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Short title

A comprehensive normative muscle morphology database

Title

A comprehensive normative reference database of muscle morphology in typically developing children aged 3-18 years – a cross sectional ultrasound study

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ABSTRACT (470 words)

During childhood, muscle growth is stimulated by gradual increase in bone length and body mass, as well as by other factors, such as physical activity, nutrition, metabolic, hormonal and genetic factors. Muscle characteristics, such as muscle volume, anatomical cross-sectional area and muscle belly length, need to continuously adapt to meet the daily functional demands. Pediatric neurological and neuromuscular disorders, like cerebral palsy and Duchenne muscular dystrophy, are characterized by impaired muscle growth, which requires treatment and close follow-up. Nowadays ultrasonography is a commonly used technique to evaluate muscle morphology in both pediatric pathologies and typically developing children, as it is a quick, easy applicable and painless method. However, large normative datasets including different muscles and a large age range are lacking, making it challenging to monitor muscle over time and estimate the level of pathology. Moreover, in order to compare individuals with different body sizes as a result of age differences or pathology, muscle morphology are often normalized to body size. Yet, the usefulness and practicality of different normalization techniques is still unknown, and clear recommendations for normalization are lacking.

In this cross-sectional cohort study, muscle morphology of four lower limb muscles (medial gastrocnemius, tibialis anterior, the distal compartment of the semitendinosus, rectus femoris) was assessed by 3D-freehand ultrasound in 118 typically developing children (mean age 10.35 ± 4.49 years) between 3 and 18 years of age. The development of muscle morphology was studied over the full age range, as well as separately for the pre-pubertal (3-10 years) and pubertal (11-18 years) cohorts. The assumptions of a simple linear regression were checked. If these assumptions were fulfilled, the cross-section growth curves were described by a simple linear regression equation. Additional ANCOVA analyses were performed to evaluate muscle- or gender-specific differences in muscle development. Furthermore, different scaling methods, to normalize muscle morphology parameters, were explored. The most appropriate scaling method was selected based on the smallest slope of the morphology parameter with respect to age, with a non-significant correlation coefficient. Additionally correlation coefficients were compared by a Steiger's Z-test to identify the most efficient scaling technique.

The current results revealed that it is valid to describe muscle volume (with exception of the rectus femoris muscle) and muscle belly length alterations over age by a simple linear regression equation till the age of 11 years. Normalizing muscle morphology data by allometric scaling was found to be most useful for comparing muscle volumes of different pediatric populations. For muscle lengths, normalization can be achieved by either allometric and ratio scaling.

This study provides a unique normative database of four lower limb muscles in typically developing children between the age of 3 and 18 years. These data can be used as a reference database for pediatric populations and may also serve as a reference frame to better understand both physiological and pathological muscle development.

KEYWORDS

Typically developing children, ultrasound, muscle morphology, muscle belly length, muscle volume

