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ORIGINAL ARTICLE

Absence of an aging-related increase in fiber type grouping in athletes and non-athletes

Guy A. M. Messa[1] | **Mathew Piasecki**[2] | **Jörn Rittweger**[3,4] | **Jamie S. McPhee**[5] | **Erika Koltai**[6] | **Zsolt Radak**[6] | **Bostjan Simunic**[7] | **Ari Heinonen**[8] | **Harri Suominen**[8] | **Marko T. Korhonen**[8] | **Hans Degens**[1,9,10]

[1]Department of Life Sciences, Research Centre for Musculoskeletal Science and Sports Medicine, Manchester Metropolitan University, Manchester, UK

[2]MRC-Versus Arthritis Centre for Musculoskeletal Ageing Research and National Institute for Health Research (NIHR) Nottingham Biomedical Research Centre, University of Nottingham, Nottingham, UK

[3]Division Space Physiology, Institute of Aerospace Medicine, German Aerospace Center, Cologne, Germany

[4]Department of Paediatric and Adolescent Medicine, University of Cologne, Cologne, Germany

[5]Department of Sport and Exercise Sciences, Manchester Metropolitan University, Manchester, UK

[6]Research Institute of Sport Science, University of Physical Education, Budapest, Hungary

[7]Science and Research Centre Koper, Institute for Kinesiology Research, Koper, Sloveni

[8]Gerontology Research Centre, Faculty of Sport and Health Sciences, University of Jyväskylä, Jyväskylä, Finland

[9]Institute of Sport Science and Innovations, Lithuanian Sports University, Kaunas, Lithuania

[10]University of Medicine and Pharmacy of Targu Mures, Targu Mures, Rumania

Correspondence
Hans Degens. Department of Life Sciences, Faculty of Science and Engineering, John Dalton Building, Manchester Metropolitan University, Manchester M1 5GD, UK.
Email: h.degens@mmu.ac.uk

The aging-related loss of muscle mass is thought to be partly attributable to motor neuron loss and motor unit remodeling that result in fiber type grouping. We examined fiber type grouping in 19- to 85-year-old athletes and non-athletes and evaluated to which extent any observed grouping is explained by the fiber type composition of the muscle. Since regular physical activity may stimulate reinnervation, we hypothesized that fiber groups are larger in master athletes than in age-matched non-athletes. Fiber type grouping was assessed in *m. vastus lateralis* biopsies from 22 young (19-27 years) and 35 healthy older (66-82 years) non-athletes, and 14 young (20-29 years), 51 middle-aged (38-65 years), and 31 older (66-85 years) athletes. An "enclosed fiber" was any muscle fiber of a particular type surrounded by fibers of the same type only. A fiber type group was defined as a group of fibers with at least one enclosed fiber. Only type II fiber cross-sectional area (FCSA) showed an age-related decline that was greater in athletes ($P < .001$) than in non-athletes ($P = .012$). There was no significant age-related effect on fiber group size or fiber group number in athletes or non-athletes, and the observed grouping was similar to that expected from the fiber type composition. At face value, these observations do 1) neither show evidence for an age-related loss and remodeling of motor units nor 2) improved reinnervation

with regular physical activity, but 3) histological examination may not reveal the full extent of aging-related motor unit remodeling.

KEYWORDS
aging, denervation, fiber type, grouping, reinnervation, vastus lateralis

1 | INTRODUCTION

Aging is associated with progressive muscle wasting and weakness, a condition referred to as sarcopenia, where almost 30% of the mass and more than 35% of the force generating capacity may be lost by the age of 70 years.[1] The gradual loss of muscle mass with advanced age is attributed to both atrophy of particularly type II fibers[2,3] and loss of fibers.[1,4,5] Consequently, older persons suffer functional and mobility limitations leading to increased fall risk, reduced quality of life, and loss of independence.[6] With people older than 65 years representing the fastest growing segment, and particularly those over 85, in the western populations, it becomes ever more important to expand knowledge on aging muscle to develop effective strategies to reverse or attenuate the aging-related decline of muscle mass and function to delay the onset of functional limitations.[7]

A motor unit consists of a single motor neuron (MN) and all of the muscle fibers that it innervates. In large muscles, such as the *m. vastus lateralis*, a single motor neuron can innervate hundreds or even thousands of fibers. The loss of muscle fibers in aged muscles is thought to be at least partly attributable to loss of motor neurons with consequent denervation of their associated fibers and ultimately disappearance if they are not reinnervated.[8-10] Many of the fibers that have become denervated because of motor neuron loss may, however, be reinnervated by the remaining motor neurons, which may result in fiber type grouping.[11,12]

Physical inactivity is an important determinant of sarcopenia progression[13] and like aging results in muscle atrophy.[14] The question thus arises to what extent muscle wasting and weakness in old age are due to reduced physical activity levels. Master athletes maintain high levels of physical activity[15,16] and are thus considered a good model to disentangle the effects aging *per se* from reduced levels of physical activity on skeletal muscle.[15,17,18] Some studies reported no aging-related loss of motor units in master athletes[19] or an attenuated loss even in octogenarians,[20,21] while others found that master athletes do suffer similar motor unit loss as seen in non-athletes.[22,23] Several studies interpreted increased motor unit size[10,23] and larger fiber type groups[24,25] as evidence for improved reinnervation in master athletes, but others found smaller motor unit size, suggested to be representative of less collateral reinnervation in master athletes.[26] Thus, the evidence is equivocal as to whether regular physical activity protects against aging-related motor neuron loss and facilitates reinnervation.

The reports on fiber type grouping in master athletes studied only old master athletes and did not compare them with young- and middle-aged athletes nor did they consider the potential impact of fiber type composition on the observed fiber type grouping. This is important because fiber type composition can vary markedly between individuals, and grouping is more likely for those with a high proportion of one single fiber type than in those with similar proportions of type I and type II fibers. Therefore, the objective of this study was to assess fiber type grouping in athletes and non-athletes of a wide age range (19-85 years). In addressing this objective, we considered the extent to which any observed grouping is explained by the fiber type composition of the muscle so that the chance occurrence of groups can be separated from the possible exercise-mediated fiber type grouping. Based on previous observations on the size of fiber groups[24,25] and the loss of motor units[22,23] in master athletes, we hypothesized that both the fiber group size and number of fiber groups increase with increasing age in master athletes beyond that expected from muscle fiber type composition. As it has been suggested that master athletes may have better reinnervation capacity than non-athletes,[23,24] we hypothesized that the group size is larger in master athletes than non-athletes.

