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Citation for published version (APA):

Document license:
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DOI:
10.1016/j.jmbbm.2018.12.026

Document status and date:
Published: 01/04/2019

Document Version:
Publisher’s PDF, also known as Version of Record (includes final page, issue and volume numbers)

Please check the document version of this publication:

• A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher’s website.
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• The final published version features the final layout of the paper including the volume, issue and page numbers.

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Myoglobin and troponin concentrations are increased in early stage deep tissue injury

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\section*{1. Introduction}

A pressure ulcer (PU) represents a local degeneration of soft tissues induced by sustained mechanical loading, usually occurring in the vicinity of bony prominences (NPUAP, 2014). The literature describes two types of PUs, those that start superficially and extend inwards and those which start in the deeper layers and gradually progress to the skin surface. It is currently accepted that both mechanisms may occur, depending on the location and the modality of the applied loading (Ankrom et al., 2005; Sibbald et al., 2011). Deep ulcers originating subcutaneously can involve fat, muscle, tendon, and bone. They are classified as deep tissue injury (DTI) if they are detected while the skin is still intact, a situation in which the anatomical depth of the wound is extremely difficult to determine (NPUAP, 2014).

Detection of DTI in an early stage is difficult, due to its concealment by the skin. It is often misdiagnosed as conditions presenting with similar symptoms (Black et al., 2016). DTI represent a small number of the total number of pressure ulcers (VanGilder et al., 2010). However, in groups at high risk of developing DTI prevalence level are high. A study by Scheel-Sailer et al. reported a total PU prevalence of 49.2% for SCI individuals in a 6 month study of which 34.5% were classified as deep PUs (Scheel-Sailer et al., 2013). These ulcers have a tremendous impact on an individual’s health and quality of life (Spilsbury et al., 2007) and often lead to extensive periods of being bedridden (Verschueren et al., 2011).

To identify individuals at risk of developing PUs several factors have been identified, including: activity and mobility limitations; skin status, including moisture; perfusion and oxygenation; nutritional status; body temperature; age; sensory perception; haematological factors; and general health (NPUAP, 2014). Risk assessment is based on clinical experience often involving risk assessment tools, such as the Braden, Norton, or Waterlow Scales (Moore and Cowman, 2014; NPUAP, 2014; Šáteková et al., 2017). Preventative measures, e.g. repositioning and specialized support surfaces, are prescribed to those considered to be at high risk (Groah et al., 2015; Mcinnes et al., 2015). Even with appropriate risk assessment and preventative measures the consensus among experts is that some PUs are unavoidable (Black et al., 2011). Therefore there will always be a need to identify PUs at an early stage. Incorporating a preferably non-invasive detection method, would clearly represent an important development.

To design an early diagnostic method, knowledge about DTIs aetiological processes is important. Mechanical loading induces pressure ulcer formation via several mechanisms occurring at different time scales. Low deformations can cause occlusion of blood and lymph...
vessels leading to a decrease in oxygen and nutrients and the associated accumulation of waste products which eventually leads to tissue damage. This process can take up to several hours, whereas high deformations can cause damage within minutes affecting the integrity of the cell membrane and the disruption of the cytoskeleton (Oomens et al., 2015). Early studies on PU development have shown that muscle tissue is more sensitive to mechanical loading than skin (Daniel et al., 1981; Nola and Vintness, 1980; Salcido et al., 1980). Several studies have shown an increase in biomarker release after muscle damage (Dahlqvist et al., 2014; Giannoglou et al., 2007; Hagisawa et al., 1988; Ide et al., 1999; Sari et al., 2008; Sorichter et al., 1997, 1998). Therefore, we hypothesize that muscle damage markers can indicate the development of DTI at an early stage.

