Effects of long-term immobilisation on endomysium of the soleus muscle in humans

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Abstract
Muscle fibres atrophy during conditions of disuse. Whilst animal data suggest an increase in endomysium content with disuse, that information is not available for humans. We hypothesised that endomysium content increases during immobilisation. To test this hypothesis, biopsy samples of the soleus muscle obtained from 21 volunteers who underwent 60 days of bed rest were analysed using immunofluorescence-labelled laminin γ-1 to delineate individual muscle fibres as well as the endomysium space. The endomysium-to-fibre-area ratio (EFAr, as a percentage) was assessed as a measure related to stiffness, and the endomysium-to-fibre-number ratio (EFNr) was calculated to determine whether any increase in EFAr was absolute, or could be attributed to muscle fibre shrinkage. As expected, we found muscle fibre atrophy ($P = 0.0031$) that amounted to shrinkage by 16.6% (SD 28.2%) on day 55 of bed rest. ENAr increased on day 55 of bed rest ($P < 0.001$). However, when analysing EFNr, no effect of bed rest was found ($P = 0.62$). These results demonstrate that an increase in EFAr is likely to be a direct effect of muscle fibre atrophy. Based on the assumption that the total number of muscle fibres remains unchanged during 55 days of bed rest, this implies that the absolute amount of connective tissue in the soleus muscle remained unchanged. The increased relative endomysium content, however, could be functionally related to an increase in muscle stiffness.

KEYWORDS
connective tissue, endomysium, immobilisation, muscle atrophy

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1 | INTRODUCTION

Muscle atrophy is a well-known result of unloading which occurs during immobilisation, spaceflight and experimental bed rest (Booth, 1994; Pavy-Le Traon et al., 2007), where muscle tissue is not used in its normal capacity. The need to understand the underlying mechanisms of muscle atrophy increases with an ageing society, and also with ambitions to travel to Mars. Experimental bed rest with a 6° head-down tilt is widely recognised as an acceptable model for microgravity conditions and has been used to investigate muscle change during disuse (Hargens & Vico, 2016; Widrick et al., 1999). Bed rest-induced muscle wasting occurs as a result of reduced myofibrillar protein synthesis (Gibson et al., 1987), and the resulting atrophy is most pronounced in the calf musculature, especially in the soleus muscle (Belavý et al., 2009).

Many different countermeasures (predominantly physical exercise regimes) have been examined in an attempt to prevent physical deconditioning during spaceflight. Making use of artificial gravity as a more generalised countermeasure may be needed in order to safely perform deep-space missions. With regards to skeletal muscle, it has already been shown that performing resistance exercise training on a short-arm centrifuge is feasible (Yang et al., 2007) and that it can at least partly prevent muscle deconditioning during experimental bed rest (Caiozzo et al., 2009; Edmonds et al., 2008). Hence, the Artificial Gravity Bed Rest Study with the European Space Agency (AGBRESA) was devised to assess the effectiveness in mitigating bed rest-induced deteriorations in the fields of cardiovascular, immunological, genetic, muscle and bone, ocular, vestibular, psychological, sleep, cognition and behavioural performance, and the present sub-study makes use of biopsy samples that were harvested from the soleus muscle within the AGBRESA study.

While it is established that muscle fibre atrophy leads to functional impairments, such as a loss of peak force and power (Fitts et al., 2010; Fitts et al., 2001), the changes transpiring within the muscular connective tissue have largely been neglected. As recently reported, the mechanical properties at whole-muscle level are at least partly influenced by connective tissue, and intramuscular connective tissue should therefore receive more attention. The smallest connective tissue component is the endomysium, which engulfs each individual muscle fibre (Borg & Caulfield, 1980; Turrina et al., 2013) and surrounds the individual cell membrane, or sarcolemma (Frontera & Ochala, 2015). As reviewed by Gillies and Lieber, endomysium has a mesh-up structure composed of equal amounts of collagen types I and III. It is interlinked with the muscle basement membrane which is in turn interlinked with the sarcolemma. The basement membrane consists mainly of type IV collagen and glycoproteins, such as laminin γ-1 (Gillies & Lieber, 2011). It has been postulated that intramuscular connective tissue is important for stability, force transmission, elasticity, as well as the nourishment of muscle fibres, and is therefore essential to physiological muscle integrity and function (Gillies & Lieber, 2011; Järvinen et al., 2002; Kjaer, 2004).

