Changes in myonuclear domain size do not precede muscle hypertrophy during prolonged resistance-type exercise training

T. Snijders,1 J. S. J. Smeets,1 J. van Kranenburg,1 A. K. Kies,2 L. J. C. van Loon1 and L. B. Verdijk1

1 NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University, Maastricht, the Netherlands
2 DSM Biotechnology Center, Applied Biochemistry Department, Delft, the Netherlands

Abstract

Aim: Muscle fibre hypertrophy is accompanied by an increase in myonuclear number, an increase in myonuclear domain size or both. It has been suggested that increases in myonuclear domain size precede myonuclear accretion and subsequent muscle fibre hypertrophy during prolonged exercise training. In this study, we assessed the changes in muscle fibre size, myonuclear and satellite cell content throughout 12 weeks of resistance-type exercise training in young men.

Methods: Twenty-two young men (23 ± 1 year) were assigned to a progressive, 12-weeks resistance-type exercise training programme (3 sessions per week). Muscle biopsies from the vastus lateralis muscle were taken before and after 2, 4, 8 and 12 weeks of exercise training. Muscle fibre size, myonuclear content, myonuclear domain size and satellite cell content were assessed by immunohistochemistry.

Results: Type I and type II muscle fibre size increased gradually throughout the 12 weeks of training (type I: 18 ± 5%, type II: 41 ± 6%, P < 0.01). Myonuclear content increased significantly over time in both the type I (P < 0.01) and type II (P < 0.001) muscle fibres. No changes in type I and type II myonuclear domain size were observed at any time point throughout the intervention. Satellite cell content increased significantly over time in both type I and type II muscle fibres (P < 0.001).

Conclusion: Increases in myonuclear domain size do not appear to drive myonuclear accretion and muscle fibre hypertrophy during prolonged resistance-type exercise training in vivo in humans.

Keywords ceiling theory, hypertrophy, myonuclei, satellite cell.
quiescent state to replenish the resident pool of satellite cells.

Whether myonuclear accretion is an obligatory step in the process of muscle fibre hypertrophy is a highly debated topic. Early γ-irradiation studies have shown that muscle fibre growth in mice is virtually non-existent when satellite cells are ablated (Rosenblatt & Parry 1992, 1993, Rosenblatt et al. 1994). However, it was later argued that the cellular specificity of γ-irradiation was too low to truly assess the absolute requirement of satellite cells in overload induced muscle fibre hypertrophy. In response, McCarthy et al. (2011) used a mouse model to conditionally and specifically ablate Pax7+ cells and reported that muscle fibre hypertrophy was similar during 2 weeks of overload when compared with wild-type mice. However, Fry et al. (2014) observed that exposing these genetically modified mice to more prolonged overload attenuated muscle hypertrophy. Although these animal studies address an important question in the context of muscle biology, they do not establish that these principles are relevant under physiological conditions in humans, like during exercise-induced muscle fibre hypertrophy. In human studies, discrepant findings have been reported. Whereas some studies (Kadi & Thornell 2000, Olsen et al. 2006, Petrella et al. 2006, Leenders et al. 2013, Bellamy et al. 2014) have shown that muscle fibre hypertrophy is accompanied by a substantial rise in myonuclear content, others failed to detect myonuclear accretion with resistance-type exercise training-induced muscle fibre growth (Kadi et al. 2004, Petrella et al. 2006, Mackey et al. 2007, Verney et al. 2008, Verdyck et al. 2009). To explain this discrepancy it has been hypothesized that in a physiological situation, such as in response to prolonged exercise training, two distinct phases of muscle fibre hypertrophy may exist (Kadi et al. 2004, Petrella et al. 2006, 2008). This ‘two-phase model’ proposes that in response to exercise training, muscle fibre hypertrophy is initially supported by an increase in myonuclear domain size. However, it is thought that the existing myonuclei can only support the underlying increase in transcriptional activity to a certain extent (Kadi et al. 2004, Petrella et al. 2008). Subsequently, the incorporation of new myonuclei may be required to allow more extensive muscle fibre growth. In other words, an initial (temporary) increase in myonuclear domain size has been hypothesized to be an important driving force for subsequent myonuclear accretion in response to prolonged resistance-type exercise training. However, there is only limited data on the time-dependent changes in myonuclear domain size and myonuclear/satellite cell content during prolonged resistance-type exercise training-induced muscle fibre hypertrophy in humans. In this study, we test the hypothesis that changes in myonuclear domain size precede myonuclear accretion and subsequent muscle fibre hypertrophy during prolonged resistance-type exercise training. Therefore, we assessed type I and type II muscle fibre size, myonuclear content, myonuclear domain size and satellite cell content in muscle biopsies taken before and after 2, 4, 8 and 12 weeks of resistance-type exercise training in healthy young men.