Clinically used markers for muscle damage are myoglobin (Mb), troponin (Tn), and creatine kinase (CK). Myoglobin, an oxygen carrying protein specific to human muscle tissue, is released within 1–6 h after muscle fibre damage from the sarcoplasm (Dahlqvist et al., 2014; Giannoglou et al., 2007; Hagisawa et al., 1988; Ide et al., 1999; Sari et al., 2008; Sorichter et al., 1997, 1998). Troponin is a complex of three small proteins (troponin I, C and T), which inhibits muscle contraction. It has different isoforms for cardiac, slow skeletal and fast skeletal muscle, enabling a differentiation between cardiac and skeletal muscle damage. As an example, troponin was shown to increase within 4 h after eccentric exercise, with elevations persisting for 2 days (Dahlqvist et al., 2014; Sorichter et al., 1997). Creatine kinase, which is present in tissues with a high metabolic demand like muscle and brain, catalyses the reversible reaction of creatine and ATP to phosphocreatine and ADP. There are relatively few studies (Hagisawa et al., 1988; Sari et al., 2008) which have measured muscle biomarkers in the early detection of DTI. These animal models revealed an increase in creatine kinase in blood at the first measurement after muscle indentation. However, since the mid-1990s the diagnostic value of creatine kinase has been debated and troponin has been proposed as the new standard (Baird et al., 2012). Therefore, this study focuses on detection of myoglobin and troponin.

To test the hypothesis that muscle damage biomarkers can be indicative of the development of deep tissue injury after sustained mechanical loading, an indentation test was performed on the tibialis anterior muscle of rats for 2 h. It should be clear that in the present setup that we are not able to separate the different damage pathways reported in literature. So we cannot distinguish between oxidative stress, ischaemic damage and deformation-induced damage. Indeed this does not represent the goal of the present research, which is designed to detect signs of damage in tissues as a first step in early detection and accept that a change requires further examination of its causation. However in this particular rat model we know from previous studies that ischemia of the lower leg does not induce damage development within a 2 h timeframe (Stekelenburg et al., 2007). Myoglobin and troponin were analysed in blood plasma and urine over a period of 5 days. The damage as detected by the release of biomarkers was compared to damage as observed with MRI to validate the response.

2. Materials and methods

2.1. Animal model

Female Sprague-Dawley rats (n = 25, 14 weeks old) were obtained from Charles River, Paris, France. These animals were part of a much larger study on DTI damage development in rats (Nelissen et al., 2018). During the acclimatization period (minimal 7 days) animals were socially housed under controlled laboratory conditions (12 h light/dark cycles) with standard food and water provided ad libitum. During the experiments the animals were individually placed on metabolic cages and weighed daily. Animals were divided in two groups, the indentation group (n = 19) and the sham group (n = 6). All experiments were approved by the Animal Care and Use Committee of Maastricht University, The Netherlands (protocol 2013–047) and performed in accordance with the European Union Directive for animal experimentation (2010/63/EU).

2.2. Blood and urine sampling

Blood samples were collected under anaesthesia, which was induced with isoflurane in 0.6 L/min medicinal air, 3–4 vol% for induction and 1–2 vol% for maintenance. The tail of the animal was carefully warmed using a hot pack to facilitate blood sampling. Blood samples (200 μl) were collected from the tail vein using a 27 G insulin syringe to minimize blood loss (Myjector, Terumo). Blood was centrifuged in K3-EDTA tubes (MiniCollect, Greiner Bio-One) for 10 min at 1600 RCF at 4°C. Chilled 24 h urine samples were collected by placing the rats individually in a metabolic cage with a collection chiller (Tecniplast, Italy). Urine was centrifuged for 10 min at 1600 RCF at 4°C. Plasma and urine samples were aliquoted and stored at −80°C until analysis.

2.3. Experimental protocol

The experimental setup and protocol have been detailed elsewhere (Nelissen et al., 2018). Briefly, anaesthesia as described above was maintained for 6 h to allow for the MR measurements. Analgesia was injected subcutaneously (Buprenorphine, 0.05 mg/kg). Ointment was applied to avoid dehydration of the eyes. The animal was placed supine on a heating pad to maintain body temperature within the physiological range (35–37 °C), which was monitored by means of a rectal probe. The hairs of the right hind limb were shaved before the leg was placed in a holder and fixed with alginate. The alginate was moulded with an opening to allow indentation of the TA. An alginate cap was applied before and after indentation to close the opening for susceptibility matching. The cylindrical indenter has a spherical head with a diameter of 3 mm. The indentation group was subjected to manual applied indentation for two hours. Blood samples were obtained before indentation and 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 1d, 2d, 3d, 4d and 5d after removal of the indentation. Urine samples were collected before, 1d, 2d, 3d, 4d, and 5d after indentation. MR scans were obtained before, during, and 90 min after indentation. The animals were sacrificed under anaesthesia, with analgesia administered, by exsanguination from the inferior vena cava.

