Comparison of Light Transmission Aggregometry With Impedance Aggregometry in Patients on Potent P2Y12 Inhibitors

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Abstract
Since data on the agreement between light transmission aggregometry (LTA) and multiple electrode aggregometry (MEA) in patients on the more potent P2Y12 inhibitors are missing so far, we investigated if the evaluation of the responsiveness to therapy by LTA can be replaced by MEA in 160 acute coronary syndrome (ACS) patients on dual antiplatelet therapy with aspirin and prasugrel or ticagrelor (n=80 each). Cut-off values for high on-treatment residual platelet reactivity (HRPR) in response to adenosine diphosphate (ADP) or arachidonic acid (AA) were defined according to previous studies showing an association of HRPR with the occurrence of adverse ischemic outcomes. ADP-inducible platelet aggregation was 33% and 37% (P=0.07) by LTA and 19 AU and 20 AU (P=0.38) by MEA in prasugrel- and ticagrelor-treated patients, respectively. AA-inducible platelet aggregation was 2% and 3% by LTA and 15 AU and 16 AU by MEA, (all P≥0.3) in patients on prasugrel and ticagrelor, respectively. By LTA, HRPR ADP and HRPR AA were seen in 5%/5% and in 4%/13% of patients receiving prasugrel- and ticagrelor, respectively. By MEA, HRPR ADP and HRPR AA were seen in 3%/25% and 0%/24% of prasugrel- and ticagrelor-treated patients, respectively. ADP-inducible platelet reactivity by MEA correlated significantly with LTA ADP in prasugrel-treated patients (r=0.4, P<0.001), but not in those receiving ticagrelor (r=0.09, P=0.45). AA-inducible platelet aggregation by LTA and MEA did not correlate in prasugrel- and ticagrelor-treated patients. Sensitivity/specificity of HRPR by MEA to detect HRPR by LTA were 25%/99% for MEA ADP and 100%/79% for MEA AA in prasugrel-treated patients, and 0%/100% for MEA ADP and 70%/83% for MEA AA in ticagrelor-treated patients. In conclusion, on-treatment residual ADP-inducible platelet reactivity by LTA and MEA shows a significant correlation in prasugrel- but not ticagrelor-treated patients. However, in both groups LTA and MEA revealed heterogeneous results regarding the classification of patients as responders or non-responders to P2Y12 inhibition.

Keywords
multiple electrode aggregometry, light transmission aggregometry, antiplatelet therapy, P2Y12 antagonists, high on-treatment residual platelet reactivity

Introduction
Despite dual antiplatelet therapy (DAPT) atherothrombotic events impair the prognosis of acute coronary syndrome (ACS) patients following percutaneous coronary intervention (PCI) with stent implantation.¹⁻³ Previous studies in clopidogrel-treated patients identified poor response to antiplatelet therapy, i.e. high on-treatment residual platelet reactivity (HRPR), as a potential reason for adverse ischemic outcomes.⁴ Meanwhile, according to current guidelines the use of prasugrel and ticagrelor is recommended over clopidogrel in ACS patients due to more favorable outcomes with regard to 1-year ischemic events in randomized clinical trials.⁵⁻⁸ However, even in patients on the more potent P2Y12 inhibitors, HRPR might play a role in the development of atherothrombotic endpoints.⁹,¹⁰ Accordingly, platelet function tests are often used to assess the response to antiplatelet therapy.
Thereby, in particular light transmission aggregometry (LTA) and multiple electrode aggregometry (MEA) have been repeatedly associated with outcomes after PCI in patients on dual antiplatelet therapy with aspirin and clopidogrel.\textsuperscript{4,11,12}

LTA was first described in the 1960s and can be performed with various agonists, e.g. adenosine diphosphate (ADP), arachidonic acid (AA), collagen, epinephrine and thrombin-receptor activating peptide (TRAP).\textsuperscript{13-15} Although LTA is a laborious technique and to a great extent dependent on different pre-analytical and procedural conditions, it is still regarded as the historical gold standard method to determine on-treatment platelet aggregation.\textsuperscript{16-18}

MEA was developed on the basis of impedance aggregometry.\textsuperscript{19,20} For MEA, a total of 4 electrodes capturing an electrical signal are used per analysis. Accordingly, optical variables (e.g. lipemia) of the sample are not relevant for the test result.\textsuperscript{21} MEA allows the rapid detection of platelet aggregation out of whole blood with highly-reproducible test results.\textsuperscript{20}