Research on this omnipresent component in skeletal muscle during muscle disuse is, however, scarce. In rat, the endo- and perimysium was increased and had lost its normal ultra-structure upon immobilisation (Järvinen et al., 2002). It has further been demonstrated in mice that immobilisation results in an increased endomysium content of the soleus muscle (Williams & Goldspink, 1984). One has to consider, though, that the latter study merely considered the relation between connective tissue and muscle fibre size, and that it does not exclude the possibility that the relative enrichment in endomysium is foremost attributable to fibre atrophy. Savolainen et al. have observed decreases in collagen biosynthetic enzyme activities in cast-immobilised rat hind-limb muscle (Savolainen et al., 1987), but increases in those enzyme activities when the hindlimb muscles were denervated (Savolainen et al., 1988a). However, intramuscular collagen degradative enzyme activity is shifted in the same direction as collagen synthetic enzyme activity (Savolainen et al., 1988b), and ex vivo enzyme activity does not necessarily reflect in vivo enzyme turnover. Moreover, the turnover of collagen is only 1–2%/day in rat skeletal muscle, whereas the fractional synthesis rate of muscle non-collagenous proteins is 12% per day (Reeds et al., 1980), all of which may explain why the total content of hydroxyproline per muscle, as a surrogate for collagen mass within an anatomical muscle, was fairly constant in another rat immobilisation study (Karpakka et al., 1991), and also in the aforementioned studies (Savolainen et al., 1987, 1988a, 1988b).

Based on the findings of Williams & Goldspink (1984), we hypothesised that the relative amount of intramuscular connective tissue in the human soleus muscle increases during long-term bed rest, which is solely attributable to muscle fibre shrinkage rather than an increase in the absolute amount of intramuscular connective tissue. The increase in the relative amount of intramuscular connective tissue could contribute to reported disproportional loss of muscle mass and muscle strength and thereby provide a step towards enhancing our understanding of the impaired function of an atrophied muscle.

New Findings

- **What is the central question of this study?**
  While muscle fibre atrophy in response to immobilisation has been extensively examined, intramuscular connective tissue, particularly endomysium, has been largely neglected: does endomysium content of the soleus muscle increase during bed rest?
- **What is the main finding and its importance?**
  Absolute endomysium content did not change, and previous studies reporting an increase are explicable by muscle fibre atrophy. It must be expected that even a relative connective tissue accumulation will lead to an increase in muscle stiffness.
2 | METHODS

2.1 | Ethical approval

The study was approved by the Ethics Committee of the North Rhine Medical Association (Ärztekammer Nordrhein, reference number 2018143) in Düsseldorf, Germany, and was registered in the German Clinical Trials Register (DRKS-ID: DRKS00015677). All subjects provided written informed consent prior to their participation in the study. The study conformed to the standards set by the Declaration of Helsinki.

2.2 | Study design

A group of 24 healthy subjects (16 men, 8 women, 33 ± 9 years; 175 ± 9 cm; 74 ± 10 kg) participated in the 60-day AGBRESA study, which was conducted jointly by the German Aerospace Center, the European Space Agency and the National Aeronautics and Space Administration. AGBRESA’s objective was to determine the effects and effectiveness of short-duration continuous (cAG) and intermittent centrifugation (iAG), i.e., artificial gravity, as a countermeasure to the negative effects of immobilisation and disuse. Subjects were pseudo-randomly assigned to the passive control (Ctrl) or to an intervention group. Subjects underwent extensive medical and psychological evaluation prior to being included in the study. More detailed information is set forth in Guidelines for standardisation of bed rest studies in the spaceflight context (Sundblad & Orlov, 2014). Details of the AGBRESA study have been published which specify the daily routines (Frett et al., 2020).