2.4. MR measurements

A 7.0 T small animal MRI scanner combined with an 86-mm inner diameter quadrature transmit coil and a 20-mm diameter surface receive coil was used to obtain MR scans (Bruker Biospin MRI GmbH, Ettlingen, Germany). Physiological changes were assessed with T2-weighted MRI with the following specifications: multi-slice-multi-echo with fat suppression, Field of view (FOV) 25 × 25 × 20 mm3, acquisition matrix (MTX) 256 × 256, number of slices 20, slice thickness 1 mm, TE 6.9–181.7 ms, number of echoes 26, TR 4000 ms.

Quantitative T2-maps were obtained by fitting the MR signal (S) of the first 16 echoes per voxel to Eq. (1).

\[
S = S_0 e^{-\frac{TE}{\sigma_T}}
\]  

(1)

A region of interest (ROI) of the TA was manually drawn on the slices of the T2-images. Voxels were included for analysis when the coefficient of determination (R2) > 0.9. When > 90% of the ROI fulfilled this criterion the slice was accepted for analysis. Animals were excluded from further analysis if less than 8 slices in the region of indentation were accepted for analysis.

Reference characteristics were obtained for each animal by calculating per slice the mean (μTE) and standard deviation (σTE) of the T2-values of the voxels inside the ROI of the scan made before indentation. Areas were considered to demonstrate elevated T2-values after indentation when at least 3 neighbouring voxels satisfied the following
\[ T_2 > \mu_{T2} + 3\sigma_{T2} \]  

2.5. Biochemical analysis

Myoglobin and troponin concentrations were measured in blood (Mb\(_b\) and Tn\(_b\)) and urine using sandwich ELISA (MYO-2, STNC, Life Diagnostics, Inc., West Chester, PA). A combined analysis of myoglobin and troponin, with sufficient dilutions, required > 80 μl of blood plasma for the indentation group and this prevented simultaneous analysis of the markers. This resulted in a total of 13 animals being analysed for myoglobin and 6 for troponin. Blood and urine were analysed in duplicate and triplicate, respectively. If an insufficient volume of plasma was obtained samples were run without replicates. If the coefficient of variation of the triplicates > 20% the outlier was removed for further analysis. The mean absorbance of the blank was subtracted from the mean absorbance calculated per samples. To determine the standard curve a linear model was used, with a fit accepted when \( R^2 > 0.985 \). The limit of detection (LOD) was defined as:

\[ \text{LOD} = \mu_B + 3\sigma_B \]

with \( \mu_B \) the mean and \( \sigma_B \) the standard deviation of the blank. Values below LOD were set to 0 in subsequent analysis. The total amount of myoglobin excreted in urine (Mb\(_u\)) was calculated by multiplying the estimated concentration with the total urinary volume of that sample.

2.6. Determination of kinetic parameters

To evaluate the amplitude of the response to indentation the maximum observed concentration in blood (C\(_{b_{\text{max}}}\)) and excretion in urine (M\(_{b_{\text{max}}}\)) were evaluated. The time-point at which C\(_{b_{\text{max}}}\) was reached could differ between animals. To also account for the possible prolonged release of the biomarkers, the area under the curve (AUC) was calculated using the trapezoidal method as defined in Eqs. 4 and 5 for blood urine samples, respectively:

\[ \text{AUC}_{\text{blood}}(\text{ng/hr/ml}) = \sum_i (C_{i+1} + C_i)(t_{i+1} - t_i) / 2 \]  

\[ \text{AUC}_{\text{urine}}(\text{ng/hr}) = \sum_i (m_{i+1} + m_i)(t_{i+1} - t_i) / 2 \]

with \( i \) the time point after indentation, \( C_i \) the concentration of the biomarkers at time point \( i \), \( m_i \) the weight of the excreted amount of protein at time point \( i \), and \( t_i \) time point in hours. Linear interpolation was used in case of missing samples. If multiple consecutive samples were missing the AUC was not calculated.