Therefore we have chosen LTA and MEA to assess platelet reactivity. Alternatively, we could have used the VASP assay, which has been shown to give reliable results in clopidogrel-treated patients.\textsuperscript{22} However, HRPR by LTA and MEA was also repeatedly linked to outcomes in clopidogrel-treated patients.\textsuperscript{4,11,12}

Several studies also used the 70% cut-off to define HRPR in response to 10 μM ADP, e.g. Buonamici et al.,\textsuperscript{23} Cuisset et al.\textsuperscript{24} and Migliorini et al.\textsuperscript{25} Frere et al determined a maximal aggregation ≥70% by LTA with 10 μM ADP as best predictor of adverse ischemic events following PCI in ACS patients using an ROC curve analysis.\textsuperscript{26} Likewise, a maximal aggregation ≥70% by LTA with 10 μM ADP was described as an independent predictor of 3-year cardiac death and stent thrombosis in 215 patients undergoing PCI for unprotected left main disease.\textsuperscript{25} Alternatively, the absolute change in maximal aggregation may be used to define HRPR. This approach however requires the assessment of platelet aggregation before and after the initiation of antiplatelet therapy, which is hardly feasible in daily clinical routine. Finally, previous studies defined platelet aggregation values in the fourth quartile as HRPR.\textsuperscript{27-29} In an additional analysis, we therefore used the fourth quartiles of LTA and MEA to define HRPR. As expected, this approach markedly affected sensitivities, specificities, positive and negative predictive values of HRPR by MEA to detect HRPR by LTA. Since the definition of thresholds for HRPR directly influences the obtained results, it would be important to establish a uniform cut-off definition for future studies in the field.

In contrast to other studies, which determined on-treatment platelet reactivity at least 12 hours after administration of a P2Y\textsubscript{12} inhibitor loading dose,\textsuperscript{24-26} we decided to perform all measurements on day 3 after PCI. This approach was chosen because 1) at this time point all patients were still at the inpatient ward, and 2) we sought to avoid procedure-related variations of the results and to assess platelet reactivity at a steady state.

Previous studies suggest a significant correlation of LTA with the more reproducible and near point-of-care assay MEA in clopidogrel-treated patients.\textsuperscript{29,30} Since data on the agreement between LTA and MEA in patients on newer P2Y\textsubscript{12} inhibitors are missing so far, we investigated if MEA reveals concordant results with LTA, therefore allowing to replace LTA by MEA in the assessment of the response to antiplatelet therapy.

Methods

Study Population

The study population consisted of 160 patients suffering from ACS on daily aspirin (100 mg/day), and either prasugrel (10mg/d, n = 80), or ticagrelor (180mg/d, n = 80) therapy. Blood sampling was performed on day 3 after acute successful PCI with stent implantation after an overnight fast. Exclusion criteria were a known aspirin, prasugrel or ticagrelor intolerance (allergic reactions, gastrointestinal bleeding), a therapy with vitamin K antagonists (warfarin, phenprocoumon, acenocoumarol) or direct oral anticoagulants (rivaroxaban, apixaban, dabigatran, edoxaban), treatment with ticlopidine, dipyridamol or nonsteroidal anti-inflammatory drugs, a family or personal history of bleeding disorders, malignant myeloproliferative disorders or heparin-induced thrombocytopenia, severe hepatic failure, known qualitative defects in platelet function, a major surgical procedure within 1 week before enrolment, a platelet count <100,000 or >450,000/μl and a hematocrit <30%.

The study protocol was approved by the Ethics Committee of the Medical University of Vienna in accordance with the declaration of Helsinki and its later amendments and all study participants signed written informed consent.

