Effect of the reaction temperature on the prothrombin time and the apparent International Normalized Ratio determined with International Standards for thromboplastins

Besselaar, A.M.H.P. van den; Cobbaert, C.M.

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ORIGINAL ARTICLE

Effect of the reaction temperature on the prothrombin time and the apparent International Normalized Ratio determined with International Standards for thromboplastins

Antonius M. H. P. Van Den Besselaar | Christa M. Cobbaert

Coagulation Reference Laboratory, Department of Clinical Chemistry and Laboratory Medicine, Leiden University Medical Center, Leiden, The Netherlands

Correspondence
Antonius M. H. P. Van Den Besselaar, Coagulation Reference Laboratory, Department of Clinical Chemistry and Laboratory Medicine, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands.
Email: a.m.h.p.van_den_besselaar@lumc.nl

Abstract

Introduction: The definition of the International Normalized Ratio (INR) depends on a reference measurement procedure for the prothrombin time (PT) determined with international standards for thromboplastins. The agreed water bath temperature for PT determination in the reference measurement procedure is 37°C. The aim of the study was to assess the influence of small deviations of the agreed reaction temperature on PT and INR determined with World Health Organization international standards for thromboplastins rTF/16 (recombinant human) and RBT/16 (rabbit brain).

Methods: Prothrombin time was determined, with a manual hook technique, in glass test tubes in a water bath at a controlled temperature. The PT reaction temperatures were varied between 28 and 40°C. Pooled normal plasma and pooled coumarin plasma (INR ≈ 2.8) were used as test plasmas. The data were fitted to a quadratic relationship between PT and temperature.

Results: Prothrombin times with rTF/16 were shortened by increasing the reaction temperature up to approximately 39-40°C. PTs with RBT/16 were shortened by increasing the reaction temperature up to approximately 34-37°C, but were prolonged at higher temperatures. The apparent INR change of the coumarin plasma at 37.0°C was 0.06/°C and 0.11/°C for rTF/16 and RBT/16, respectively.

Conclusions: Reaction temperature had a significant effect on PT and the apparent INR with the International Standards. At 37.0°C, the apparent INR of coumarin plasma determined with RBT/16 was more responsive to temperature change than the apparent INR determined with rTF/16. The required accuracy of the water bath temperature should be 37.0 ± 0.1°C.

KEYWORDS

international normalized ratio, prothrombin time, reference measurement procedure, temperature, thromboplastin

1 | INTRODUCTION

The prothrombin time (PT) test was invented by Armand Quick in 1935.[1] The scientific name for the PT is tissue factor-induced coagulation time.[2] The PT test is the main laboratory test used for monitoring therapy with vitamin K antagonists (VKA). PT test results are influenced by both the thromboplastin reagent and the instrument used for end-point detection. It is possible to express PT results on a common scale, ie the international normalized ratio (INR), if the international sensitivity index (ISI) and mean normal PT (MNPT) of the reagent/instrument combination are known.[3]The measurement uncertainty of the PT/INR depends on variations of several quantities such as the pipette volumes of reagent and plasma, the timing of the start and end-point of the reaction, and the reaction temperature. According to the World Health Organization (WHO) guidelines, the PT is the time taken for the reaction mixture to clot when maintained at a temperature of between 36.5 and 37.5°C.[3] The PT measured with some commercial thromboplastin reagents depends on the reaction temperature.[4,5] We wondered to what extent the PT/INR determined with WHO international standards for thromboplastins would vary with the reaction temperature. In the present study, we assessed the influence of the reaction temperature on the PT determined with the current international standards for rabbit thromboplastin (RBT/16) and recombinant human thromboplastin (rTF/16). The proposed reference measurement procedure (RMP) for the PT is the manual tilt tube technique.[6] In the present study, a manual hook technique has been used. The rationale for using a manual hook technique is the possibility of maintaining a constant and accurately measured reaction temperature during the test. The purpose of our study was to estimate the contribution of temperature deviations on PT measurement uncertainty and its effect on the uncertainty of INR.