Methods

Subjects

Twenty-two healthy young men were recruited to participate in a 12-weeks resistance-type exercise intervention programme. Participants were included in an age range of 18–30 years. During an initial screening visit, medical history was evaluated, and a blood sample was taken to assess blood HbA1c and fasting plasma glucose levels. Participants were excluded when HbA1C levels exceeded 6.5% or fasted plasma glucose levels were higher than 7 mm. All participants were recreationally active, performing sports on a non-competitive basis between 2 and 5 h per week. None of the participants had a history of participating in a structured resistance-type exercise training programme over the past 2 years. During the intervention period, one participant dropped out because of pneumonia. In addition, one participant was excluded from the analysis because he missed too many training sessions (more than 10% was the predefined exclusion criterion). All participants were informed on the nature, and possible risks of the experimental procedures before their written informed consent were obtained. This study was approved by the Medical Ethics Committee of Maastricht University Medical Centre and complied with the guidelines set out in the Declaration of Helsinki. This study was part of a greater project investigating the effect of nutritional supplementation on muscle mass and strength gains during prolonged exercise training (Snijders et al. 2015).

Exercise intervention programme

Supervised resistance-type exercise was performed three times a week for a 12-weeks period. All training sessions were performed in the evening between 8.00–9.00 pm and 9.00–10.00 pm. During the first week of the training period, the workload was gradually increased from 70% (10–15 repetitions) of 1RM to 80% of 1RM (8–10 repetitions). Thereafter, training was always performed at 80% 1RM. Workload intensity was adjusted based on the 1-RM tests (performed at weeks 4 and 8). In addition, workload was increased when more than eight repetitions could be
performed in three of four sets. Training consisted of a 5-min warm-up on a cycle ergometer, followed by four sets on both leg press and leg extension machines (Technogym, Rotterdam, the Netherlands); these two exercises were performed every training session. Two sets on the chest press and horizontal row were alternated with two sets on the vertical pull-down and shoulder press between every training session. Resting periods of 1.5 and 3 min were allowed between sets and exercises respectively. Each session ended with a 5-min cooling down period on the cycle ergometer.

**Post-exercise nutrition**

After every exercise session participants received a snack, including a cheese sandwich, an apple and a non-caloric beverage (total energy intake, 1151KJ; 37 g of carbohydrates, 10 g of protein and 9 g of fat). In addition, throughout the 12-weeks intervention period, participants consumed a protein-based beverage daily before going to sleep (total energy intake 746KJ; 13.75 g casein hydrolysate (Peptopro, DSM, Delft, the Netherlands), 13.75 g intact casein, 15 g carbohydrate (Sucrose, Suikerunie, the Netherlands) and 0.1 g fat) to maximize the hypertrophic response to the training programme (Snijders et al. 2015).

**Muscle biopsy sampling**

Seven days before the start of the intervention and after 2, 4, 8 and 12 weeks of intervention, muscle biopsies were taken from the right leg of each participant in the morning after an overnight fast. To avoid sample collection from a previous biopsy site, all consecutive incisions for the muscle biopsy sampling were made ~2 cm apart from each other, in a distal to proximal direction. Simply by visual inspection, we observed no effect of a previous biopsy sampling in any of the follow-up biopsy measurements (i.e. no clusters of myonuclei, irregular laminin outlines). This was also confirmed by the low number of regenerating muscle fibres at baseline which did not change over time (Table 1). To prevent any acute effect of the last exercise bout, the exercise sessions planned 3 days prior to muscle biopsy collection at week 2, 4 and 8 were not performed. After local anaesthesia was induced in the skin, percutaneous needle biopsy samples (50–80 mg) were collected from the *vastus lateralis* muscle, approx. 15 cm above the patella (Bergstrom 1975). Any visible non-muscle tissue was removed immediately, and biopsy samples were embedded in Tissue-Tek (Sakura Finetek, Zoeterwoude, the Netherlands), frozen in liquid nitrogen-cooled isopentane and stored at −80 °C until further analyses. For each subject, all biopsy samples were available for each time point and none of the biopsies were excluded from analyses.