Light Transmission Aggregometry (LTA)

LTA was performed on a PAP-8E aggregometer (Bio-Data, Horsham, PA USA) as previously described.\textsuperscript{29,31,32} Citrate-anticoagulated whole blood was allowed to “rest” in a tilt position at room temperature for 20 min before centrifugation. Blood tubes were centrifuged at 150 × g for 10 minutes (min) at room temperature to acquire platelet rich plasma (PRP). To obtain platelet-poor plasma (PPP) remaining specimens were re-centrifuged at 2,000 × g for 10 min. Platelet counts were not adjusted as the median platelet count was 226 G/l (interquartile range 188-262 G/l) for the overall study population. The optical density of PPP was set as 100% aggregation. Platelet aggregation was initiated by the following agonists: ADP (10 μM) and arachidonic acid (AA; 0.5mg/mL). Optical density changes were recorded photoelectrically for 10 min as platelets began to aggregate to obtain maximal aggregation values. Maximal aggregation % was automatically calculated by the PAP-8E aggregometer by comparing the increase of light transmission through PRP after addition of an agonist to the baseline optical density that was set with PPP and considered as 100% platelet aggregation.
Multiple Electrode Aggregometry (MEA)

Whole blood impedance aggregometry was performed with the Multiplate analyzer (Roche Diagnostics, Mannheim, Germany) as previously described.\textsuperscript{29,33} One Multiplate test cell contains 2 independent sensor units and 1 unit consists of 2 silver-coated highly conductive copper wires with a length of 3.2 mm. After dilution (1:2 with 0.9% NaCl solution) of hirudin-anticoagulated whole blood and stirring in the test cuvettes for 3 minutes at 37°C, ADP (6.4 μM, Roche Diagnostics, Mannheim, Germany) or arachidonic acid (AA; final concentration of 0.5 mM; Roche Diagnostics, Mannheim, Germany), was added and aggregation was continuously recorded for 6 minutes. The adhesion of activated platelets to the electrodes led to an increase of impedance, which was detected for each sensor unit separately and transformed to aggregation units (AU) that were plotted against time. The AU at 6 minutes were used for calculations. One AU corresponds to 10 AU*min (area under the curve of AU).

Previous studies reported an influence of sample transportation by a pneumatic tube system on platelet activation by MEA.\textsuperscript{34} Therefore, all samples were brought to the laboratory in person immediately after blood sampling. Both tests were performed by the same laboratory technician in order to avoid operator-dependent variations of the results. As LTA requires pre-processing, like centrifugation and resting thereafter, samples were immediately prepared for LTA and MEA was performed while aggregation by LTA was recorded. The intra-assay coefficient of variation (CV) in our laboratory was below 15\% for both test methods.\textsuperscript{35}

Statistical Analyses

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS version 24.0, SPSS, Chicago, Illinois, USA). Median and interquartile range of continuous variables are shown. Categorical variables are given as number (%). We performed the non-parametric Mann Whitney U test to detect differences in continuous variables. The chi-square test was used to assess differences in categorical variables. Spearman correlation was used to test for correlations between platelet aggregation by LTA and MEA in response to the different agonists. Two-sided \( P \)-values <0.05 were considered statistically significant.

The cut-off values for HRPR were based on previous studies showing an association of test results with clinical outcomes in patients on dual antiplatelet therapy with aspirin and clopidogrel. By LTA, a maximal aggregation \( \geq 70\% \) in response to ADP and \( \geq 20\% \) in response to AA were considered as HRPR ADP and HRPR AA, respectively.\textsuperscript{23,26,36,37}

By MEA, AU \( \geq 46 \) in response to ADP and \( \geq 21 \) in response to AA were considered as HRPR ADP and HRPR AA, respectively.\textsuperscript{4,38} Sensitivities and specificities of MEA to detect HRPR based on the results by LTA were calculated.

Since the above-mentioned cut-off values were only established for clopidogrel-treated patients, we performed an additional analysis in which platelet aggregation values in the fourth quartiles by LTA and MEA were considered as HRPR (online-only supplement).

Results

Clinical and laboratory characteristics of ticagrelor (n = 80)-and prasugrel (n = 80)-treated patients are given in Table 1.

ADP-inducible platelet aggregation was 35\% (27-42.8\%) by LTA and 20 AU (15-23.8 AU) by MEA in the overall study population. In prasugrel-treated patients, ADP-inducible platelet aggregation was 33\% (25-41.8\%) by LTA, which was numerically lower but not significantly different from ticagrelor-treated patients (37\% [28-43.8\%], \( P = 0.07 \)). ADP-inducible platelet aggregation by MEA was 19 AU (15-23 AU) in prasugrel-treated patients and 20 AU (15-24.8 AU) in ticagrelor-treated patients (\( P = 0.38 \)).