MAIN BODY

Introduction

During childhood, the gradual increase in bone length and body mass will stimulate muscle growth (Haines, 1932; Schiaffino *et al.*, 2013; Apibantaweesakul *et al.*, 2021; Kruse *et al.*, 2021). Muscle characteristics such as muscle volume, anatomical cross-sectional area and muscle belly length need to continuously adapt to meet the daily functional demands of growing children (Kubo *et al.*, 2001, 2014; O'Brien *et al.*, 2010a, 2010b; Sparre *et al.*, 2015; Tumkur Anil Kumar *et al.*, 2021). Muscle growth is related to changes in age, body mass and height (Pitcher *et al.*, 2012; Apibantaweesakul *et al.*, 2021; S. Obst *et al.*, 2022) and is also influenced by physical activity, metabolic and hormonal factors, genetic factors and nutrition (Verschuren *et al.*, 2018; Millward, 2021). Muscle volume is often seen as a measure of muscular fitness and is related to muscle power and force generation (Tonson *et al.*, 2008; O'Brien *et al.*, 2009; Pitcher *et al.*, 2012; Orsso *et al.*, 2019). Impaired muscle development may result in limited functioning and participation. Pediatric neurological and neuromuscular disorders, like cerebral palsy and Duchenne muscular dystrophy, are characterized by impaired muscle growth and muscle degeneration, for example reduced muscle volumes, which requires treatment (Graham *et al.*, 2016; Herskind *et al.*, 2016; Barber *et al.*, 2017; Schless *et al.*, 2018; Duan *et al.*, 2021; Evans *et al.*, 2021; Handsfield *et al.*, 2022). Unfortunately, treatment planning is challenging due to the heterogeneous nature of symptoms and natural course of the disorder. Hence, close follow-up is needed to improve the insight in muscle development and muscle-specific problems in different pediatric neurological conditions. Proper quantification of the level of pathology and irregularity in muscle growth requires a comprehensive normative reference database of muscle morphology (Barber *et al.*, 2013b; De Beukelaer *et al.*, 2022).

Nowadays ultrasonography is a technique to evaluate muscle morphology in both pediatric pathologies and typically developing children, as it is a quick, easy and painless method. Three-dimensional freehand ultrasound (3DfUS), combining conventional B-mode ultrasonography with three-dimensional motion tracking, is frequently used to estimate morphological parameters like muscle volume, muscle belly length and tendon length (Cenni, Monari, *et al.*, 2018; Cenni, S. H. Schless, *et al.*, 2018; Hanssen *et al.*, 2021). Due to the accessibility, validity and reliability, ultrasonography and more specifically 3DfUS, seems a promising tool to use for follow-up of muscle development and potential treatment selection (Cenni *et al.*, 2016; Cenni, S. H. Schless, *et al.*, 2018; Schless *et al.*, 2019; Peeters *et al.*, 2020; Vill *et al.*, 2020; De Beukelaer *et al.*, 2021, 2022).

In order to compare individuals with different body sizes as a result of age differences or pathology, muscle morphology data is commonly normalized to body size (Williams *et al.*, 2021). Normalizing muscle morphology data can be done by ratio or allometric scaling (Nuzzo and Mayer, 2013). Ratio scaling, or isometric scaling, is applied by dividing the muscle morphology parameter by a measure of body size, like body mass. Therefore, ratio scaling assumes a simple linear relationship between muscle morphology and measure of body size (Jaric, Mirkov and Markovic, 2005). Allometric scaling, on the other hand, assumes a curvilinear relationship between muscle morphology and body size (i.e. the ratio of change between the two variables does not need to be constant) and is based on the theory of geometric symmetry, stating that all humans have the same architecture and shape, but differ in size (Jaric, Mirkov and Markovic, 2005). Multiple studies in cerebral palsy have reported different ratio scaling methods. Muscle volume is often normalized to body mass (Fry *et al.*, 2007; Malaiya *et al.*, 2007; McNee *et al.*, 2009; Barber *et al.*, 2011, 2016; Pierce *et al.*, 2012; Noble *et al.*, 2014, 2017; Pitcher *et al.*, 2018; Schless *et al.*, 2018, 2019; Cenni, Monari, *et al.*, 2018; Noble, Gough and Shortland, 2019; De Beukelaer *et al.*, 2021; S. J. Obst *et al.*, 2022), the product of body mass and height (Handsfield *et al.*, 2016; Obst *et al.*, 2017; Massaad *et al.*, 2019; De Beukelaer *et al.*, 2022) or bone length (Sîan A Williams *et al.*, 2013; Sîan A. Williams *et al.*, 2013; Haberfehlner *et al.*, 2016; Alexander *et al.*, 2018), whereas, muscle and or tendon lengths are often normalized to bone length (Fry, Gough and Shortland, 2004; Fry *et al.*, 2007; Malaiya *et al.*, 2007; Wren *et al.*, 2010; Hösl *et al.*, 2015; Haberfehlner