**Immunochemistry**

Frozen muscle biopsies were cut into 5 μm thick cryosections using a cryostat at −20 °C, and thaw mounted on uncoated pre-cleaned glass slides. Samples from baseline and after 2, 4, 8 and 12 weeks of resistance-type exercise training were mounted together on the same glass slide. Care was taken to properly align the samples for cross-sectional muscle fibre analyses. Histochemical methods were adapted from previous published methods (Snijders et al. 2014). Muscle cross sections were stained with antibodies against laminin (polyclonal rabbit antimamin, dilution 1:50; Sigma, Zwijndrecht, the Netherlands), myosin heavy chain (MHC)-I (A4.840, dilution 1:25; Developmental Studies Hybridoma Bank, Iowa City, IA) and CD56 (dilution 1:40, BD Biosciences, San Jose, CA). Appropriate secondary antibodies were applied: goat anti-rabbit IgG Alexa647, goat anti-mouse IgM Alexa 555 and Streptavidin Alexa 488 (dilution 1:400, 1:500 and 1:200, respectively; Molecular Probes, Invitrogen, Breda, the Netherlands). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, 0.238 μM; Molecular Probes). Images were visualized and automatically captured at 10× magnification with a fluorescent microscope equipped with an automatic stage (IX81 motorized inverted microscope, Olympus, Hamburg, Germany), an EXi Aqua CCD camera (QImaging). Micromanager 1.4 software was used for image acquisition (Edelstein et al. 2010). Quantitative analyses were performed using ImageJ version 1.46d software package (version 1.46d, National Institute of

**Table 1** Regenerating type I and type II muscle fibres

<table>
<thead>
<tr>
<th>Fibre type</th>
<th>Pre</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regenerating muscle fibres (%)</td>
<td>I</td>
<td>0.7 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0.9 ± 0.3</td>
<td>0.7 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>1.2 ± 0.4</td>
</tr>
</tbody>
</table>

Data represent means ± SEM. Regenerating muscle fibres defined as the number of type I and type II muscle fibres with one or more central myonuclei expressed as a proportion to total number of type I or type II muscle fibres.
Health, MD (Strandberg et al. 2010)). For fibre cross-sectional area (muscle fibre size), laminin was used to automatically detect the outline of the individual muscle fibres; corrections were made by hand where necessary. Fibre size was automatically determined for each muscle fibre and was used to calculate mean type I and type II muscle fibre size. Type I and type II myonuclei enumerations were performed (semi) automatically (i.e. correction were made by hand if necessary). DAPI+ cells were automatically detected by the ImageJ macro and only DAPI+ cells located within the laminin outline were included to determine type I and type II myonuclear content, which was corrected for the number of satellite cells (i.e. only nuclei within cell border and not expressing CD56 were counted as myonuclei). As a proxy measure of regenerating muscle fibres (Carlson & Faulkner 1983), the number of type I and type II muscle fibres with a central myonuclei (defined as a DAPI+ cell within the muscle fibre disconnected from laminin) were determined and expressed as a proportion to the total number of type I or type II muscle fibres included into the analyses. Type I and type II muscle fibre satellite cell enumerations were performed manually. A muscle satellite cell was identified as a DAPI+/CD56+ cell located within the muscle fibre (i.e. laminin staining). The number of satellite cells per muscle fibre, the number of satellite cells per mm² of muscle fibre and the number of satellite cells relative to the total number of nuclei (number of satellite cells/number of myonuclei + number of satellite cells)*100%) were assessed for type I and type II muscle fibres separately. All image recordings and analyses were performed by an investigator blinded to participant coding. Mean numbers of 352/C629, 326/C629, 374/C643, 326/C628 and 311/C623 muscle fibres were analysed in the biopsy samples collected prior to and after 2, 4, 8 and 12 weeks of resistance-type exercise training. Representative images of the immunohistological analyses are provided in Figure 1.