2 | MATERIALS AND METHODS

2.1 | Subjects

The study population consisted of 22 young (19-27 year; men n = 14; women n = 8) and 35 healthy older (66-82 year; men n = 27; women n = 8) non-athletes who participated in the MYOAGE study[3,27] and athletes. The athletes were divided into three groups: 20-29 year (men n = 14), 38 - 65 year (men n = 44; women n = 7), and 66-85 year (men n = 32; women n = 3). Athletes were recruited at the European Veteran Athletics Championship 2008 (Ljubljana, Slovenia), World Master Athletics Championships in 2009 (Lahti, Finland), the European Veterans Athletics Championships in 2010 (Nyíregyháza, Hungary), or by means of personal letters from among the members of Finnish track and field organizations as described in Korhonen et al.[28] The event specialities ranged from sprint and power events to middle- and long-distance running events (200m, 400m, 110m hurdles, long

jump, hammer, javelin, 1500 m and 5000 m). The studies were approved by the ethical committee of the Manchester Metropolitan University (UK), the Republic of Slovenia National Medical Ethics Committee (Slovenia), and the ethics committees of the University of Jyväskylä (Finland) and the Semmelweis Institute (Budapest, Hungary) and have, therefore, been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All participants gave written informed consent.

2.2 | Anthropometry and muscle size

For each participant, the body mass and height were measured and the body mass index (BMI: kg m^{-2}) calculated. In 100 athletes, the thickness of the *m. vastus lateralis* (VL) was determined, while lying on a bed, at 50% thigh length with a 5-cm linear-array 7.5-MHz ultrasound probe as described previously.[28]

2.3 | Biopsy sampling and histochemistry

Biopsies were obtained from the middle portion of the *m. vastus lateralis* at 40% of the distance from the patella to greater trochanter under aseptic conditions by using either a conchotome or Bergström needle after local anesthesia with 1%-2% lidocaine. The biopsy was placed on cork with Optimum Cutting Temperature compound (Scigen® Gardena) and immediately frozen in isopentane cooled in liquid nitrogen, or with vigorous shaking in liquid nitrogen, and stored at −80°C for histochemistry.

Serial 10-μm cross sections were cut on a cryostat (Leica CM 3050S) at −21°C and stained for myofibrillar ATPase after acid (pH 4.30) pre-incubation as described by Brook and Kaiser.[29] Type I fibers stained dark, type II fibers light, and type I/II fibers intermediate (Figure 1A).

2.4 | Morphometry

The stained cross sections were photographed under a light microscope (Carl Zeiss Vision GmbH, Aalen, Germany) at 10x objective with a digital camera (Zeiss AxioCam MRc, Göttingen, Germany). The contours of each fiber were drawn using a digitizing program (Program Btablet, BaLoH Software, Ooij, The Netherlands), and the coordinates of the outlines stored for further analysis with AnaTis (BaLoH software, Ooij, NL). In each biopsy, 58-718 complete fibers were analyzed (only 3 samples had fewer than 100 fibers). The fiber type proportion (expressed as fiber number percentage) and the fiber cross-sectional area (FCSA) of each fiber were assessed. The variation in FCSA was given as the standard deviation of the FCSA (SD FCSA). The shape factor of the fiber was calculated as follows: $\text{perimeter}^2/(4\pi \times \text{FCSA})$, where an increased shape factor represents increased angularity.[3]

2.5 | Analysis of fiber type grouping

To assess the extent of fiber type grouping in a cross section, the method of Jennekens et al[30] was used. An "enclosed fiber" is any muscle fiber of a given type surrounded by fibers of the same type only.[30,31] A fiber type group was defined as a group of fibers with at least one enclosed fiber, similar to that used by others.[24] In each cross section, the number of enclosed fibers for each type was counted manually. The prevalence of enclosed fibers (%), reflecting fiber type grouping, was calculated as: $100\% \times n_{enclosed}/n_{total}$, where "$n_{enclosed}$" is the number of enclosed fibers of a given type, and "n_{total}" the total number of muscle fibers of the same type in a region of interest.[30] An "enclosing fiber" was defined as a fiber that surrounds an "enclosed" fiber. The remaining fibers that were neither "enclosed" nor "enclosing" fibers were called "remaining" fibers.

To determine the extent to which the muscle fibers were enclosed by chance, a mathematical model was used that has been

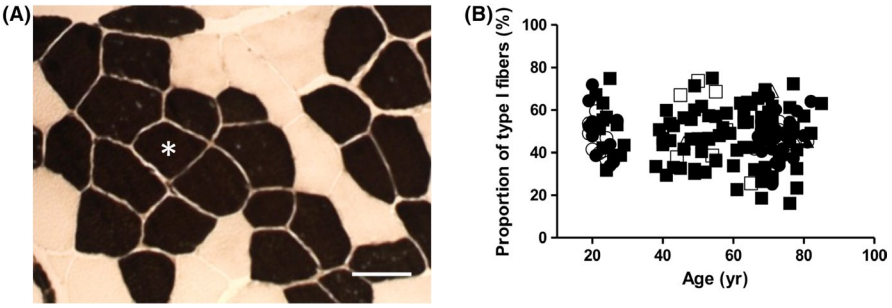

FIGURE 1 A, Example of a m. vastus lateralis muscle biopsy cross-section obtained from an 85-year-old man and stained for myosin ATPase after pre-incubation at pH 4.30. Type I fibers were stained black and type II stained light. *: an enclosed type I fiber. Scale bar = 50 μm. B, The relationship between age and proportion of type I fibers in male (●) and female (○) control, male (■) and female (□) power, and male (▲) and female (△) endurance athletes (n = 157)

applied previously to 36 different human muscles with a large difference in fiber type composition.[32] The model assumes a random spatial arrangement of the fibers of the two main histochemical types (type I and type II fibers). Additionally, it is assumed that the proportion of type I fibers is constant throughout a cross section. The number of neighbors for each fiber in a cross section was counted. The prevalence of the expected fiber type grouping (%) was calculated as follows:

$$\%\ \text{Fibers of a given type enclosed by chance} = 100 \times P^{n+1}$$

"P" the proportion of a given fiber type in the cross section and "n" the number of fibers surrounding a fiber of a particular type in the cross section. Only very occasionally enclosing fibers were on the edge of the region of interest and considered enclosing fibers.