2 | MATERIALS AND METHODS

The international standards for rabbit thromboplastin (coded RBT/16) and recombinant human thromboplastin (coded rTF/16) were obtained from the National Institute for Biological Standards and Control (Potters Bar, UK). Frozen pooled normal plasma and frozen pooled coumarin plasma were prepared as described previously.[7] Borosilicate glass test tubes, 12 × 75 mm, were obtained from Duran Wheaton Kimble (product nr 73500-1275). A glass tank (40 × 26 × 17 cm) filled with water was used as a thermostat bath. The temperature of the thermostat bath was regulated with a Heating Immersion Circulator model MB (Julabo Labortechnik GmbH) with a heater capacity of 2000 W and temperature stability of ±0.02°C. A calibrated mercury expansion thermometer was used to measure the temperature of the thermostat bath. The temperature was read to one decimal °C. Glass test tubes were placed in a rack in the thermostat bath for at least 4 minutes. Thromboplastin (0.2 mL) was pipetted into a test tube and incubated for 2 minutes. Next, plasma (0.1 mL) was pipetted into the test tube and a digital stopwatch (Traceable, VWR International) was started. Prothrombin times were determined manually with a stainless steel Kolle hook.[8] The hook was moved manually into and out of the reaction mixture two times per second. The reaction mixture was observed by the operator horizontally from the side through the wall of the tank. When the clot was pulled up with the hook, the end-point was timed with the digital stopwatch. Analytical imprecision was assessed as the coefficient of variation (CV) from four replicate PT determinations on normal and coumarin plasmas at each reaction temperature.

Prothrombin times were plotted versus reaction temperature. A quadratic regression line was calculated using SPSS version 25 (IBM). The slope of the line, i.e. the change of PT per degree centigrade, was calculated from the first derivative of the equation. PT ratios were calculated by dividing the PT of the coumarin plasma at each temperature by the mean PT of the normal plasma pool at the same temperature. PT ratios were transformed to "Apparent INR" using the established ISI values of 1.11 and 1.21 for rTF/16 and RBT/16, respectively.[9]

3 | RESULTS

The PT determined at various reaction temperatures are shown in Figure 1 −4. Analytical imprecision (CV) of the PTs determined with rTF/16 varied between 0.8% and 3.0% (median: 1.0%). Analytical imprecision (CV) of the PTs determined with RBT/16 varied between 0.9% and 5.0% (median: 1.4%). The PTs of the normal plasma pool determined with rTF/16 were decreasing with increasing temperature (Figure 1). The change of PT of this plasma at the target temperature of 37°C was −0.35 s/°C. The PTs of the coumarin plasma pool determined with rTF/16 showed a local minimum at 39.2°C (Figure 2). The change of PT of this plasma at the target temperature of 37°C was −0.34 s/°C.

The PTs of the normal plasma pool determined with RBT/16 showed a local minimum at 37.4°C (Figure 3). The change of PT of this plasma at the target temperature of 37°C was −0.05 s/°C. The PTs of the coumarin plasma pool determined with RBT/16 showed a local minimum at 33.8°C (Figure 4). The change of PT of this plasma at the target temperature of 37°C was 1.32 s/°C.

The PT of the coumarin plasma pool may be transformed to a PT ratio R by dividing by the PT of the normal plasma pool determined at the same temperature. We estimated R as a function of the reaction temperature. For rTF/16, the following equation was obtained: $R_{rTF/16} = 3.61 - 0.102\ t + 0.00197t^2$, where t is the reaction temperature in °C. At 37°C, the estimated change of the PT ratio per degree centigrade is 0.044. For RBT/16, the following equation was obtained: $R_{RBT/16} = 4.23 - 0.18\ t + 0.0034t^2$. At 37°C, the estimated change of the PT ratio per degree centigrade is 0.076. The ratios of PT were transformed to "Apparent INR" using the established ISI values of 1.11 and 1.21 for rTF/16 and RBT/16, respectively. The "Apparent INR" is equal to the true INR at 37°C only because the established ISI values have been determined at 37°C. At other temperatures, the "Apparent INR" is different from the true