AA-inducible platelet aggregation in the overall study population was 2.5\% (2-5\%) by LTA and 15.5 AU (11-20 AU) by
MEA. In prasugrel-treated patients AA-inducible platelet aggregation was 2\% (1.3-4\%) and 15 AU (11-20.8 AU) by LTA and MEA, respectively. In ticagrelor-treated patients AA-inducible platelet aggregation was 3\% (2-5\%) by LTA and 16 AU (11-20 AU) by MEA, which was not significantly different from prasugrel-treated patients (both \( P \geq 0.3 \)).

A significant correlation between ADP-inducible platelet aggregation by LTA and MEA was discernible in the overall cohort (\( r = 0.25, P = 0.002 \)). When prasugrel-treated patients were considered separately from ticagrelor-treated patients, there was a stronger correlation between LTA ADP and MEA ADP (Figure 1A; \( r = 0.4, P < 0.001 \)). In contrast, ADP-inducible platelet aggregation by LTA did not correlate with MEA ADP in ticagrelor-treated patients (Figure 1B; \( r = 0.09, P = 0.45 \)).

After platelet activation with AA, there was a significant correlation between LTA and MEA in the overall study population (\( r = 0.16, P = 0.04 \)). There was no correlation between LTA AA and MEA AA, if patients on prasugrel or ticagrelor were considered separately (Figure 1C and D).

By LTA ADP and LTA AA HRPR was seen in 7 (4\%) and 14 (9\%) of the overall study population, respectively. By MEA ADP and MEA AA HRPR was seen in 2 (1\%) and 39 (24\%) of the overall study population, respectively.

Sensitivities, specificities, PPV and NPV of HRPR by MEA to detect HRPR by LTA are reported in Table 2.

Out of the 7 patients with HRPR by LTA ADP, HRPR was present in 1 (14\%) patient by MEA ADP. Further, out of the 14 patients with HRPR by LTA AA, HRPR was present in 11 (79\%) patients by MEA AA.

By LTA HRPR ADP and HRPR AA were seen in 4 (5\%) prasugrel-treated patients, and in 0 and 19 (24\%) ticagrelor-treated patients, respectively.

By MEA HRPR ADP and HRPR AA were seen in 2 (3\%) prasugrel-treated patients, and in 0 and 19 (24\%) ticagrelor-treated patients, respectively.

Figure 1. Correlations between light transmission aggregometry (LTA) and multiple electrode aggregometry (MEA) (A) in response to adenosine diphosphate (ADP) in prasugrel-treated patients, (B) in response to ADP in ticagrelor-treated patients, (C) in response to arachidonic acid (AA) in prasugrel-treated patients, and (D) in response to AA in ticagrelor-treated patients. Circles represent individual measurements. Cut-off values for high on-treatment residual platelet reactivity are indicated by the dotted lines.

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Table 2. Sensitivities, Specificities, Positive (PPV) and Negative (NPV) Predictive Values of High On-Treatment Residual Platelet Reactivity (HRPR) by Multiple Electrode Aggregometry (MEA) in Response to Adenosine Diphosphate (ADP) or Arachidonic Acid (AA) for HRPR by Light Transmission Aggregometry (LTA) in the Overall Study Cohort (O) and in Patients on Prasugrel (P) or Ticagrelor (T) Therapy.

| Test     | LTA ADP | MEA ADP | LTA AA | MEA AA |
|----------|---------|---------|--------|--------|
| Sensitivity (%) | O | 14 | 99 | 79 |
| Specificity (%) | P | 25 | 99 | 100 |
| PPV (%) | T | 0 | 100 | 80 |
| NPV (%) | O | 50 | 96 | 72 |
| PPV (%) | T | 0 | 96 | 80 |
| NPV (%) | O | 28 | 100 | 80 |
| PPV (%) | T | 37 | 95 | 80 |

In prasugrel-treated patients, out of the 4 patients with HRPR ADP by LTA, 1 (25%) patient was non-responsive to prasugrel by MEA ADP. Further, all 4 patients with HRPR AA by LTA were non-responsive to prasugrel by MEA AA.

In ticagrelor-treated patients, out of the 3 patients with HRPR by LTA ADP, none was non-responsive to ticagrelor by MEA ADP. Further, out of the 10 patients with HRPR by LTA AA, 7 (70%) patients were non-responsive to ticagrelor by MEA AA.