2.6 | Statistical analyses

All data were analyzed with IBM SPSS version 25. The Kolmogorov-Smirnov test showed that all data had a normal distribution.

For height, body mass, BMI, muscle thickness, and fiber type composition, a two-way ANOVA was applied with as factors sex and group (young (YC) and old non-athletes (OC), and young (YA), middle-aged (MA), and old (OA) athletes). For FCSA, % enclosed fibers (grouping) and the shape factor a repeated-measures ANOVA was used with as within-factor fiber type and between-factor sex and group. To assess whether the fiber type grouping was more than expected by chance, we performed a similar analysis, but with the addition of expected grouping as a within factor. To assess whether the size of enclosed, enclosing, and remaining fibers differed from each other and between groups, a repeated-measures ANOVA was performed with as within-factor 1) the condition of the fiber (three levels: enclosed, enclosing, and remaining) and as between-factors 2) fiber type (as in some cases only type I or type II grouping was present), sex and group. In the case of significant age effects or interactions, a Bonferroni *post hoc* analysis was performed to identify the significant differences between groups. Unless otherwise specified, all data are expressed as mean ± SEM. Differences were considered significant at $P < .05$.

3 | RESULTS

3.1 | Participant characteristics

In the figures, data from endurance athletes are illustrated where indicated, but not included in the statistical analyses as there were only 5 endurance athletes in the population.

Physical characteristics are shown in Table 1. Women were shorter than men ($P < .001$) and had a lower body mass ($P = .001$). It can be seen that athletes were in general taller than non-athletes ($P < .05$), and the OA were shorter

TABLE 1 Participant characteristics and *m. vastus lateralis* thickness of male and female athletes and non-athletes in different age groups

		YC	OC	YA	MA	OA	Effects (*P*-values)		
N Men/women		**N = 22 14/8**	**N = 35 27/8**	**N = 14 14/0**	**N = 51 44/7**	**N = 35 32/3**	**Sex**	**Group**	**Sex x Group**
Age (yr)		22.0 (2.7)	72.9 (4.0)	23.9 (3.0)	52.3 (8.3)	71.8 (4.9)			
Height (m)	M	1.72 (0.07)	1.69 (0.08)	1.77 (0.05)[a,b]	1.77 (0.06)[a,b]	1.71 (0.05)[b,c,d]	<.001	<.001	0.354
	W	1.66 (0.05)	1.61 (0.07)		1.73 (0.07)	1.63 (0.03)			
Mass (kg)	M	69.2 (13.4)	77.7 (15.6)[a]	76.5 (9.9)[a]	76.5 (9.5)[a]	68.6 (6.6)[b,c,d]	< .01	.002	0.091
	W	61.3 (3.9)	63.2 (8.8)		72.7 (14.9)	51.0 (4.6)			
BMI ($kg \cdot m^{-2}$)	M	23.2 (3.2)	26.8 (3.6)[a]	24.3 (3.1)[b]	24.3 (3.1)[a,b]	23.5 (1.9)[b]	<.001	.003	0.074
	W	22.3 (1.8)	24.3 (1.5)		24.1 (3.3)	19 (1.3)			
Muscle thickness (cm)	M			2.70 (0.27)	2.18 (0.35)[c]	1.86 (0.40)[c,d]	NA		NA
Grouping	M	18 (82%)	31 (89%)	13 (93%)	44 (86%)	34 (97%)			

Note: BMI: body mass index. M = men; W = women. Young (YC) and older (OC) non-athletes, young athletes (YA), middle-aged athletes (MA) and older master athletes (OA). Muscle thickness was only available for male athletes (6 young, 35 middle-aged and 26 older athletes). Grouping indicates the number of participants in the group with fiber type grouping. Data are expressed as mean ± SD.

[a]Different from young non-athletes at $P \leq .029$;

[b]Different from old non-athletes at $P < .05$;

[c]Different from young athletes at $P \leq .002$;

[d]Different from middle-aged athletes at $P \leq .002$; NA: not applicable.

than the YA and MA ($P \leq .002$). The body mass was lowest in the YC and OA ($P < .015$). The BMI of OC was larger than that of YC and all athlete groups ($P < .05$). The muscle thickness of VL was greater in YA than in the MA and OA ($P \leq .002$). Vastus lateralis muscle cross-sectional area correlated negatively with age in the athletes ($r^2 = 0.29$, $P < .001$).

3.2 | Fiber type composition

An example of the myosin ATPase staining for an 85-year-old man is shown in Figure 1A. The proportion of hybrid fibers (intermediate staining intensity) represented less than 1% of the fiber population, and they were excluded from analysis. There was no aging-related difference in fiber type composition (Figure 1B) in either men, women, athletes, or non-athletes.

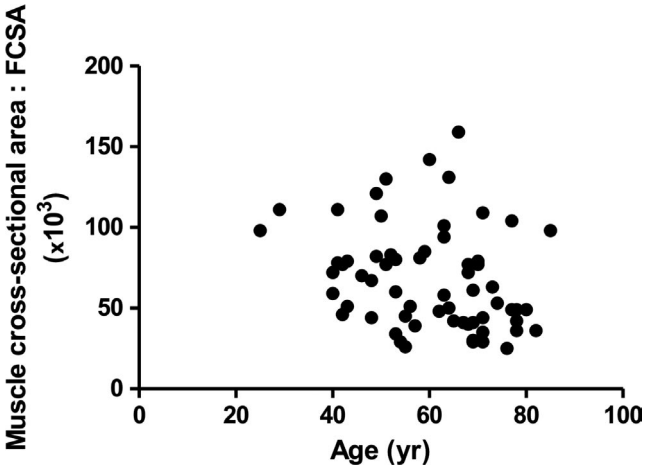

FIGURE 2 Relationship between age and the ratio of muscle cross-sectional area: fiber cross-sectional area (FCSA) in male power athletes (n = 67)

3.3 | Muscle fiber cross-sectional area and fiber size variation

3.3.1 | Fiber cross-sectional area (FCSA)

