjusted PRP may also result in variation between healthy volunteers. Another study shows that LTA with platelet count adjusted PRP is superior to native PRP for detecting bleeding disorders, although the benefit is small and may not be clinically significant [41]. On the other hand, Althaus et al. [36] used also non-adjusted PRP and published lower CV's in healthy PRPs compared to our findings.

A limitation of our study is that healthy control subjects differ in 2013 (before standardization) and 2016 (after standardization).

Furthermore, there are limited data for some agonists, e.g. less than 10 donors per hospital. This makes our research largely observational. We only investigated healthy volunteers and not patients with thrombocytopathies. Advantages of standardization could be larger in this target population; patient suspected of bleeding disorder.

The CLSI guideline requires a minimum of 120 normal subjects to establish reference intervals. For a technique such as LTA this is expensive, labor-intensive and not feasible for each individual laboratory. The proposed ranges in [Table III](#) can give guidance for laboratories and are directorial. Each laboratory must decide and substantiate whether they can use these values in daily patient care.

Taken together, our findings illustrate that standardization of LTA procedures does not necessarily lead to less variation, especially for low agonist concentrations. For most agonist concentrations (intermediate and high ADP, low and high collagen, low and high ristocetin, epinephrine and arachidonic acid) variability decreased in healthy subjects. The VHL still encourages all LTA users to adhere to the new guideline.

Acknowledgements

The authors thank C. Klopper, S. Busscher, H. Nieman-Teunis, T. de Boer, J. van Empel, N. van Dijk and C. van den Nieuwenhof for performing the LTA.

Declaration Of Interest Statement

The authors report no declarations of interest.

Supplementary Material

Supplemental data for this article can be accessed on the [publisher's website](#)

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