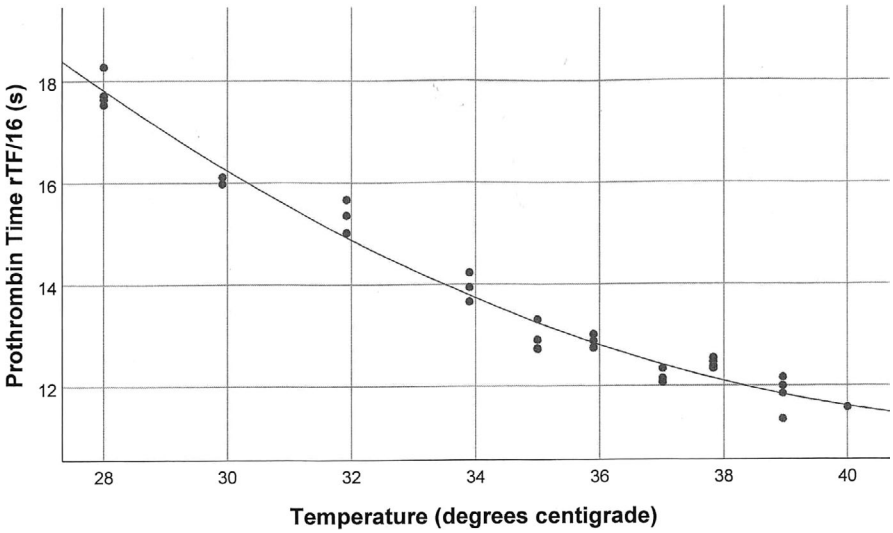

FIGURE 1 The PT of normal plasma pool determined with rTF/16 as a function of reaction temperature. The curve represents a regression line $y = 62.52 - 2.35x + 0.027x^2$, where y and x denote the PT in seconds and the reaction temperature in degrees centigrade, respectively

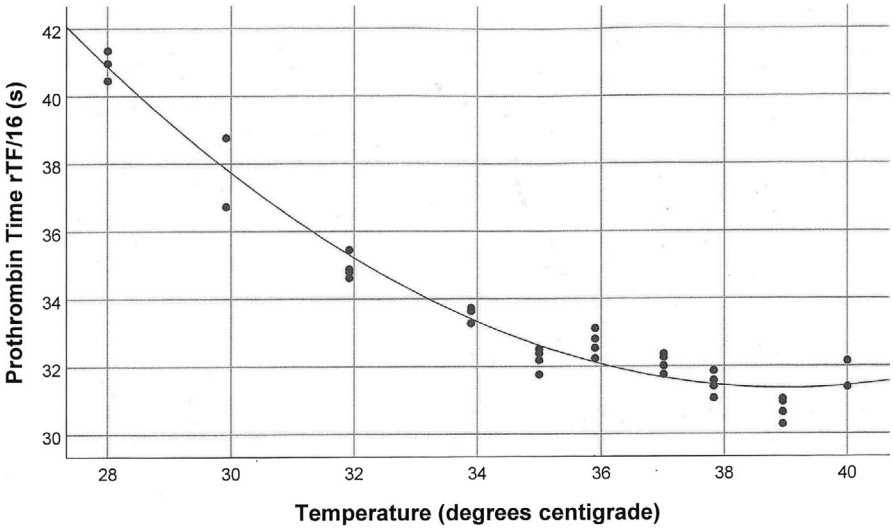

FIGURE 2 The PT of coumarin plasma pool determined with rTF/16 as a function of reaction temperature. The curve represents a regression line $y = 151 - 6.11x + 0.078x^2$, where y and x denote the PT in seconds and the reaction temperature in degrees centigrade, respectively

INR. The relationship between the "Apparent INR" and the temperature was: $INR_{rTF/16} = 4.05 - 0.12\ t + 0.00245t^2$ and $INR_{RBT/16} = 6.02 - 0.29\ t + 0.00544t^2$ for rTF/16 and RBT/16, respectively (Figure 5). At 37°C, the change of "Apparent INR" per degree centigrade was 0.06 and 0.11 for rTF/16 and RBT/16, respectively.