et al., 2016; Handsfield *et al.*, 2016; Pitcher *et al.*, 2018; Kruse *et al.*, 2018, 2019; Massaad *et al.*, 2019; De Beukelaer *et al.*, 2022) or muscle tendon unit length (Wren *et al.*, 2010; Hösl *et al.*, 2015; Kruse *et al.*, 2018, 2019; Schless *et al.*, 2018). The different scaling methods make it difficult to compare results and the most useful scaling technique has yet to be defined. Furthermore, clear recommendations are lacking (Williams *et al.*, 2021). To the best of our knowledge, allometric scaling has been rarely used to normalize lower limb muscle morphology in pediatric populations.

Today, only limited studies have investigated muscle morphology of typically developing children. Most previous studies only included normative data as a control group or investigated muscle morphology in 2D only (Scholten *et al.*, 2003; Maurits *et al.*, 2004; Barber *et al.*, 2013b; Jacobs *et al.*, 2013; Lori *et al.*, 2018; Vanmechelen, Shortland and Noble, 2018; Schless *et al.*, 2019; De Beukelaer *et al.*, 2021, 2022; Mogi and Wakahara, 2022). Additionally, some studies calculated or predicted growth rates and growth deficits, by assuming that muscle growth can be described by a simple linear regression (Barber *et al.*, 2013a; De Beukelaer *et al.*, 2022; S. J. Obst *et al.*, 2022; S. Obst *et al.*, 2022). Furthermore, most ultrasound studies have only investigated one muscle, most often the medial gastrocnemius and in limited age ranges. There is limited knowledge on the change in lower limb muscle morphology during puberty and whether boys and girls show differences before and during puberty. In summary, there is need for an accessible comprehensive normative database of muscle morphology data of multiple lower limb muscles in a large cohort of typically developing children with a wide age range, including pubertal ages. Additionally, the success of normalization and whether the selection of scaling method should be muscle-specific needs to be investigated.

In this cross-sectional study, 3DfUS data of four lower limb muscles have been collected for an extended group of children with a typical development and these data have been made available online. The primary aim of this cross-sectional study was to cross-sectionally describe muscle morphology development of the medial gastrocnemius (MG), tibialis anterior (TA), semitendinosus (ST) and rectus femoris (RF) of typically developing children, between the ages of 3 and 18 years. Considering puberty, we expected that muscle development would be more heterogeneous after the age of 11, since the average onset of puberty in girls is 11 years of age, while for boys the average age is 12 years (Tanner and Buckler, 1997; National Health Service (NHS), 2019; Wood, Lane and Cheetham, 2019). Therefore, it was hypothesized that the relation between muscle volume and age and muscle belly length and age can be described by a simple linear equation until the age of 11 years.
The second study aim was to investigate whether there were differences in muscle development (change in muscle volume and muscle belly length) between the four muscles or between boys and girls. It was hypothesized that muscle development is similar between boys and girls before the age of 11 years, due to the limited impact of hormonal changes at these pre-pubertal ages. Additionally, it was expected that muscle development is muscle-specific, due to differences in biomechanical loading, function, muscle stretch and cellular processes between muscles (Braun and Gautel, 2011; Radnor *et al.*, 2018).
Finally, an exploration of ratio and allometric scaling techniques to normalize muscle volume and muscle belly length was performed. It was hypothesized that muscle morphology data can be successfully normalized for differences in body dimensions.

Methods

Participants

This study was approved by the local ethics committees of the University Hospitals Leuven (s59945 TAMTA project, s62187 and s62645 3D-MMAP project) and the University of Ghent (EC/2017/0526) (Ethische Commissie Onderzoek KU Leuven/UZ Leuven, Commissie voor Medische Ethiek - Universiteit Gent). Written informed consent was given by parents or legal guardian or participants of 18 years old. Children above the age of 12 signed an additional informed assent form.