2.3 | Biopsy acquisition

Biopsies were obtained from the soleus muscle of 21 of the AGBRESA subjects (14 men, 7 women) under sterile conditions at baseline (BDC), at day 6 and at day 55 head down tilt bed rest (HDT6 and HDT55). Acquisition followed skin disinfection and local anaesthesia with lidocaine. Muscle tissue was extracted using a rongeur (diameter of 4 mm) which extracted approximately 150 mg of muscle tissue. Muscle biopsy specimens were mounted with Tissue-Tek O.C.T. Compound (Sakura, Torrance, CA, USA) with a fibre orientation adjusted for transversal sectioning and immediately shock-frozen in liquid nitrogen and stored at −80°C. Frozen, unfixed muscle biopsies were sectioned at −20°C on a cryostat (Leica CM 1850 UV; Leica Biosystems, Wetzlar, Germany). Slices of 8 μm were transferred to adhesive microscope slides for myopathological analysis and stored at −20°C until staining.

2.4 | Staining protocol

Indirect immunofluorescence staining was conducted at room temperature in a humid environment protected from light. Slides were let to equilibrate to room temperature, fixed in formaldehyde prepared from 0.5% paraformaldehyde, and washed three times in phosphate-buffered saline (PBS) for 5 min and briefly in distilled water. Slides were subsequently blocked with 5% normal goat serum in PBS/0.2% Tween-20 for 30 min. Anti-laminin γ1 antibody (polyclonal rabbit anti-mouse laminin γ1 chain, Immundiagnostik, Bensheim, Germany, cat. no. AP1001.1, RRID:AB_227233) at a dilution of 1:200 in blocking buffer was applied to the slides and allowed to incubate for 1 h. After washing in PBS three times, the slides were incubated with the secondary antibody (polyclonal Cy2-conjugated AffiniPure goat anti-rabbit antibody, Jackson ImmunoResearch Laboratories, West Grove, PA, USA, cat. no. 111-225-144, RRID:AB_2338021) at a dilution of 1:500 in blocking buffer for 45 min. After incubation, the slides were washed in PBS three times, washed briefly in distilled water, mounted with ProLong Gold Antifade Mountant with 4′,6-diamidino-2-phenylindole (Thermo Fisher Scientific, Waltham, MA, USA, P36941), and stored at 4°C.

2.5 | Image analysis

Tiled images were captured using a Zeiss Axio Observer.Z1 microscope (Carl Zeiss Microscopy, Oberkochen, Germany) with a ×25 objective (NA 0.8). The observer was blinded to the operational day and group allocation when analysing the images. Laminin γ-1-stained images were stitched and analysed using Zeiss ZEN Desk 3.1 software. Each measurement frame (called region of interest (ROI) for readability) included at least 50 muscle fibres, and ROIs were carefully placed within the stitched image, i.e., the stained section, to exclude artefacts and perimysium, thereby containing muscle fibres and endomysium and its enclosures (capillaries, nerve fibres) only. For each muscle fibre that is bordered by its laminin γ-1-positive basement membrane, the minimal and maximal Feret-diameters, muscle fibre perimeter (PeriFibre) and muscle cell area (A_fibre) were computed with Zeiss ZEN software (version 3.1, blue edition). The endomysium space is hereby delineated by the laminin γ-1-stained basement membranes of neighbouring muscle fibres. We assume that endomysium area is correlated with endomysium content and therefore used endomysium area as a parameter to estimate endomysium content. Notably, the ROIs’ borders contained truncated fibres. Hence, in order to adjust for truncation effects upon endomysium readouts, fibres adjacent to the border were identified being away from the border by less than the median of half the maximal Feret diameter. The validity of this approach was confirmed by visual inspection with custom-made R scripts (R Foundation for Statistical Computing, Vienna, Austria). For statistical analyses, diameter, cross-section and perimeter values were averaged over only fibres that were not truncated by the ROI borders.