4 | DISCUSSION

The PT is the overall result of several sequential enzyme reactions. The effect of temperature on the velocity of enzyme reactions may be due to several different causes, eg an effect on the stability of the enzyme, an effect on the enzyme reaction itself or conformational changes.[10,11] The PT is influenced by several plasma coagulation factors, ie Factors I, II, V, VII, X and by the tissue factor. At present, it is not possible to derive a theoretical model describing the temperature dependence of a complex enzymatic system as the PT. We used an empirical model in which the temperature dependence was described by a quadratic equation. The quadratic equation appeared to fit the measured PT very well. The scatter of the measurements around the regression line may be due to the analytical imprecision of the manual technique and preanalytical instability of the plasma samples. We used the first derivative of the regression lines to estimate the change of the PT induced by the temperature change near the target temperature of 37°C. Using pooled normal plasma and RBT/16, we observed a minimum PT near 38°C (Figure 3). A minimum PT at a similar temperature was observed in previous studies with other commercial thromboplastin reagents prepared from rabbit tissues.[4,5] Using pooled coumarin plasma and RBT/16, we observed a minimum PT near 34°C (Figure 4). A minimum PT near 34°C was also observed by Uldall using human brain thromboplastin.[4] The temperature dependence of the PT determined with recombinant human thromboplastin was different (Figures 1 and 2). The difference in temperature dependence must be due to the different chemical compositions of RBT/16 and rTF/16. The observed minimum in the PT-temperature plot may be the result of two opposing effects: by increasing the temperature the enzymatic reactions are accelerated resulting in shorter clotting times, but further increase in the temperature may affect the thermal stability of the coagulation factors resulting in longer clotting times. Further studies are needed

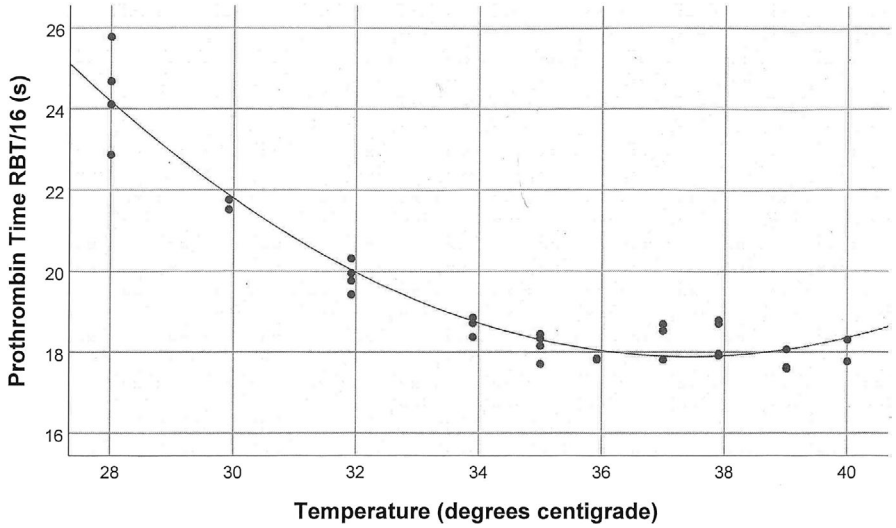

FIGURE 3 The PT of normal plasma pool determined with RBT/16 as a function of reaction temperature. The curve represents a regression line $y = 117 - 5.3x + 0.071x^2$, where y and x denote the PT in seconds and the reaction temperature in degrees centigrade, respectively

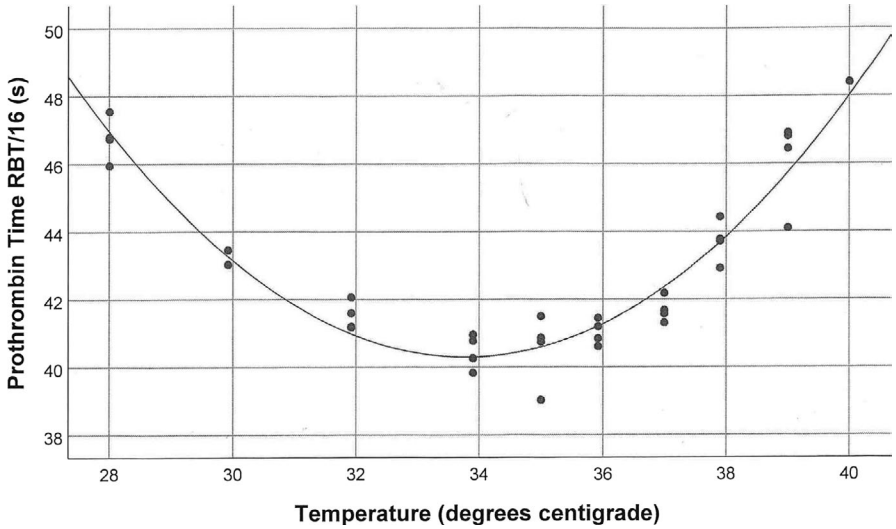

FIGURE 4 The PT of coumarin plasma pool determined with RBT/16 as a function of reaction temperature. The curve represents a regression line $y = 268 - 13.48x + 0.20x^2$, where y and x denote the PT in seconds and the reaction temperature in degrees centigrade, respectively

to determine the heat stability of the various components of the thromboplastin reagents as well as the substrate plasmas.