Due to the numerical baseline difference in the presence of diabetes between prasugrel- and ticagrelor-treated patients and as the metabolic syndrome and diabetes were associated with increased platelet reactivity in previous studies,39,40 we performed subgroup analyses to reveal the incidence of HRPR in patients with and without diabetes. Diabetes was present in 43 study participants, whereas there were 117 patients without diabetes.

HRPR by LTA ADP occurred in 2 out of 43 (5%) diabetic patients (1 patient on prasugrel and 1 patient on ticagrelor), and in 5 out of 117 (4%) patients without diabetes (P = 0.92). HRPR by LTA AA was present in 3 (7%) diabetic patients and in 11 (9%) patients without diabetes (P = 0.63). By MEA, HRPR ADP was seen in none of the diabetic patients, but in 2 (2%) patients without diabetes (P = 0.39) and HRPR AA occurred in 6 (14%) diabetic and 33 (28%) non-diabetic study participants (P = 0.06).

A detailed additional analysis in which the fourth quartiles of platelet aggregation by LTA and MEA were defined as HRPR is presented in the online-only supplement. The corresponding cut-off values were a maximal aggregation ≥42.8% for LTA ADP, AU ≥24 for MEA ADP, a maximal aggregation ≥5% for LTA AA and AU ≥20 for MEA AA. With use of these alternative cut-offs, sensitivity/specificity of HRPR by MEA to detect HRPR by LTA were 44%/89% for MEA ADP and 33%/75% for MEA AA in prasugrel-treated patients, and 27%/67% for MEA ADP and 42%/78% for MEA AA in ticagrelor-treated patients.

Discussion

To the best of our knowledge, this is the first study comparing LTA and MEA in ACS patients on the potent P2Y12 inhibitors prasugrel and ticagrelor. Although ADP-inducible platelet aggregation did not differ between patients with prasugrel and ticagrelor, a positive correlation between LTA and MEA was only seen in prasugrel-treated patients but not in those on ticagrelor therapy.

In previous reports, Helten et al described a significant correlation between platelet aggregation by LTA and MEA as well as between the VASP assay and LTA or MEA in 23 patients on DAPT with aspirin and clopidogrel.41 However, HRPR occurred in 57%, 43% and 13% of the patients as measured by LTA, the VASP assay and MEA, respectively.41 In a preceding study we also observed a significant correlation between ADP-inducible platelet aggregation by LTA and MEA in 80 clopidogrel-treated patients following endovascular stent implantation.29 The present findings in prasugrel-treated patients are in line with these previous results, but those revealed in patients on ticagrelor are unexpected. The positive correlation between LTA and MEA in prasugrel- and clopidogrel-treated patients is most likely due to their similar pharmacokinetics and -dynamics. While both thienopyridines are prodrugs that need to be metabolized before exerting their antiplatelet effect, the cyclopropyl-triazolopyrimidine ticagrelor acts directly without prior biotransformation.42 Furthermore, the active metabolites of clopidogrel and prasugrel irreversibly block the P2Y12 receptor, whereas ticagrelor inhibits ADP-induced signaling reversibly in a non-competitive manner at a different binding site.42-45

Moreover, ticagrelor acts as an inverse agonist at the P2Y12 receptor and limits basal Gi-coupled signaling thereby increasing cAMP levels.46 In addition, pleiotropic effects like the inhibition of the type 1 equilibrative nucleoside transporter (ENT1) and in consequence increase of the extracellular concentration of adenosine are described.47,48

Further pointing at in vitro differences between prasugrel and ticagrelor-inhibited platelets are our previous findings that ticagrelor exerts a significantly stronger inhibitory effect on TLR-1/2 and PAR mediated platelet activation in ACS patients than prasugrel.49

On the contrary, the thienopyridines clopidogrel and prasugrel are converted into active metabolites in the liver and intestines and bind covalently to the P2Y12 receptor.50 The P2Y12 receptor enables full and stable platelet aggregation in response to ADP after initiation of platelet aggregation via P2Y12.51,52 Differences of the in vitro response of prasugrel- and ticagrelor-inhibited platelets due to the measurements in plasma (LTA) versus whole blood (MEA) may also play a role. In detail, following platelet activation by ADP-P2Y12 interaction in LTA, fibrinogen binds to adjacent platelets via the activated GPIIb/IIIa receptor leading to homotypic aggregation in PRP.53 In contrast, in MEA, activated platelets bind to electrodes initially through negatively-charged phosphatidylserine surface followed by platelet-platelet aggregation. Hence, differences in platelet aggregation results due to the reversible binding of ticagrelor to platelets may be more pronounced in platelet aggregation measured ex vivo.
Thus, there are various possibilities that may abolish the correlation between the results obtained by LTA and MEA in ticagrelor-inhibited platelets.