Statistics

All data are expressed as means ± SEMs. Because all data were normally distributed, training-induced changes were analysed with a two-way repeated-measures ANOVA with time (before, and after 2, 4, 8, and 12 weeks of exercise training) and fibre type (type I and type II) as within-subjects factors. Fibre type was included as a within-subjects factor as resistance-type exercise training is generally known to induce a more profound effect in type II muscle fibres. In case of a significant main effect of time, without a significant fibre type x time interaction, a Bonferroni post hoc test was performed. In case of a significant fibre type x time interaction, type I and type II muscle fibres were analysed separately using one-way repeated-measures ANOVA. In case a main effect of...
time was observed within a single muscle fibre type, a Bonferroni post hoc test was performed. All analyses were performed using SPSS version 20.0 (Chicago, IL, USA). An α-level of 0.05 was used to determine statistical significance.

**Results**

**Muscle fibre size**

A significant time x fibre type interaction was observed for muscle fibre size ($P < 0.001$), as such, type I and type II muscle fibres were analysed separately. Type I muscle fibre size increased significantly in response to 12 weeks of resistance-type exercise training (from $5907 \pm 249 \text{ \mu m}^2$ to $6983 \pm 375 \text{ \mu m}^2$, $P < 0.05$; Figure 2a). In addition, we observed a gradual increase in type II muscle fibre size over time ($P < 0.05$). A significant increase in type II muscle fibre size from pre-exercise training ($6048 \pm 208 \text{ \mu m}^2$) was observed after 2 weeks ($7173 \pm 252 \text{ \mu m}^2$, $P < 0.05$), 8 weeks ($7562 \pm 354 \text{ \mu m}^2$, $P < 0.01$) and 12 weeks ($8415 \pm 360 \text{ \mu m}^2$, $P < 0.001$) of resistance-type exercise training (Figure 2a). Furthermore, type II muscle fibre size tended to be greater at 4 weeks of resistance-type exercise training compared with baseline ($P = 0.075$).

**Myonuclear content and domain size**

Type I and type II myonuclear content were analysed separately as a significant time x fibre type interaction was observed ($P = 0.032$). Type I muscle fibre myonuclear content was significantly increased at 12 weeks of resistance-type exercise training (Figure 2b). Furthermore, the number of myonuclei per type II muscle fibre tended ($P = 0.063$) to be increased at 8 weeks and was significantly increased at 12 weeks of exercise training compared with baseline ($P < 0.001$; Figure 2b). For myonuclear domain size, a significant effect of muscle fibre type was observed ($P < 0.001$). Myonuclear domain size was significantly larger in type II compared with type I muscle fibres at all time points ($P < 0.05$). No significant changes in type I and type II myonuclear domain size were observed at any time point (main effect of time $P = 0.81$; Figure 2c). No significant difference was observed between the proportion of muscle fibres holding a central myonucleus between type I and type II muscle fibres. In addition, the proportion of type I and type II muscle fibres

---

**Figure 2** Muscle fibre size (a), number of myonuclei (b), myonuclear domain size (c) and number of satellite cells (SC) per muscle fibre (d) in type I and type II muscle fibres before and after 2, 4, 8 and 12 weeks of resistance-type exercise training in healthy young men. Data are expressed as means ± SEM. *significantly different compared with pre-exercise values ($P < 0.05$); **significantly different compared with pre, 2 and 4 weeks after exercise training ($P < 0.05$). Bar indicates the effect is present in both type I and type II muscle fibres. #: significant effect of fibre type ($P < 0.05$).
with a central myonucleus remained unchanged during 12 weeks of resistance-type exercise training (Table 1).

**Satellite cell content**

No significant difference was observed in satellite cell content between type I and type II muscle fibres. We observed a robust increase in both type I and type II muscle fibre satellite cell content after exercise training (main effect of time $P < 0.05$). Compared with baseline values, the number of type I and type II muscle satellite cells per muscle fibre was significantly increased at 8 and 12 weeks of exercise training (Figure 2d). The number of satellite cells expressed relative to the number of myonuclei or per mm$^2$ did not change over time in both the type I and type II muscle fibres (Table 2).