Next, we assessed, the endomysium-to-fibre-area ratio (EFAR). To this purpose, total fibre area (A_Tot_Fibre, which includes border-adjacent and thus truncated fibres) was computed, and the total endomysium area (A_Tot_Endo, visible as the area delineated by two laminin γ-1-positive basement membranes, see Figure 1) was computed as the difference between the ROI’s area and A_Tot_Fibre, and EFAR was
FIGURE 1  (a–c) Typical staining from one representative subject before (BDC, a), at day 6 (HDT6, b) and at day 55 (HDT55, c).
(d) Segmentation of muscle fibres shown in a part of the region of interest (ROI) in a biopsy.
(e) The area of endomysium if the muscle fibre area (coloured) is subtracted. The model used assumed that muscle fibres are approximately round and can be approximated by using parameters of the minimal (red) and maximal (blue) Feret-diameters (f). Scale bars 100 µm

A probability as:

\[ \text{EFAr} = 100 \cdot \frac{A_{\text{Tot_Endo}}}{A_{\text{Tot_Fibre}}} \]

We specifically chose to assess the endomysium-to-fibre-area ratio, and not the fractional area of the endomysium within the entire ROI, firstly because this describes the ratio of passive-to-active constituents of muscle tissue, and secondly because this measure yields a stronger contrast to the measure of endomysium-to-fibre number (see below).

Endomysium thickness was calculated, using the summed perimeters of all fibres (ΣPeri, including border-adjacent fibres) as:

\[ A_{\text{Tot_Endo}} = 0.5 \cdot \Sigma \text{Peri} - \text{Peri}_{\text{ROI}} \]

where Peri_{ROI} is the ROI’s perimeter. The factor 0.5 in the denominator is necessary to account for the fact that each endomysium strut is outlined by two muscle fibre perimeters, and subtraction of Peri_{ROI} adjusts for the border-truncated fibres.

For computation of the endomysium-to-fibre-number ratio (EFNr), we also had to take care of the border-truncated fibres. It turns out that this is well feasible by adjusting the fibre number for the border-adjacent fibres of the ROI (N_{Border_Adjusted})

\[ N_{\text{Border_Adjusted}} = \frac{A_{\text{Tot_Fibre_Border}}}{A_{\text{Fibre_Mean}}} \]

where A_{Tot_Fibre_Border} is the total area of all border-adjacent fibres and A_{Fibre_Mean} is the average area of all fibres not adjacent to the ROI border. Accordingly, EFNr was computed as

\[ \text{EFNr} = \frac{A_{\text{Tot_Endo}}}{N_{\text{Non_Border}} + N_{\text{Border_Adjusted}}} \]

where N_{Non_Border} is the number of fibres that are entirely located within the ROI.

2.6 Statistical analyses

Statistical analyses were performed using R version 3.6.1. All calculations were performed using an analysis of variance with a linear mixed-effects model. Results are shown as means and standard deviations. The level of significance was set at \( P < 0.05 \). Four
biopsies were excluded from analysis due to poor biopsy quality or an insufficient number of analysed fibres (<50 fibres).

3 | RESULTS

There were no significant differences at baseline across groups in either anthropometric characteristics or in biopsy data (all \( P > 0.20 \), see Table 1).

### 3.1 | Muscle fibre atrophy

We first analysed the fibre area (see Table 2) to determine the presence of muscle fibre atrophy. We found a significant effect of time (BDC, HDT6 and HDT55) pertaining to the decrease of muscle fibre area (\( F(2,34) = 6.9 \) and \( P = 0.0031 \)), but no interaction between group (iAG, cAG or Ctrl) and time (\( F(2,34) = 1.03 \) and \( P = 0.41 \)). Following up the time effect by a priori contrasts revealed a shrinkage of 16.6% (SD 28.2%) at HDT55 (\( P = 0.028 \)), but no change at HDT6 (\( P = 0.90 \)). The minimal Feret-diameter showed a main effect for time (\( F(2,34) = 8.42 \) and \( P = 0.0011 \)), but not for the interaction between time and group (\( F(4,34) = 0.67 \) and \( P = 0.62 \)). The main effect for time was again significant at HDT55 (\( P < 0.001 \)), but not at HDT6 (\( P = 0.93 \)).

### 3.2 | Intramuscular connective tissue

Endomysium-to-fibre-area ratio (EFAr, Table 2) showed a main effect of time (\( F(2,34) = 10.4 \) and \( P < 0.001 \)), but no group-by-time-interaction (\( F(4,34) = 1.07 \) and \( P = 0.39 \)). A priori contrast testing showed that EFAr was increased on day HDT55 (\( P < 0.001 \), see Figure 2a), but not on day HDT6 (\( P = 0.43 \)). We furthermore computed the thickness of the intramuscular connective tissue. There was no main effect of time (\( F(2,34) = 2.30 \) and \( P = 0.12 \)), and also no interaction between time and group (\( F(4,34) = 1.56 \) and \( P = 0.21 \)). Likewise discordant from the EFAr, there was no effect of time for EFNr (\( F(2,34) = 0.48 \) and \( P = 0.62 \), Figure 2b), and also no interaction effect between time and group (\( F(4,34) = 0.79 \) and \( P = 0.54 \)).