2.7. Statistical analysis

Statistical analysis was performed with the R environment for statistical computing and graphics (R Version 3.5.0, The R Foundation for Statistical Computing, Vienna, Austria). One-tailed Mann Whitney U tests were performed for AUC, C\(_b_{\text{max}}\) and M\(_b_{\text{max}}\) to assess if there was an increase in biomarkers due to indentation. The relationship between the two damage responses, as measured by elevated T\(_2\) values and biomarkers, was evaluated using Spearman’s correlation coefficient (r\(_s\)) using the one-tailed Spearman test. P-values below 0.05 were considered statistically significant.

3. Results

MRI T\(_2\)-maps were used to assess muscle damage, oedema, and inflammation non-invasively. Fig. 1 presents T\(_2\)-maps before, during, and 90 min after indentation of three animals with different volumes of elevated T\(_2\) ranging from low to high (top-bottom respectively). Before indentation a homogenous area of T\(_2\)-values was evident in the TA of each animal (Fig. 1, A-D-G). During indentation the position of the indenter was clearly visible (Fig. 1, B-E-H, marked with an *). After indentation the region of elevated T\(_2\) resembled an epi-, peri-mysium-like structure (Fig. 1, C-F-I). In some animals, oedema formed underneath the skin (Fig. 1I). Fig. 2, which depicts the volume of elevated T\(_2\), clearly reveals a considerable variation in response to indentation across the experimental animals.

The temporal profile of the biomarkers following indentation also revealed considerable variability (Figs. 3–4). Mb\(_b\) increased in concentration immediately after the load was removed with a maximum elevation evident at 2 h and a return to baseline levels after 24 h (Fig. 3C). The increase of Tn\(_b\) was delayed compared to Mb\(_b\), starting at 2 h with a maximum generally after 4 h and a return to baseline after 24 h (Fig. 3A). Excretion of myoglobin in urine (Mb\(_u\)) was highest in the first 24 h after load removal (Fig. 3A). Troponin was undetectable in urine.

The sham group revealed smaller increases in both markers compared to the indentation group. Both Mb\(_b\) and Tn\(_b\) revealed a delay in this increase compared to the indentation group (Fig. 3B,D). Mb\(_b\) started to increase 1 h after the time point equivalent to the load removal in the indentation group, with its peak occurring between 2 and 8 h. Tn\(_b\) started to increase 4 h after load removal with its peak occurring between 4 and 24 h. Mb\(_b\) exhibited a small increase at 24 h after load removal (Fig. 3B).

Fig. 5 indicates the individual values for C\(_b_{\text{max}}\) and AUC for both groups of animals. The trends for troponin were fairly similar (Fig. 5A–B). By contrast, myoglobin exhibited peaks in the AUC, e.g. animal #12, which were not present for the C\(_b_{\text{max}}\). (Fig. 5C–D) However, in urine the trends of C\(_b_{\text{max}}\) and AUC for myoglobin were similar (Fig. 6). When examining the effects of indentation on the kinetic parameters, group comparisons revealed statistically significant increases in C\(_b_{\text{max}}\) for Tn\(_b\) (U = 5, p = 0.02) and Mb\(_b\) (U = 14, p = 0.014), but not for Mb\(_u\) (U = 23, p = 0.09). The differences in AUC were statistically significant for Tn\(_b\) (U = 6, p = 0.03) and Mb\(_b\) (U = 14, p = 0.02), but not with respect to Mb\(_u\) (U = 27, p = 0.16).