In addition, as a reversible agent requiring twice daily administration, ticagrelor may be more affected by a decrease in drug potency during incubation of the sample on the bench than the irreversible antiplatelet agent prasugrel with once daily administration. However, since both, LTA and MEA, were performed at the same time point in our study population this should not have affected the correlation between MEA and LTA in ticagrelor-treated patients.

In the overall patient cohort, we found a high specificity and NPV of MEA for HRPR by LTA but a rather low sensitivity and PPV of MEA for HRPR based on the results by LTA. The obtained values are in line with our previous results obtained in clopidogrel-treated patients, where HRPR ADP by MEA had a sensitivity and PPV of 35% and a specificity and NPV of 78.3% for HRPR ADP by LTA.

On the contrary, MEA AA achieved a high sensitivity, specificity and NPV, but low PPV, when compared to LTA AA regarding HRPR in the overall study population.

It is known that P2Y_{12} inhibition also decreases the in vitro response to AA via an inhibition of amplification pathways. Therefore, it is difficult to attribute a decreased response to AA to cyclooxygenase inhibition alone if patients are on aspirin. Accordingly, one can expect some false negative results in LTA AA due to the inhibition of the P2Y_{12} signaling pathway, which may be less observed with clopidogrel than with prasugrel and ticagrelor.

Overall, the test results were not concordant. We therefore think that for the detection of patients with HRPR or adequate platelet inhibition LTA and MEA are not interchangeable.

As the higher proportion of diabetic patients in the ticagrelor group could affect the utility of platelet aggregation tests in this group, we performed subgroup analyses comparing platelet aggregation and HRPR between diabetics and non-diabetics. However, there was no difference in HRPR between ticagrelor- and prasugrel-treated patients with and without diabetes.

Our study is limited to prasugrel and ticagrelor, which carry a higher bleeding risk and a lower risk of ischemic events than clopidogrel. This approach was chosen because numerous previous studies have already assessed the comparability of different platelet function tests in clopidogrel-treated patients. Another limitation of our study is the lack of clinical outcome data and the lack of randomization between patients receiving ticagrelor and prasugrel. To date, there are no approved cut-offs for HRPR by LTA and MEA in patients treated with prasugrel- or ticagrelor. We therefore applied cut-offs for HRPR that have been established for clopidogrel-treated patients, keeping in mind that different levels may be required for the more powerful P2Y_{12} inhibitors. The fact that even prasugrel and ticagrelor therapy have been associated with the occurrence of adverse ischemic events in 6.6% and 5.7% of the patients, respectively, suggests a role for in vitro testing of antiplatelet drug response even in patients on these potent P2Y_{12} inhibitors. Furthermore, in previous studies we have shown that alternative platelet activation pathways, i.e. toll-like (TLR) and protease-activated receptor (PAR) signaling, remain active despite P2Y_{12} inhibition with prasugrel or ticagrelor, pointing at the possibility that even these potent drugs are associated with HRPR.

In conclusion, on-treatment residual ADP-inducible platelet reactivity by LTA and MEA shows a significant correlation in prasugrel- but not ticagrelor-treated patients. However, in both groups, LTA and MEA revealed heterogeneous results regarding the classification of patients as responders or non-responders to P2Y_{12} inhibition. Accordingly, these tests are not interchangeable in the assessment of the response to antiplatelet therapy in ACS patients undergoing PCI.

Author Contributions
P.P. Wadowski: data collection, performance of measurements, statistical analysis, writing of the manuscript. J. Pultar: data collection, critical revision and final approval of the manuscript. C. Weikert: data collection, critical revision and final approval of the manuscript. B. Eichelberger: performance of measurements, critical revision and final approval of the manuscript. I.M. Lang: critical revision and final approval of the manuscript. R. Koppensteiner: critical revision and final approval of the manuscript. S. Panzer: study design, critical revision and final approval of the manuscript. T. Gremmel: study design, statistical analysis, writing of the manuscript, critical revision and final approval of the manuscript.

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Supplemental Material
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