Muscle cross-sectional area divided by FCSA provides a rough estimate of fiber number. In 67 male power athletes, we were also able to calculate muscle cross-sectional area (from ultrasound obtained thickness) to FCSA ratio. This ratio did not differ significantly between YA, MA and OA, and was not correlated with age (Figure 2). Figure 3 shows the FCSA. For all fibers combined, the FCSA was larger in muscles from men than from women ($P < .001$). A sex × fiber type interaction ($P = .002$) for FCSA was reflected by the larger FCSA for type II than type I in men ($P < .001$), while in women type I fibers were larger than type II fibers ($P = .022$). There was a significant main effect of group (YC, OC, YA, MA, OA) on FCSA of pooled fibers ($P = .001$), but the group x fiber type interaction ($P = .001$) indicated that the effects of group on FCSA differed between fiber types. Indeed, the FCSA of type I fibers did not show a significant association with age in both non-athletes (Figure 3A) and athletes (Figure 3C), but there was a progressive age-related decrement in the FCSA of type II fibers for both non-athletes ($P = .029$; Figure 3B) and athletes ($P \leq .015$; Figure 3D). The absolute age-related decrement in type II FCSA was larger in athletes than in non-athletes (Figure 3B vs. 3D). This was further supported by the observation that while YA had larger fibers than YC ($P < .005$), while there was no significant difference in FCSA between OA and OC (Figure 3; Figure 9A,B).

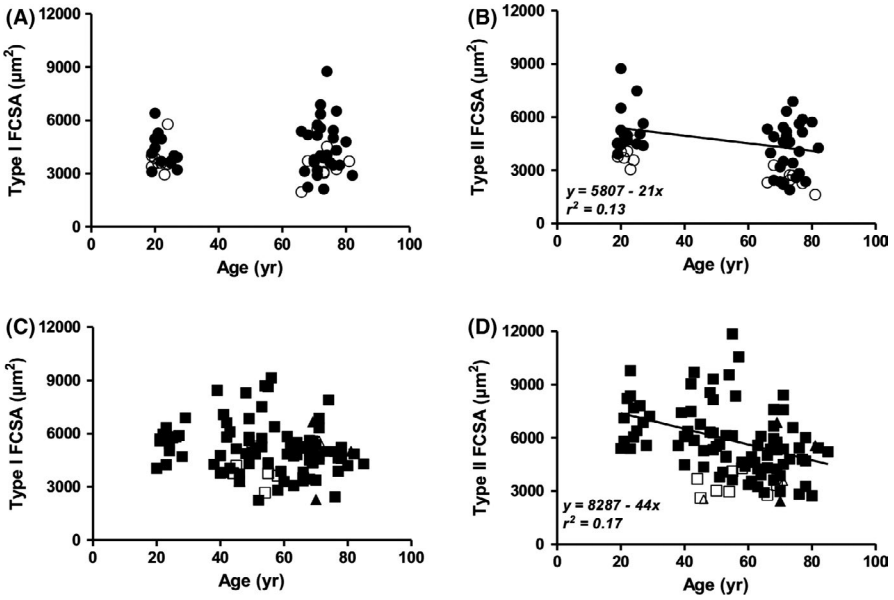

FIGURE 3 Relationship between age and fiber cross-sectional area (FCSA) of type I (A), type II (B) fibers in male (●) and female (○) control (n = 57). C and D, indicate the same relationship in male (■) and female (□) power athletes, and male (▲) and female (△) endurance athletes (n = 100)

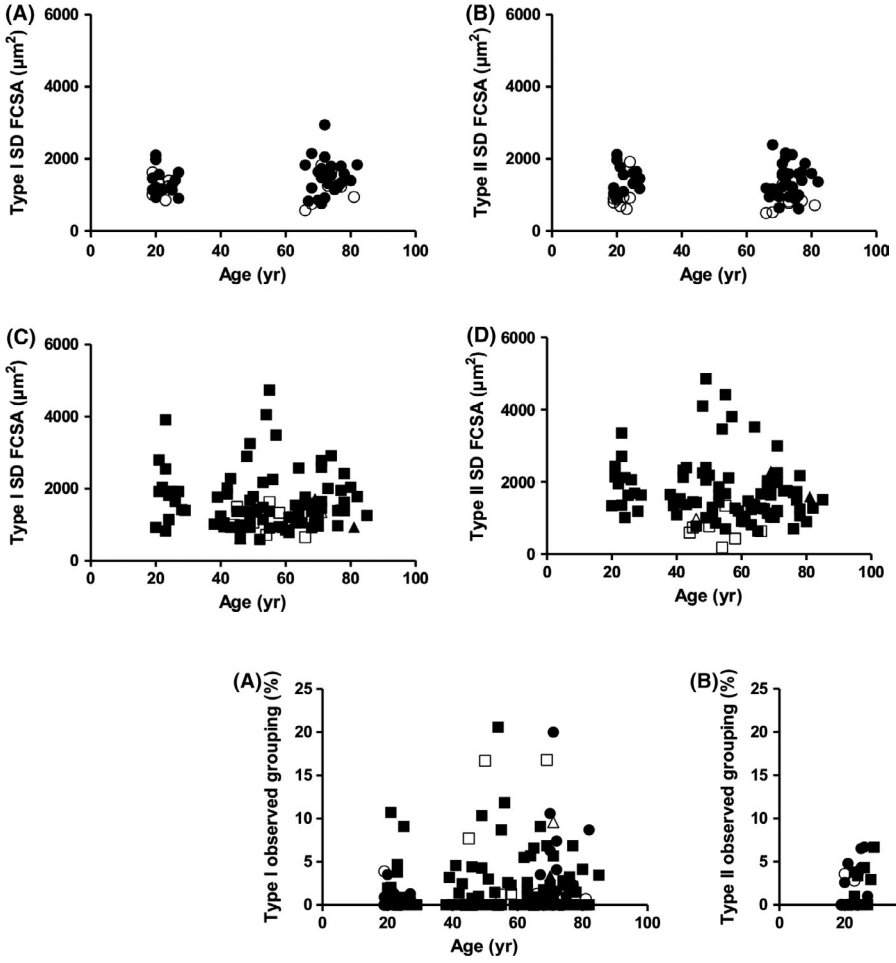

FIGURE 4 Relationship between age and the standard deviation of the fiber cross-sectional area (SD FCSA) of type I (A) and type II (B) fibers in male (●) and female (○) control (n = 57). C and D, indicate the same relationship in male (■) and female (□) power, and male (▲) and female (△) endurance athletes (n = 100)