To conclude, we did not find any group effects in any outcome variable (muscle fibre atrophy, EFAr, endomysium thickness and EFNr).

4 | DISCUSSION

The purpose of this study was to determine whether endomysium content, assessed through the endomysium area, increases during long-term immobilisation, and whether this increase could possibly be a missing factor in explaining the seemingly exaggerated loss in muscle function compared to muscle atrophy in disuse models. In line with the hypotheses, results showed an increase in the relative amount of intramuscular connective tissue, but no increase in the absolute amount of endomysium during immobilisation. As the absolute amount of connective tissue remained unchanged, but fibres atrophied, the relative thickness of endomysium increased.
TABLE 2  Muscle fibre morphology and intramuscular connective tissue before (BDC) and after 6 (HDT6) and 55 (HDT55) days of head-down tilt bed rest for the control (Ctrl) and intervention groups (iAG and cAG)

| Variable                  | Group | BDC         | HDT6        | HDT55        |
|---------------------------|-------|-------------|-------------|--------------|
| Fibre CSA (μm²)           | Ctrl  | 6011 (2962) | 6255 (1951) | 4104 (1526)  |
|                           | iAG   | 4983 (991)  | 5037 (989)  | 4074 (2006)  |
|                           | cAG   | 5150 (962)  | 4978 (1272) | 4758 (1895)**|
|                           | All   | 5340 (1734) | 5362 (1465) | 4336 (1753)  |
| Feret Min (μm)            | Ctrl  | 69.5 (14.2) | 69.7 (7.9)  | 57 (8.4)     |
|                           | iAG   | 63.1 (5.8)  | 63.1 (5.8)  | 55.6 (15.5)  |
|                           | cAG   | 63.6 (6.8)  | 64 (9.1)    | 59.5 (13.1)  |
|                           | All   | 65.1 (9.2)  | 65.3 (7.9)  | 57.5 (12.1)**|
| EFA ratio (% of fibre CSA)| Ctrl  | 21.9 (3.6)  | 23.4 (3.6)  | 30.7 (6.1)   |
|                           | iAG   | 24.2 (2.6)  | 26.1 (6.0)  | 34.4 (12.7)  |
|                           | cAG   | 27.1 (7.4)  | 25.8 (5.3)  | 28.7 (7.3)   |
|                           | All   | 24.6 (5.4)  | 25.2 (5.0)  | 31.1 (8.9)** |
| Endomysium thickness (μm)| Ctrl  | 8.73 (3.77) | 9.45 (2.06) | 9.18 (1.22)  |
|                           | iAG   | 8.55 (1.42) | 9.32 (2.71) | 10.17 (1.56) |
|                           | cAG   | 9.78 (2.08) | 8.99 (1.3)  | 9.23 (1.49)  |
|                           | All   | 9.07 (2.45) | 9.23 (1.97) | 9.51 (1.43)  |
| EFN ratio (μm³/fibre)     | Ctrl  | 1369 (928)  | 1439 (370)  | 1203 (282)   |
|                           | iAG   | 1201 (249)  | 1306 (372)  | 1212 (354)   |
|                           | cAG   | 1342 (195)  | 1252 (250)  | 1295 (382)   |
|                           | All   | 1303 (503)  | 1323 (322)  | 1240 (328)   |

Values are means ± SD. Fibre area given as cross-sectional area (CSA), and minimal fibre diameter as Feret Min. Significances from baseline: **P < 0.01 ***P < 0.001.

Naturally, the present technical approach has not directly assessed the constituents of endomysium, but rather the limits of the endomysium space (defined by the laminin γ-1-stained basement membranes). However, to do so would be difficult as the exact biochemical composition of endomysium is generally not well established (Gillies & Lieber, 2011), and it is possible that single constituents, e.g., certain collagens, are differentially expressed during the atrophy response. In keeping with the primary aim of our study, we therefore approached the endomysium space histologically.