All children who received muscle morphology assessments as part of the ongoing studies (s59945, s62187 and s62645) were enrolled in the database. The aim was to include good-quality 3DfUS images of approximately 100 children for each of the four lower limb muscle. Eventually, a total of 118 typically developing children were recruited through acquaintances and graduate students of the Clinical Motion Analysis Laboratory of the University Hospitals Leuven, KU Leuven and the University of Ghent. All children were aged between 3 and 18 years and had to be fully cooperative and understand the instructions. Children with neurological, neuromuscular or orthopedic disorders, previous lower limb surgery or performing organized sports at an intensive level (over 6 hours a week) were excluded. All measurements were performed at the Clinical Motion Analysis Laboratory of the University Hospitals Leuven and University of Ghent.

Data acquisition

Measures of body mass, height and lower-limb segment lengths were taken. Leg length was measured with a tape measure between the lower edge of the anterior superior iliac spine and the lower border of the medial malleolus. Lower leg length was measured from the medial side of the knee cleft till the lower border of the medial malleolus and for the upper leg length between the greater trochanter and the medial side of the knee cleft, with at tape measure. Additionally, the average duration of sport participation per week was queried. Participants who participated in more than six hours sports per week were excluded.

Only one leg per participant was assessed. In order to have an equal distribution of non-dominant and dominant limbs in the database alternately dominant and non-dominant legs were assessed. Leg dominance was determined by kicking a ball.

Muscle morphology of the MG, TA, distal compartment of the ST and RF was assessed by a previously published 3D-freehand ultrasound (3DfUS) technique (Cenni *et al.*, 2016; Cenni, Monari, *et al.*, 2018; Cenni, S. H. Schless, *et al.*, 2018). Due to the deep origin of the ST, it was difficult to reliably visualize the muscle borders of proximal compartment by ultrasonography for all recruited children. Therefore, to define ST muscle volume, only the distal compartment of the ST was included in the analyses. Ultrasound images were acquired by a 59 mm 10 MHz linear transducer connected to a conventional B-mode US machine (Telemed Echoblaster 128 Ext-1Z, Telemed Ltd., Vilnius, Lithuania). With four reflective markers attached to the transducer, position and orientation of the ultrasound images were tracked by a portable motion tracking device with three fixed optical cameras (Optitrack, V120:Trio, Naturalpoint, Corvallis, OR, USA). Ultrasound settings were kept constant for all acquisitions at a frequency of 8 MHz, a focus of 3 cm, a gain of 64%, a dynamic range of 56 dB and unaltered time-gain compensation. Default settings of image depth was 5 cm. However, image depth was adjusted up to 7 cm in order to visualize the deep muscle border of larger muscles (i.e. the MG and RF in older children). A custom made gel pad, named Portico, was used to limit muscle deformation during acquisition (Cenni, S. Schless, *et al.*, 2018).

The 3DfUS images were acquired by an experienced assessor using the Stradwin software package (version 6.0; Mechanical Engineering, Cambridge University, Cambridge, UK). The muscles were assessed in a resting condition, while the participants were positioned prone or supine. A triangular cushion supported the lower limb to avoid bi-articular stretch on the investigated muscles (images of measurement setup can be found in Schless et al. 2019 (Schless *et al.*, 2019)). A second assessor, controlling the data acquisition computer, performed a quick real-time quality check including visibility of all bony landmarks and muscle borders throughout the full recording. Ultrasound recordings were repeated in case of bad quality data, movement of the subject or observed muscle contraction. In case the muscle size exceeded the transducer's width, a multiple sweep technique was applied, capturing two parallel sweeps to visualize the full width of the muscle. The 3DfUS technique is proven to be reliable to quantify the size and length of all four muscles. A recent reliability study by our research group showed intra-class correlation coefficients (ICCs) values between 0.933 and 0.998 with relative standard error of measurement (%SEM) of 1.6 to 12.6% for muscle volume estimates, and ICCs ranging

between 0.934 and 0.997 with relative SEMs of 0.9 to 3.2% for muscle belly length estimates(Hanssen et al. unpublished data – under review)(Hanssen *et al.*, 2022).