FIGURE 5 Relationship between age and observed prevalence of grouping of (A) type I and (B) type II fibers in the vastus lateralis muscle of male (●) and female (○) non-athletes, male (■) and female (□) power athletes, male (▲) and female (△) endurance athletes for all subjects combined (n = 157)

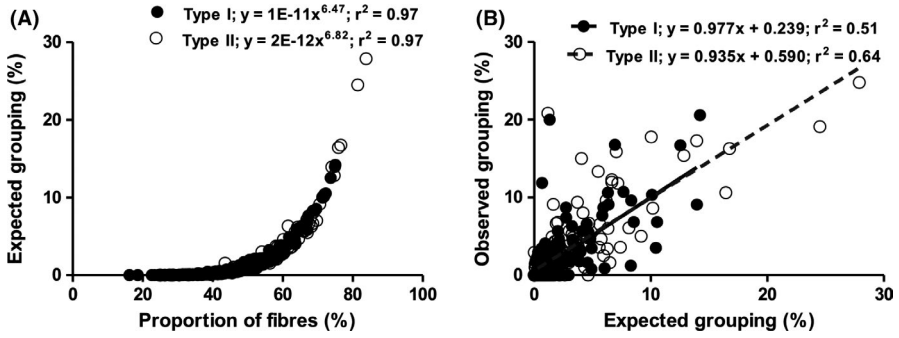

FIGURE 6 A, Exponential correlation between the prevalence of expected grouping of type I and type II fibers for the vastus lateralis muscle and fiber type proportions for all subjects combined. B, Relationship between observed and expected grouping of type I and type II fibers for all subjects combined (n = 157). It can be seen that the regression lines for type I (solid line) and II (dashed line) fibers are similar and close to the line of identity

3.3.2 | Fiber size variation

The fiber size variation, reflected by the standard deviation of the fiber cross-sectional area (SD FCSA), did not significantly correlate with age (Figure 4). There was a main effect of fiber type on SD FCSA (P = .014), but the sex x fiber type interaction (P = .002) was reflected by the higher SD FCSA for type I than type II in women

($P < .001$), but no significant difference between types in men.

3.4 | Fiber type grouping

3.4.1 | Number of groups

No significant difference was observed in the proportion of enclosed fibers between men and women or between type I and type II fibers. There was also no significant age-related increase in the observed proportion of enclosed fibers in athletes and non-athletes of either type I (Figure 5A) or type II (Figure 5B) fibers, with at any age participants showing no grouping (Table 1).

There was an exponential correlation between the expected proportion of enclosed fibers and fiber type proportion (Figure 6A). In Figure 6B, the regression analyses showed similar linear correlations between the observed and expected proportion of enclosed fibers for type I and type II fibers that were close to the line of identity ($P < .001$).

The expected grouping did not differ significantly between men and women or between type I and type II fibers. Similar to the observed grouping, also the prevalence of the expected fiber type grouping showed no significant age-related increase (Figure 7A,B), and there was no significant difference between the observed and the expected grouping (Figure 7A,B).

The fiber group size (Figure 8A,B) and number of fiber groups per 1000 fibers (Figure 8C,D) did not differ significantly between groups, men and women, or between type I and type II fibers.

3.5 | Characteristics of grouped fibers

There was no significant difference in FCSA between the enclosed, enclosing and remaining type I (Figure 9A) or type II (Figure 9B) fibers.

The deviation from the normal shape of a fiber is reflected by the shape factor. Although there were main effects of type ($P < .001$) and group (YC, OC, YA, MA, OA;$P = .001$), there were also sex x fiber type ($P = .002$) and group x fiber type ($P = .004$) interactions. Post hoc comparison showed that in both men and women, the shape factor was larger in type II than type I fibers ($P \leq .006$), with no significant sex differences in the shape factor in fibers of each type (Figure 10A-D).

The group x type interaction ($P = .004$) was reflected by the absence of significant differences between groups in the shape factor of type I fibers (Figure 11A), while for type II fibers (Figure 10B) the shape factor was higher in OC than YC ($P < .001$). For type II fibers only, the OC had a larger shape factor than any of the athlete groups ($P \leq .025$), and while OA had a higher shape factor than MA ($P = .007$), it was not significantly different from that in YA.

There was a significant group x condition interaction ($P = .022$). Post hoc tests showed that in the YC, YA, MA, and OA, there were no significant differences in shape factor between the enclosed, enclosing, and remaining fibers (Figure 11A,B). In the OC, the enclosed fibers had a larger shape factor than the enclosing and remaining fibers ($P = .044$).

4 | DISCUSSION

The main observation of the present cross-sectional study was that in contrast to our expectations and despite indications of preferential type II fiber atrophy in both athletes and non-athletes, there was no age-related increases in the number and size of fiber type groups in either controls or athletes. The observed proportion of enclosed fibers, indicative of grouping, was explained by the fiber type composition of the muscle, which showed no significant age-related differences. These histological observations and the similar muscle cross-sectional area to fiber size ratio in young, middle-aged and old athletes do not provide evidence for the common concepts of 1) age-related motor unit remodeling and 2) improved reinnervation with regular physical activity. However, the age-related motor unit loss and enlargement in motor unit size previously reported with electromyographic

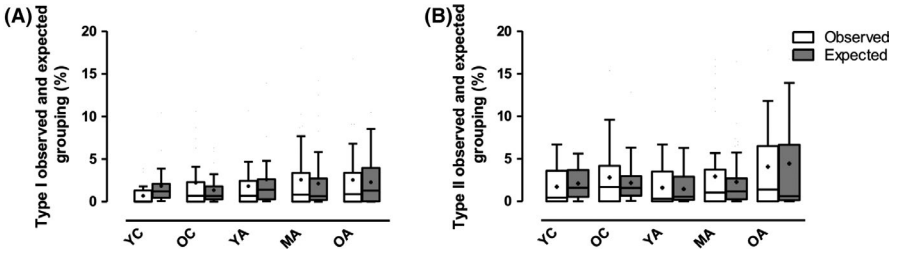

FIGURE 7 Observed and expected grouping for (A) Type I and (B) Type II fibers in the vastus lateralis muscle of young (YC: n = 22) and healthy older (OC: n = 35) non-athletes, and young (YA: n = 14), middle-aged (MA: n = 51) and older athletes (OA: n = 35). Center lines indicate the medians; box limits show the 25th and 75th percentiles. Crosses represent means