We used one pooled plasma obtained from healthy adults and another one from patients treated with VKA (coumarin plasma) with an INR of approximately 2.8 to estimate the change of the PT ratio and the "Apparent INR" (INR_A) with the reaction temperature. The use of pooled plasmas is justifiable because the previous studies have shown that pooled plasmas may replace individual plasmas for ISI determination.[7,12] The change of INR_A per degree centigrade increased with the increasing temperature. The relationship between INR_A and reaction temperature could be described by a quadratic equation. At 37°C, ie the recommended target reaction temperature for the test, the change of the INR_A with rTF/16 and RBT/16 was 0.06/°C and 0.11/°C, respectively. Therefore, it seems that rTF/16 is less responsive to temperature change than RBT/16. For estimating the INR_A change with temperature, we used a fixed ISI value independent of the temperature. It is obvious that an operator not being aware of performing the PT test at a reaction temperature (slightly) different from the target temperature of 37°C, will use the established ISI for the calculation of the INR. On the contrary, one could argue that the INR, as a measure of a patient's anticoagulation intensity, should be independent of the reaction temperature. Theoretically, temperature-independent INR could be achieved using temperature-dependent ISI and MNPT values. However, we do not recommend that an operator performing the PT test will adjust the ISI of the international standard thromboplastin for the actual reaction temperature. We do recommend that the operator shall adjust the temperature of the thermostat as closely to 37°C as possible, using a calibrated and certified thermometer. The temperature scale of many (digital) laboratory thermometers is in °C with one decimal. The temperature of the water bath can be easily adjusted to values between 36.9 and 37.1°C. The temperature stability of our water bath was ±0.02°C (see Materials and Methods). Our results demonstrate that the PT and INR are not significantly influenced by temperature fluctuations of ±0.02°C.

The International Standards for thromboplastins should be used with a harmonized manual tilt tube technique (MTT) at a thermostat temperature of 37°C.[6] In the present study, we did not use the manual tilt tube technique but the manual hook technique. With the hook technique, the reaction temperature is equal to that of