Data analyses

The 3DfUS data were analyzed with the above specified Stradwin software. Extensive quality checks were performed before processing the files. Quality was judged based on the smoothness of the 3D reconstruction, potential gaps in the data due to limited visibility of the reflective markers or losing contact with the skin, visibility of muscle borders, and visibility of bony landmarks. In case of bad-quality, the data were excluded from further analyses. Per participant one good quality scan per muscle was used for the final analysis.

Muscle belly and tendon lengths were calculated as the Euclidean distance between anatomical landmarks, which are summarized in Table 1. The muscle belly length was defined between the origin (origin of the distal compartment for the ST) and muscle-tendon junction, while the tendon length was estimated between muscle-tendon junction and tendon insertion. No tendon length was defined for the TA and ST, as the used technique and position of the subject made it difficult to visualize the osteotendinous junction. Tendon lengths were included in the normative database, but were not further discussed in this manuscript as this manuscript focused on the muscle belly only.

Estimates of muscle volume were created based on manual transverse segmentations along the inside of the muscle border in approximately 5% of the collected transverse B-mode ultrasound images following the cubic planimetry technique (Treece *et al.*, 1999; Cenni, S. H. Schless, *et al.*, 2018).

[Table 1.]

Statistical analyses

Statistical analyses were performed in a SPSS software package (version 28; IBM). Data distribution was explored by a Kolmogorov–Smirnov test and visual inspection of the histograms. As all participant characteristics were normally distributed, descriptive statistics were presented by means and accompanying standard deviations.

To address the first hypothesis, the presence of a simple linear relationship was explored in the entire cohort (including all ages) and in two age subgroups referred to as the pre-pubertal (<11 years of age) and pubertal cohort (≥11 years). Additionally, sub-analyses in other age-subgroups were explored to evaluate the age of 10 and 12 years as potential breakpoint caused by puberty onset, by checking the assumptions of a simple linear regression in the age groups up to and after 10 and 12 years.

The assumptions of a simple linear relationship, i.e. normality, linearity, and homoscedasticity were tested. The distribution of the residuals was explored by visually inspecting the Predicted Probability (P-P) plots. Homoscedasticity of the residuals was checked by plotting the predicted values and residuals on a scatterplot. The distribution of residuals had to be random, i.e. no presence of clusters or trends. The P-P and scatter plots were independently judged by two assessors. If the residuals were both normally distributed and homoscedastic, the relation was considered linear.

To address the second hypothesis, slopes per gender or per muscle were compared. In case of a confirmed simple linear relationship between muscle volume or muscle belly length and age in the total, pre-pubertal (<11 years) or pubertal cohort (≥11 years), differences in slope between muscles and between gender were explored by an analysis of covariance (ANCOVA). An interaction variable describing the relationship between the dependent variable (muscle volume or muscle belly length) and the categorical variable (muscle or gender) was created and applied in the ANCOVA to define any significant differences between the slopes of the equations.

To address the third hypothesis, allometric scaling and different previously applied ratio scaling techniques were explored (Williams *et al.*, 2021). Allometric scaling was done by using the equation:

$a = \frac{S}{m^b}$ (Jaric, Mirkov and Markovic, 2005; Nuzzo and Mayer, 2013) ,

more specifically: $a = \frac{muscle\ volume\ (mL)}{body\ mass(kg)^b}$ or $a = \frac{muscle\ belly\ lengt\quad (mm)}{body\ mass(kg)^b}$

a = the allometric scaled muscle morphology parameter, S = the muscle morphology parameter, m = measure of body size (in this stud body mass or height) and b = the derived allometric parameter.