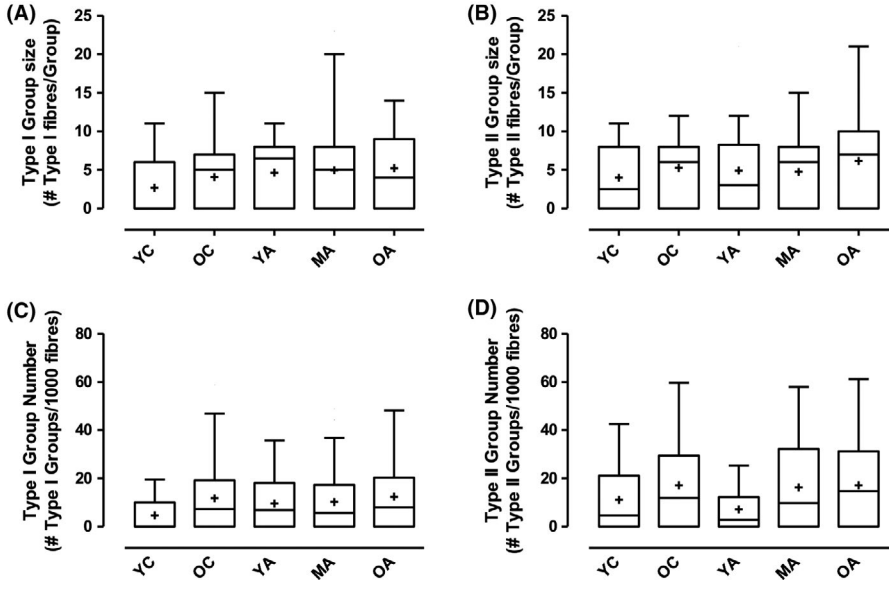

FIGURE 8 A and B, show fiber group size and (C) and (D) indicate number of fiber groups in the vastus lateralis muscle of young (YC: n = 22) and healthy older (OC: n = 35) non-athletes, and young (YA: n = 14), middle-aged (MA: n = 51), and older master athletes (OA: n = 35). Center lines indicate the medians; box limits show the 25th and 75th percentiles. Whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. Crosses represent means.

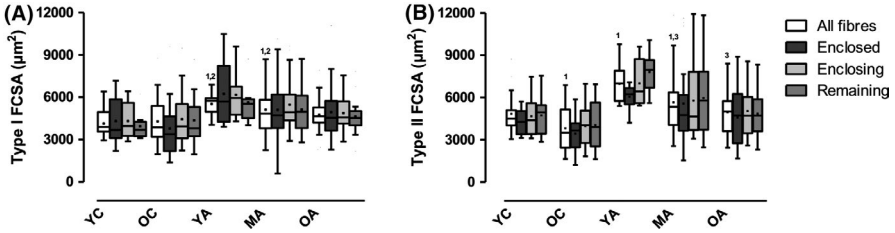

FIGURE 9 Fiber cross-sectional area (FCSA) of (A) type I and (B) type II fibers, respectively in the vastus lateralis muscle of young (YC: n = 22) and older (OC: n = 35) non-athletes, and young (YA: n = 14), middle-aged (MA: n = 51) and older athletes (OA: n = 35). Center lines indicate the medians; box limits show the 25th and 75th percentiles. Crosses represent sample means. Enclosed fibers: fibers surrounded by fibers of the same type only; enclosing fibers: fibers that surround an enclosed fiber; remaining fibers: fibers that are neither an enclosing nor an enclosed fiber. [1] different from YC at $P \leq .045$; [2] different from OC at $P \leq .004$; [3] different from YA at $P \leq .015$

analyses may be reconciled with these histological observations if denervation and reinnervation following motor neuron loss does not necessarily lead to fiber type grouping.

4.1 | Fiber type composition

In line with previous observations,[3,4,28,33-36] the proportion of type I and type II fibers in the *m. vastus lateralis* in both athletes and non-athletes was unaltered with age. It has been reported that older muscles have a higher percentage of hybrid fibers than young muscles (those coexpressing both slow and fast myosin heavy chain (MHC) isoforms),[37] but in this study the proportion of hybrid fibers was negligible in muscles from both old and young-adult athletes and non-athletes.

4.2 | Muscle fiber cross-sectional area (FCSA)

We observed that in athletes, there was an age-related decrease in the size (thickness) of the *m. vastus lateralis*. The absence of a significant age-related decline in the estimated number of fibers (muscle cross-sectional area divided by FCSA) in the *m. vastus lateralis* of athletes suggests that the observed muscle atrophy is explained by muscle fiber atrophy. With the caveat that this is a very rough indicator of fiber number that is affected also by changes in muscle architecture, this is in contrast to previous reports suggesting a similar contribution of fiber atrophy and fiber loss to the age-related atrophy in non-athletes,[1,4] but in line with a previous study showing no age-related loss of fibers.[38] In rat plantaris muscle, it has been reported that the aging-related loss of fibers is attenuated by intensive running exercise[39] and strength training.[40] The discrepancy may therefore be related to differences in physical activity patterns, with higher levels of physical activity of the non-sarcopenic participants[38] and our athletes than that of the recreationally active sarcopenic older people in our previous work.[1] Another possible explanation is that ultrasound underestimates the actual difference in muscle size between young and old when compared with MRI work.[1]

Whatever the cause of the discrepancy, we and previous studies in untrained[1-4,38,41-43] and trained humans[28,44-47] found