The endomysium-to-fibre-number ratio in this study was calculated to unambiguously determine whether previously reported increases in endomysium content with immobilisation are solely an effect of muscle fibre atrophy, or whether it is attributable to an actual net gain in intramuscular connective tissue. Since an increase was only shown in endomysium-to-fibre-area and not in endomysium-to-fibre-number, the results clearly demonstrate that the previously reported increase in endomysium content with disuse is most likely an effect of muscle fibre shrinkage. Williams and Goldspink did in fact calculate the endomysium-to-fibre-area ratio between day 1 and 3 weeks of immobilisation (Williams & Goldspink, 1984) and concluded that a direct effect of muscle fibre atrophy could not be excluded. If indeed the endomysium content per fibre remains constant, and if the number of muscle fibres in a given muscle remains likewise unaffected by bed rest, then one would expect the total collagen content to be unchanged in atrophied rat muscles mentioned above (Savolainen et al., 1987, 1988a, 1988b). It is of interest in this context that muscle collagen synthesis rate is comparable to muscle myofibrillar protein synthesis rate, but lower than tendon collagen synthesis rate in ambulatory younger and older people (Babraj et al., 2005). Under steady state conditions, one would expect, accordingly, that protein breakdown rates are comparable between myofibrillar proteins and muscle collagen. Immobilisation-related fibre atrophy is mostly due to reductions in myofibrillar protein synthesis rate, with protein breakdown being mainly unaffected (Paddon-Jones et al., 2006), and tendon collagen synthesis reductions in limb immobilisation seem to match the reductions in myofibrillar protein synthesis (de Boer et al., 2007). Therefore, if indeed the endomysium content of the muscle remains constant in bed rest, there would have to be a reduction in endomysium protein breakdown that is commensurate to reductions in endomysium protein synthesis.

An increase in the endomysium content has also been hypothesised to take place during other circumstances, for example, ageing and spasticity (Kjaer, 2004). Many different muscle disorders include muscle fibrosis, meaning an abnormal accumulation of connective tissue (Lieber & Ward, 2013). Although the consequences
of exaggerated endomysium content remain to be elucidated in general, it has been found that the enhanced stiffness in contractured hamstring muscles of patients with cerebral palsy is attributable to enhancement of extracellular matrix content (Smith et al., 2011), and very similar effects have been demonstrated for older age (Pavan et al., 2020). Ward et al. (2020) demonstrated that passive modulus increases non-linearly when examining properties of single fibres, fibre bundles, fascicles and whole muscles, indicating the role of the extracellular matrix. Of note, generation of force and of stiffness are two different mechanical functions of muscle (Lai et al., 2019). Whilst strength, which is related to the sarcomeres-in-parallel, is necessary to generate force, and thereby provides movement ability, stiffness, which is related to connective tissue, allows elastic storage and return of energy. Extracellular matrix of the muscle is assumed to have a modulus up to 25 times that of muscle fibres (Lieber & Ward, 2013). Future studies should therefore address the question of whether the relative enrichment of endomysium and the other constituents of muscular connective tissue do indeed affect muscle strength and stiffness differentially.

In conclusion, we found that the endomysium of the intramuscular connective tissue does not increase in relation to fibre number, but is enhanced in relation to fibre size in response to long-term immobilisation in humans. These changes must be expected to differentially affect the muscle’s abilities to generate force or to store elastic energy, thereby potentially increasing the relative stiffness of the atrophied skeletal muscle.

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COMPETING INTERESTS
Authors declare no competing interests.

AUTHOR CONTRIBUTIONS
J.R. conceived and designed the study. G.K.T., C.B., I.G., J.R. and B.G. performed and processed the muscle biopsies. G.K.T. immunostained the samples. Y.L. provided support on microscopic settings. G.K.T. and J.R. analysed the data. C.S.C. participated in final data presentation. All authors contributed to the interpretation of data. G.K.T. drafted the manuscript. C.S.C. supported figure preparation. All authors contributed to the revision of the manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

DATA AVAILABILITY STATEMENT
Data will be made available upon reasonable request to the corresponding author.

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