**Discussion**

In the present study, we show that muscle fibre hypertrophy is accompanied by a time-dependent increase in myonuclear and satellite cell content in response to 12 weeks of resistance-type exercise training in young men. In addition, we show that the exercise training-induced muscle fibre hypertrophy is not accompanied by any temporary or permanent increase in myonuclear domain size.

According to the myonuclear domain theory, every myonucleus controls a certain amount of cytoplasm, referred to as the myonuclear domain (Cheek 1985). Accordingly, skeletal muscle fibre hypertrophy can be accomplished by a rise in the number of domains (by the incorporation of new myonuclei) or an increase in the size of the existing domains (Edgerton & Roy 1991). It has been well established that muscle protein synthesis rates are increased for up to 24 to 48 h after a single bout of resistance-type exercise (Burd et al. 2009). A post-exercise increase in muscle protein synthesis rate indicates that the pre-existing myonuclei have the ability to rapidly respond to the anabolic stimuli by enhancing their transcriptional activity. The cumulative effects of performing repeated bouts of exercise during a more prolonged exercise training programme should increase the size of each domain, resulting in fibre hypertrophy. However, such a progressive increase in myonuclear domain size would put the existing myonuclei under progressively more strain. Therefore, it is assumed that additional myonuclei are required to allow more extensive muscle fibre hypertrophy during prolonged exercise training. However, discrepant findings have been reported on the impact of resistance-type exercise training-induced muscle fibre hypertrophy with respect to changes in myonuclear domain size and, as such, myonuclear accretion. Whereas some studies (Kadi & Thornell 2000, Olsen et al. 2006, Petrella et al. 2006, Leenders et al. 2013, Bellamy et al. 2014) have shown that muscle fibre hypertrophy is accompanied by a substantial rise in myonuclear content, others failed to detect myonuclear accretion with resistance-type exercise training-induced muscle fibre growth (Kadi et al. 2004, Petrella et al. 2006, Mackey et al. 2007, Verney et al. 2008, Verdijk et al. 2009). To explain this apparent discrepancy, it was hypothesized that the myonuclear domain size can increase to a certain extent (e.g. $\sim 2250 \mu m^2$) before the incorporation of new myonuclei becomes prerequisite, also referred to as the ‘ceiling theory’ (Petrella et al. 2006, 2008). In the present study, baseline myonuclear domain size was 1866 ± 70 and 2009 ± 65 $\mu m^2$ in type I and type II muscle fibres, respectively, which is close to the suggested theoretical maximum. In the present study, we assessed whether (temporal) changes in myonuclear domain size, even beyond the theoretical maximum, may act as a driving factor in the generation and subsequent incorporation of new myonuclei. To provide more information on the proposed timeline of events that take place during the gradual process of muscle fibre growth, we analysed muscle biopsy samples taken at regular time point throughout a 12-weeks resistance-type exercise training programme. We clearly show that both type I and type II muscle fibre hypertrophy are accompanied by a significant gradual increase in myonuclear content in response to 12 weeks of exercise training (Figure 2a and 2b). However, the repeated-measures ANOVA
analyses did not reveal any changes in muscle fibre myonuclear domain size at any time point during the resistance-type exercise training intervention (Figure 2c). Even when performing single paired-samples t-test, (i.e. without the relatively conservative Bonferroni correction for multiple t-tests), no differences were detected between any of the time points. Not a single parameter was observed to be as stable as myonuclear domain size over the entire 12-weeks training period. These data suggest that in a physiological situation, increases in myonuclear domain size are not warranted for the generation of new myonuclei in support of extensive muscle fibre hypertrophy during resistance-type exercise training. As myonuclear domain size did not increase beyond approx. 2200 μm² during exercise-induced muscle fibre hypertrophy in young adults, it could be argued that our findings are in line with the ceiling theory (Petrella et al. 2006, 2008). However, providing a single absolute threshold for myonuclear domain size is likely impossible, given the substantial variation between subjects. Furthermore, Karlsten et al. (2015) recently reported that myonuclear domain size may also be different between smaller and bigger muscle fibres within an individual muscle biopsy sample. In the present study, we were only able to present our results expressed as a mean of the entire muscle cross section. Whether changes in myonuclear number and/or domain size during exercise-induced muscle fibre hypertrophy are affected on the individual level by the range of muscle fibre size remains to be determined.