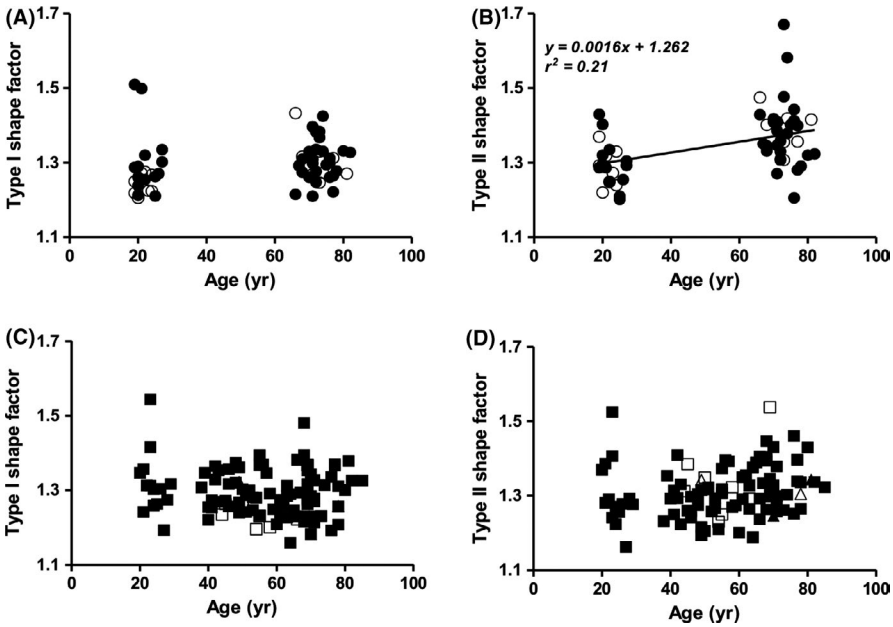

FIGURE 10 Relationship between age and shape factor of (A) type I and (B) type II fibers in male (●) and female (○) non-athletes (n = 57). C and D, indicate the same relationship in male (■) and female (□) power athletes, and male (▲) and female (△) endurance athletes (n = 100). Regression line is indicated for the shape factor of type II fibers in the control group

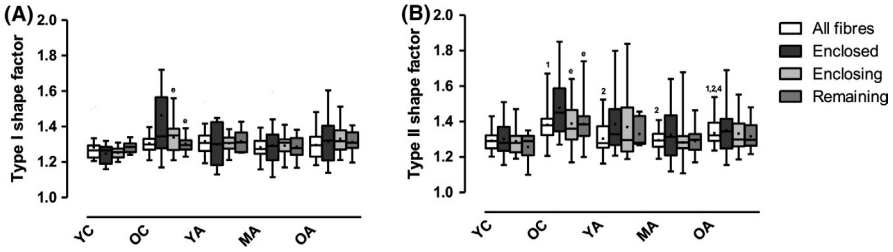

FIGURE 11 The shape factor of (A) type I and (B) type II fibers in the vastus lateralis muscle of young (YC: n = 22) and older (OC: n = 35) non-athletes, and young (YA: n = 14), middle-aged (MA: n = 51) and older (OA: n = 35) athletes. Center lines indicate the medians; box limits show the 25th and 75th percentiles. Crosses represent sample means. [e] different from enclosed fibers within the same group at $P \leq .044$. [1] different from YC at $P \leq .018$; [2] different from OC at $P \leq .025$; [4] different from MA at $P < .007$

that the FCSA of type II fibers was significantly reduced with increasing age, while the size of type I fibers was unaffected. In fact, the absolute slope of the aging-related decrease in type II FCSA was higher in athletes than non-athletes and explains that while young athletes had larger muscle fibers than young non-athletes, no such difference in FCSA was seen between old athletes and non-athletes. Thus, in contrast to previous suggestions that regular physical activity preserves muscle mass and morphology[24,25,48,49] the absolute age-related gains in type II FCSA by regular physical activity were diminished in old age, but the relative gains were most likely similar.

4.3 | Fiber type grouping

4.3.1 | Number of groups

The common assumption is that the aging-related loss of motor neurons[50] is associated with motor unit remodeling that will be apparent as an increase in the number and size of fiber type groups.[51,52] While many studies do report an age-related increase in the prevalence of grouping in both animal[53] and human muscle,[12,54] and even more so in older athletes,[24,55] our data showed no age-related increase in the proportion of enclosed fibers in either non-athletes or athletes. Our study does not stand alone, since others have also reported no age-related change in the prevalence of groups in the human *m. vastus lateralis*.[56]

Although in theory part of the discrepancy between studies may be due to differences in the sarcopenic state of the muscle, no differences in motor unit numbers have been observed in pre-sarcopenic, sarcopenic and severe sarcopenic groups.[57] Another possibility is the age of the participants where the likelihood of grouping has been reported to appear after the age of 70 years.[52] We contend, however, that the discrepancy between our observations and those in many, but not all, of the previous studies is attributable to not taking into account the chance occurrence of fiber type grouping due to the fiber type composition. This is an important consideration as we illustrate an exponential increase in the expected grouping of a fiber of a given type with increasing

proportion of that particular type (Figure 5), where the same pattern occurred when the data of Johnson et al[32] was plotted. Another study reported that 58% of the variance of grouping is explained by fiber type composition.[56] In this context, it is interesting to note that the more pronounced type I grouping in master athletes was associated with a larger proportion of type I fibers than in age-matched non-athletes.[24,55] In fact, Mosole et al[24] show that there is a positive relationship between the proportion of type I fibers and the observed type I grouping, and hence, the observed grouping can be explained by differences in fiber type composition between athletes and non-athletes. In further support of this explanation, we found no significant difference between the expected and observed grouping in young and old athletes, and non-athletes. The absence of an aging-related change in fiber type composition and number of groups challenges the concept of an aging-related denervation-reinnervation process that is thought to lead to fiber type grouping.

4.3.2 | Fiber group size

It is thought that denervation-reinnervation is not only reflected by an increased prevalence of fiber type grouping, but also by an increase in the motor unit size, which may also be reflected in the fiber group sizes. Indeed, previous electromyographic studies have shown an age-related increase in the size of motor units[22,23] and group size in histology.[56] It has been suggested that the larger group number[55] and group size[24] in master athletes than age-matched non-athletes is indicative for a better reinnervation capacity. Yet, in our study, we did not see evidence for an aging-related increase in group size in either athletes or non-athletes. While we did not investigate this relationship, the chance of a large group size increases with increasing proportion of a given fiber type. This can readily be seen in the extreme situation of a muscle consisting of only type I fibers that would present as one extremely large group. Thus, the absence of any differences in fiber group size between any of the groups is explained by the similar fiber type composition between ages and training status. Thus, similar to the absence of an age-related increase in the number of fiber type groups, also the data on group size do not confirm the current concept of an age-related denervation-reinnervation process that is suggested to lead to grouping.