To evaluate whether the markers could ultimately provide an early detection of DTL, the correlation between increased T\(_2\) volume and the kinetic parameters of the biomarkers was assessed. C\(_b_{\text{max}}\) and Mb\(_b\) provided similar results as AUC (AUC data not shown). Two trends were present in the troponin levels in blood (Fig. 7A). Five animals demonstrated similar elevated T\(_2\) volume associated with a considerable range of troponin levels, whereas one animal (#2) did not exhibit any damage. In the myoglobin group the damage response was more variable (Fig. 7B–C). At low volumes of elevated T\(_2\) values small increases in myoglobin levels were evident. Myoglobin levels were elevated in concert with the volume of elevated T\(_2\)-values. This effect was most pronounced in urinary myoglobin (Fig. 7C). There was a positive correlation for all kinetic parameters with the volume of elevated T\(_2\). Tn\(_b\) had a strong and significant correlation for both the AUC and C\(_b_{\text{max}}\) (r\(_s\) = 0.94, p = 0.008). Mb\(_b\) revealed a strong and significant correlation for C\(_b_{\text{max}}\) (r\(_s\) = 0.75, p = 0.006) and a reasonable correlation, which was significant for AUC (r\(_s\) = 0.57, p = 0.04). In addition, Mb\(_u\) demonstrated a strong and significant correlation for both AUC and M\(_b_{\text{max}}\) (r\(_s\) = 0.91, p < 0.001 and r\(_s\) = 0.96, p < 0.001 respectively).

4. Discussion

The present study examined the potential of two muscle biomarkers for the early detection of deep tissue injury in a rat model. An early stage pressure ulcer was induced by performing an indentation test on the TA of the individual rat. The resulting damage development was assessed with MRI and correlated to the biomarker concentrations in blood and urine. Both myoglobin and troponin were released in blood as a result of indentation. Elevated myoglobin levels were also observed in urine. Furthermore, a strong positive correlation was demonstrated...
between the biomarkers and damage parameter associated with MRI.

Previous studies by the host group reported a large variation in the damage response of the animals in response to mechanical loading (Bosboom et al., 2003; Loerakker et al., 2010). This is mainly caused by biological variation leading to differences in geometry of the leg and, as a result, different deformations at a specific indentation. Indeed, MRI data in the present study support these findings (Fig. 2) with, for example, increase in T2 values ranging from as low as 0.3% of the TA volume, to a severe response affecting 79.2% of the TA volume. Clearly, it is not feasible to apply an equivalent magnitude of indentation in experiments with different animals. Due to this variability the total range of damage responses is presented in this study.

This study showed that in the case of a damage response, whereas myoglobin increases rapidly after load removal, troponin exhibited a more a delayed response (Figs. 3–4). These trends are similar to previous studies on skeletal muscle damage in rat models (Kerkweg et al., 2010; Tonomura et al., 2012; Vassallo et al., 2009). For example, Kerkweg et al. reported that following a crush test there was an increase in myoglobin levels in blood after 30 min which remained elevated throughout the 4 h experiment (Kerkweg et al., 2010). Other studies induced skeletal muscle damage by the intraperitoneal injection of myotoxins. Vasallo et al. reported an increase in both urinary myoglobin and skeletal troponin levels 24 h after the last injection (Vassallo et al., 2009). Tonomaru et al. used a similar approach and revealed an increase in skeletal troponin levels at the earlier time points of 4–8 h (Tonomura et al., 2012). The sham group showed a small increase in myoglobin and troponin compared to the indentation group (Figs. 3–4), which is comparable to a previous study (Kerkweg et al., 2010). This release is unlikely caused by the anaesthetic. Isoflurane has been shown to have a beneficial effect on myocardial tissue in rabbits (Kitagawa et al., 2008). However, the prolonged anaesthetic time of 6 h, might induce some deep or superficial tissue injury. Indeed time in an operating room is a well-known risk factor for developing surgically acquired PU (Primiano et al., 2011).

Careful consideration needs to be made when comparing the two groups. First of all some animals in the indentation group did not develop tissue damage due to indentation (Figs. 2 and 7). It is already well established that, because of individual differences between animals, it is impossible to predict beforehand how much tissue deformation will occur. This is exactly the reason why we have started to create animal specific models to retrospectively determine the strain field. This means that in the loaded group it is possible that some animals did not experience deformation to induce damage. By directly comparing the elevated T2 volume with excreted myoglobin and troponin, as seen in Fig. 7, this variability does not represent a major issue, although it will of course affect the interpretation of Figs. 3 and 4 considerably. Secondly, reducing the overall profile to single kinetic parameters will inevitably reduce the amount of temporal information. As an example, sham animal 25 exhibits a transient spike of troponin at 15 min, which is not seen in any other animal. For the sham group this results in a higher variance of the Cmax data as shown in Fig. 5A.