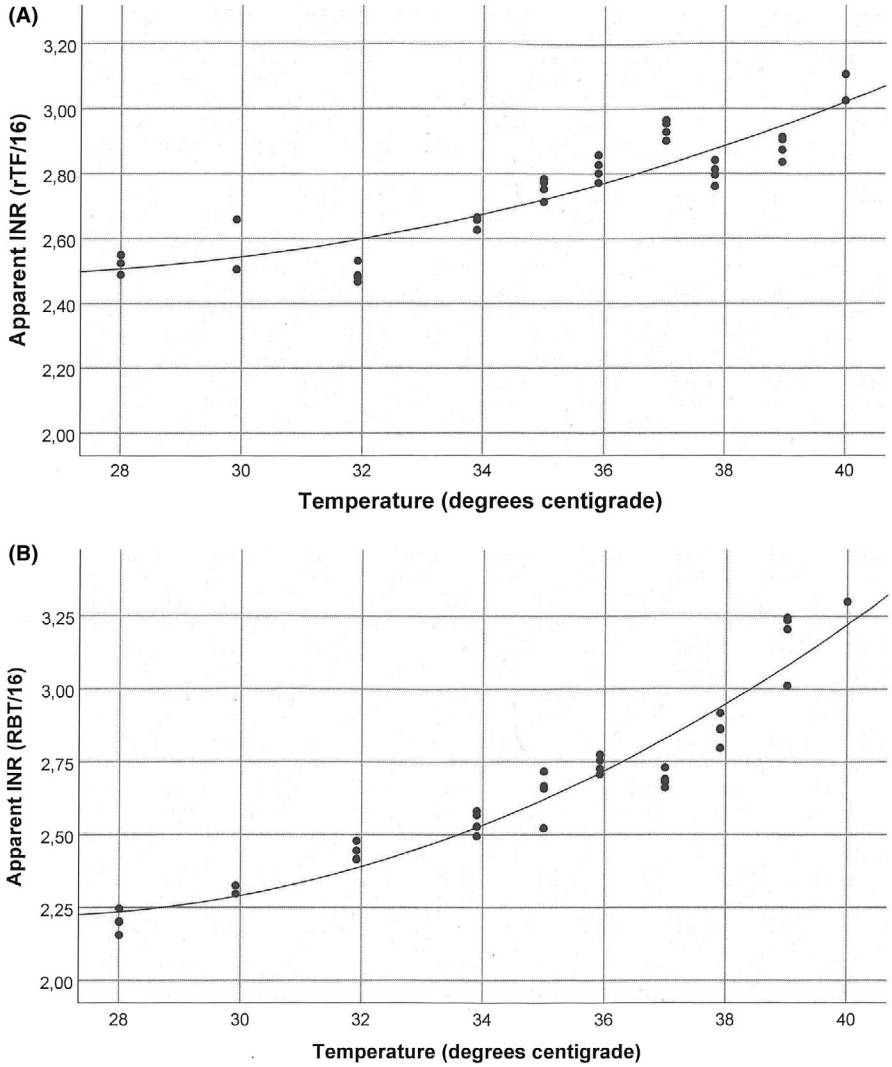

FIGURE 5 Apparent INR of coumarin plasma pool as a function of reaction temperature. Panel A, The curve represents a regression line $y = 4.05 - 0.12x + 0.00245x^2$, where y and x denote the apparent INR determined with rTF/16 and the reaction temperature in degrees centigrade, respectively. Panel B, The curve represents a regression line $y = 6.02 - 0.29x + 0.00544x^2$, where y and x denote the apparent INR determined with RBT/16 and the reaction temperature in degrees centigrade, respectively

the thermostat bath. Using the manual tilt tube technique, the reaction temperature is not equal to the thermostat but slightly lower because the test tube is regularly taken out of the bath at a higher temperature than the surroundings. In the manual tilt tube technique, the average temperature of the reaction mixture in the test tube during tilting is approximately 0.4°C lower when the thermostat temperature is 37°C and the room temperature is 21°C.[6] The fluctuation of the temperature of the reaction mixture during the tilting cycle is not known. If all operators use the same harmonized MTT, the average temperature of the reaction mixture will not be 37°C, but approximately 36.6°C. It is essential that all operators use the same harmonized MTT, so that the average reaction temperature is the same even though it is not equal to the thermostat temperature of 37°C. We calculated the INR difference between 37 and 36.5°C for the pooled coumarin plasma using the respective quadratic equations for rTF/16 and RBT/16:0.03 INR and 0.055 INR, respectively. These are no large differences, but one may wonder whether they are acceptable or not. According to WHO and ISO guidelines, the overall INR bias at a target INR of 3.0 should be ±0.3 or less, for a commercial PT reagent or a commercial point-of-care PT-INR device in the hands of the end-users.[3,13] In the present study, we are dealing with the international standards which are to be used with RMP, and the allowable INR bias should be a fraction of the total allowable INR bias for a commercial PT reagent. It should be realized that the INR bias associated with an international standard is not only due to the temperature effects but also due to the operators' variation in visual end-point detection.[6] For example, when the harmonized manual tilt-tube technique with rTF/16 was used by seven different operators, the INR of a common control plasma varied between 2.95 and 3.13.[6] These results suggest that the effect of minor deviations (ie <0.5°C) from the target reaction temperature as observed in the present study is small compared to the between-operator differences. To limit the measurement uncertainty as much as possible, we recommend adjusting the temperature of the water bath to 37.0 ± 0.1°C. This can be easily achieved using modern thermo-regulators and certified laboratory thermometers.

We conclude that the PT and INR determined with the current international standards rTF/16 and RBT/16 are influenced by the reaction temperature and that the temperature of the thermostat bath

shall be maintained at a temperature of 37.0 ± 0.1°C, and shall be measured with a calibrated and certified thermometer.

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CONFLICT OF INTEREST

The authors have no competing interests.

ORCID

Antonius M. H. P. Van Den Besselaar https://orcid.org/0000-0002-0794-4982

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