4.3.3 | Characteristic of grouped fibers

The development of fiber type grouping over time is suggested to be a consequence of reinnervation of denervated fibers by adjacent axons from remaining motor neurons. In this scenario, the adopted fiber (s) may change type as they become reinnervated by a motor neuron that innervates fibers of a different type. In line with such a concept, it has been shown that many grouped type I fibers show characteristics, presumably reminiscent, of type II fibers.[56] One of these characteristics was the larger size of grouped than non-grouped type I fibers.[56] We, however, did not find any significant differences in size of type I or type II fibers between grouped and non-grouped fibers. Fiber type- and size-selective denervation-reinnervation therefore appears unlikely to us, and the difference in phenotype seen by Kelly and colleagues[56] may be more a reflection of the continuum of fiber type phenotypes[58] rather than being a legacy of denervation-reinnervation cycles.

As grouped fibers may be the result of denervation-reinnervation, one might argue that in particular these fibers may show signs of denervation or being in the process of reinnervation. It has been suggested that angulated fibers—that can appear flattened, crushed or crescent-shaped—are reflective of such a phenomenon, and they have particularly been reported in muscles of older people.[51,52] In the present study, we calculated the shape factor (an increased value shows deviation from the usual polyhedral shape) to indicate abnormality of fiber morphology. In line with previous observations,[3,59] our data showed that in non-athletes, but not in athletes, the shape factor of type II fibers increased with increasing age, whereas the shape factor of type I fibers remained unaffected. The cause of such a preferential increase in angularity of type II fibers is uncertain, but has been suggested to be a first sign of denervation[60,61] or reorganisation of motor units.[2,59] We consider this unlikely as the absence of significant differences in shape factor between grouped and non-grouped type II fibers suggests that it affects all type II fibers indiscriminately. Further support for another cause than denervation comes from the observation that type II fibers were more angular than type I fibers, even in the young athletes and non-athletes, and the absence of an increase in angularity in denervated rat muscles.[62] Overall, the aging-related increase in angularity of type II fibers may be due to disuse,[63] rather than a denervation-reinnervation process.

5 | IN CONTEXT

Overall, the absence of an aging-related increase in the number and size of fiber type groups challenges the common idea that much of the aging-related muscle wasting is attributable to an ongoing denervation-reinnervation process. The support for such a process is significant and reflected by an age-related loss of fibers in both human[1,4] and rodent muscles.[64] This is, however, not unequivocal, as others report no significant aging-related decrease in fiber number in both human[38] and rodent muscles.[65] The major limitation of all of these studies is that they are cross-sectional designs. Nevertheless,

the aging-related reductions in axons in nerves[8] and loss of motor neurons[50] do suggest that motor neuron loss does indeed occur. Electromyographic studies also suggest almost invariably that motor unit loss occurs during aging as well as an increase in individual motor unit sizes in both athletes and non-athletes,[10,19-21,23,66,67] the latter suggestive of reinnervation. The question thus arises how such an apparent discrepancy between our data and the overwhelming evidence for motor unit remodeling during aging can be reconciled.

Perhaps most important is that an ongoing denervation-reinnervation process is not necessarily unidirectional (eg, only type II fibers being denervated and reinnervated by a type I motor neuron), and in that instance, the fiber type composition remains unaltered. Grouping may then still occur when neighboring axons reinnervate the denervated fibers, which then would result in clusters even when the fiber type composition is unaltered. However, glycogen depletion studies on the distribution of motor unit fibers in aging rats have shown that an increase in the number of fibers per motor unit is accompanied by an increase in the motor unit territory, without evidence of fiber type clustering.[68] In addition, whereas biopsy studies can clearly identify significant remodeling that has caused fiber type transformations (seen as type grouping), it cannot show remodeling that has not resulted in type transformations. These observations and limitations then would reconcile our observations of an absence of fiber type grouping and yet an increase in motor unit size seen in electromyographic studies. If so, histological examination alone may not be sufficient to assess aging-related motor unit remodeling. Many studies have drawn conclusions concerning fiber type grouping in master athletes.[24,56] However, the considerations above and our observation that the fiber type grouping is similar to that expected from fiber type composition indicate that perhaps we need to be careful with drawing firm conclusions from muscle biopsies on age-related motor unit remodeling, unless pathological grouping is evident.

Another important consideration is that almost all, if not all, reports of motor unit loss in old age are cross-sectional and show a large variation in the number of motor units within a muscle between people of the same age.[66,69] It could be that part of the age-related reduction in motor units and motor neurons in cross-sectional studies is not presenting a real loss, but rather a lower number of motor units and motor neurons at birth due to differences in lifestyle (eg, pre/post-natal diet) over the past decades. In fact, in piglets it has been shown that limited intrauterine protein supply has both a negative effect on myogenesis and muscle growth potential,[70] and in rats, the motor neuron survival during embryonic development appears dependent on the number of muscle fibers available for innervation.[71] If this is the explanation, then there is perhaps no age-related motor unit loss, and in this context, it is interesting to note that in a cross-sectional study both motor unit size and number did not differ between middle-aged and old people as reflected by unchanged myelinated axons and muscle fiber number in the intrinsic laryngeal muscles.[72]

6 | PERSPECTIVE

We observed no age-related differences in fiber type grouping in either athletes or non-athletes, and any grouping observed was similar to that predicted by the fiber type composition of the muscle. Thus, previously observed age-related increases in complete fiber type grouping may not so much be a reflection of the aging process, but rather due to underlying pathologies. The absence of any difference in grouping between athletes and non-athletes does not support the concept that exercise increases grouping, which is often employed as an indicator of reinnervation of muscle fibers that have become denervated as a consequence of motor neuron death.

7 | CONCLUSION

In the present study, we found no evidence for an age-related increase in fiber type grouping, and no difference in grouping between athletes or non-athletes. The prevalence of fiber type grouping that was observed was similar to that expected based on the fiber type composition of the muscles. Older age was, however, associated with a smaller fiber cross-sectional area of type II fibers, and this age-related decrement was in absolute terms even more pronounced in master athletes. These findings do not support the common notion of 1) an aging-related motor unit remodeling 2) nor that reinnervation is enhanced with prolonged physical training. The aging-related motor unit loss and increase in motor unit size often seen with electromyographic analyses may be reconciled with these histological observations if denervation and reinnervation following motor neuron loss does not necessarily lead to fiber type grouping.

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CONFLICT OF INTEREST

There is no conflict of interest.

ORCID

Guy A. M. Messa https://orcid.org/0000-0003-1974-1987
Mathew Piasecki https://orcid.org/0000-0002-7804-4631
Ari Heinonen https://orcid.org/0000-0002-3681-9953
Hans Degens https://orcid.org/0000-0001-7399-4841

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