Besides the ceiling theory, which suggests an absolute threshold for myonuclear domain size (Petrella et al. 2006, 2008), others have speculated that it is the relative extent of muscle fibre hypertrophy that should exceed a certain threshold (±26%) before incorporation of new myonuclei becomes evident (Kadi et al. 2004). Our data do not seem to support that rationale as the observed increases in type I and type II muscle fibre size (18% and 41%, respectively) were both achieved by incorporating new myonuclei (Figure 2a,b), without any changes in myonuclear domain size throughout the 12 weeks of exercise training (Figure 2c). However, in the study by Kadi et al. (2004), baseline myonuclear domain size (1522 μm²) was substantially smaller compared in the current study (1866 ± 70 and 2009 ± 63 μm² in type I and type II muscle fibres respectively). Exercise-induced muscle fibre growth could be speculated to be supported without myonuclear addition when initial myonuclear domain size is relatively small. However, it would be difficult to define whether a certain relative hypertrophy threshold should be reached before additional myonuclei become prerequisite as this may very well depend on baseline muscle fibre characteristics. Moreover, based on the present findings, an increase in myonuclear domain size does not represent a necessary step before the generation of new myonuclei is accomplished to support muscle fibre hypertrophy.

As myonuclei are post-mitotic, exercise training-induced myonuclear accretion is dependent on a pool of myogenic precursor cells, also known as skeletal muscle satellite cells. Animal studies have clearly shown that satellite cells are essential in muscle fibre repair (Lepper et al. 2011, McCarthy et al. 2011, Murphy et al. 2011, Sambasivan et al. 2011) and extensive muscle fibre growth (Fry et al. 2014). In accordance, a positive correlation between the increase in muscle fibre size and satellite cell content has been shown numerous times during prolonged exercise training in human skeletal muscle (Petrella et al. 2006, Verdiik et al. 2010, 2014, Mackey et al. 2011a, Bellamy et al. 2014). In addition, mixed muscle fibre satellite cell content has been reported to increase substantially in response to different exercise intervention programmes in young adults (Kadi & Thornell 2000, Kadi et al. 2004, Olsen et al. 2006, Petrella et al. 2006, 2008, Mackey et al. 2011b). This increase in satellite cell content has also been reported in both type I and type II muscle fibres during exercise training in healthy young men (Bellamy et al. 2014) and female myalgia patients (Mackey et al. 2011a). In the present study, we confirm these results by showing that muscle fibre hypertrophy is accompanied by a time-dependent increase in the number of both type I and type II satellite cells per muscle fibre during exercise training (Figure 2d). In contrast, the number of satellite cells expressed per mm² of muscle fibre remained constant throughout all time points. This indicates that fibre size and satellite cell content appear to be tightly regulated throughout exercise-induced muscle fibre growth. The direction, however, of this regulation remains unknown. Unfortunately, due to technical limitations, we were not able to differentiate satellite cell content between type I/type II and hybrid muscle fibres which has been of particular interest in a recent published article by Joannis and colleagues (Joannis et al. 2013). Nonetheless, the overall data presented in this study provide further support for the hypothesis that an increase in satellite cell pool size is an important supportive factor in the process of muscle fibre hypertrophy during prolonged resistance-type exercise training in vivo in humans, facilitating the incorporation of newly formed myonuclei.

In conclusion, resistance-type exercise training-induced muscle fibre hypertrophy is accompanied by an increase in myonuclear and satellite cell content. Myonuclear domain size does not change throughout prolonged resistance-type exercise training and, as
such, does not appear to be critical in myonuclear accretion to facilitate subsequent muscle fibre hypertrophy in healthy, young males.

Author contribution
The study was performed at Maastricht University, Maastricht, the Netherlands. TS, AKK, LJCvL and LBV did the conception and design of the study; TS and JSJS performed the experiments; TS, JSJS, JvK, AKK and LBV analysed the data; TS, JSJS, JvK, LJCvL and LBV interpreted the results; TS drafted the manuscript; TS, JSJS, JvK, LJCvL and LBV edited and revised the manuscript. All authors approved the final version of the manuscript.

Conflict of interest
No conflict of interest is declared by the authors.

References