The small sample size precluded a direct comparison of the
indentation group to the sham at all time points. Accordingly two kinetic parameters were used to evaluate biomarker performance (Figs. 5–6), the first of which focused on the amplitude of the response of the biomarkers regardless of the time it occurred. The second parameter, AUC, involved associated time effects, and enabled smaller increases over a longer period to be detected. In blood, the AUC and \(C_{t_{\text{max}}}\) of troponin gave similar results in contrast to myoglobin. This indicates that in order to evaluate myoglobin in blood a comprehensive kinetic profile would be required. This is undesirable in clinical practice because of the increased burden of multiple blood withdrawals from the patient. In a similar manner, the limited period of increased \(C_{t_{\text{max}}}\) levels for troponin would also necessitate multiple blood withdrawals within 24 h. By contrast, myoglobin measured in urine does not involve the issue of multiple invasive measurements. Furthermore, the 24 h collection period results in a cumulative assessment, ensuring that any notable increases in myoglobin levels are not missed due to low sampling frequency. Taking into account the focus group of this study, the spinal cord injured who generally collect urine via a catheter bag, it is tempting to suggest that for practical purposes the biomarker of choice is myoglobin measured in urine.

There are clear differences between the response of the two biomarkers when correlated to the increase in \(T_2\) (Fig. 7). Myoglobin increases gradually, whereas troponin, albeit with a few animals presenting with a limited damage response, exhibited a more stepwise response. This response may be attributed to the nature of the proteins. Myoglobin is a cytoplasmic protein and can be released easier from muscle fibres than troponin, which is a structural protein with a low cytoplasmic pool (Sorichter et al., 1997). We therefore hypothesize that increases in troponin levels coincide with structural damage. A previous study demonstrated that increased \(T_2\) values correlate to signs of muscle

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**Fig. 3.** Kinetic profiles for individual animals for troponin (A-B) and myoglobin (C-D) in blood plasma. Left column; indentation group (A-C) right column; sham group (B-D).

**Fig. 4.** Excreted amount of myoglobin in urine for individual animals in the A) indentation group; B) sham group.
damage on histology over 14 day period (Nelissen et al., 2018). Indeed the animals that initially demonstrated an increase in troponin levels still reveal a marked increase in $T_2$ value in 19–60% of the volume of the leg (data not shown).

ROI was restricted to 8 slices a limited volume of the muscle tissue was evaluated in the $T_2$-analysis. The $T_2$-scans revealed that muscle damage could extend to an area outside the ROI volume. Therefore it is likely that myoglobin/troponin is released by a larger volume of the muscle tissue than included in the $T_2$-analysis.

The current study presented with some challenges and limitations.
There were several practical issues related to the blood samples. The first blood samples that were collected after induration were difficult to obtain due to an anaesthesia time of approximately 4 h. Indeed isoflurane causes a decrease in blood pressure (Bencze et al., 2013) and, regardless of the controlled core temperature, the tail of the animal cooled down. In 6 animals these issues could not be overcome within 15 min, precluding the collection of these first blood samples. When these issues were overcome, the time to collect a blood sample was prolonged, increasing the risk on haemolysis and blood clotting within the syringe. Premature termination of blood collection occurred 4 times, which resulted in an insufficient blood volume for duplicate analysis.

The present findings indicate that myoglobin is a suitable marker for early detection of deep tissue injury. To evaluate the predictive validity of the muscle damage markers in clinical practice prospective studies should be performed. Muscle damage markers can be incorporated in a non-invasive monitoring method for the early detection of deep tissue injury. Such a method will aid in identifying individuals at risk and help to prevent deterioration of the developing wound, thereby minimizing the associated negative effects.

Acknowledgements

This work was supported by the Dutch Technology Foundation (STW) under grant 12398. STW is part of the Netherlands Organisation for Scientific Research (NWO) and is partly funded by the Ministry of Economic Affairs. The authors gratefully acknowledge David Verraart, Leonie Niessen, Carlijn Tijsen-van Helvert, Roy Lucassen and Marije Janssen for their biotechnical assistance.

Declarations of interest

None.

References