The allometric parameter was the slope of a linear regression between the log-transformed body size measure and log-transformed muscle-morphology parameter. Data on these allometric parameters for muscle volume and muscle belly length are enclosed in the supplementary appendix (Table S7).
In agreement with previous studies that applied ratio-scaling, muscle volume was normalized to body mass (Fry *et al.*, 2007; Malaiya *et al.*, 2007; McNee *et al.*, 2009; Barber *et al.*, 2011, 2016; Pierce *et al.*, 2012; Noble *et al.*, 2014, 2017; Pitcher *et al.*, 2018; Schless *et al.*, 2018, 2019; Cenni, Monari, *et al.*, 2018; Noble, Gough and Shortland, 2019; De Beukelaer *et al.*, 2021; S. J. Obst *et al.*, 2022), height*body mass (Handsfield *et al.*, 2016; Obst *et al.*, 2017; Massaad *et al.*, 2019; De Beukelaer *et al.*, 2022) and height^3(Sîan A Williams *et al.*, 2013; Sîan A. Williams *et al.*, 2013; Haberfehlner *et al.*, 2016; Alexander *et al.*, 2018). Muscle belly length was normalized to height, leg length or segment length (Fry, Gough and Shortland, 2004; Fry *et al.*, 2007; Malaiya *et al.*, 2007; Wren *et al.*, 2010; Hösl *et al.*, 2015; Haberfehlner *et al.*, 2016; Handsfield *et al.*, 2016; Pitcher *et al.*, 2018; Kruse *et al.*, 2018, 2019; Massaad *et al.*, 2019; De Beukelaer *et al.*, 2022). Allometric scaling was applied to normalize muscle volume to body mass and muscle belly length to height. Following the approach of previous studies, normalization techniques were explored for all four muscles over the full age-range.
The best normalization technique was selected based on the smallest slope of the relation (simple linear regression line) between the normalized muscle morphology parameter (volume or muscle belly length) and age, since a perfect normalization was expected to exclude growth, resulting in a horizontal linear regression line (slope=0). Additionally, the correlation between age and scaled muscle morphology parameter had to be non-significant. All non-significant correlation coefficients were compared by a Steiger's Z-test, in order to compare the different ratio and allometric scaling methods (Weiss, 2011; Nuzzo and Mayer, 2013).

Results

The flow chart in Figure 1 summarizes all included, missing and excluded data. The missing data were defined by the quality check. Specifications of these quality problems are described in Figure 1. Missing data only occurred in the MG (3.6% of the parameters, mainly for muscle volume) and the RF (1.7% of the parameters, mainly for tendon length). Due to these missing data, the sample sizes differed between muscles and between outcomes.

[Figure 1.]

Participant characteristics are summarized in Table 2 for the total group of participants and for the four investigated muscles separately.

[Table 2.]

The cross-sectional data on muscle volume and muscle belly length of the MG, TA, distal compartment of the ST and RF with respect to age, body mass and body height are presented in Figure 2 and Figure 3.

[Figure 2.]

[Figure 3.]

Examination of assumptions simple linear relation

The assumptions of a simple linear regression were checked by visual inspection of the P-P and scatter plots in the total population as in both the pre-pubertal (<11 years of age) and pubertal cohort (≥11 years). All findings are summarized in Table 3. An overview of P-P and scatter plots is enclosed in the supplementary data (Table S1-S6). Additionally, we analyzed subpopulations before and after the age of 10 and 12 years, respectively. For subgroups based on 12 years of age as cut-off, the assumptions for simple linear relations were not fulfilled in the group below the age of 12 years. These data confirmed that 11 years of age was the most valid estimation of onset of increased data variability. Linearity was confirmed in the pre-pubertal cohort for the muscle volumes of the MG, TA and ST and muscle belly length of all muscles, but not for RF volume, where increased variability was observed from approximately 9 years of age onwards. Additional analyses revealed that RF volume increase was linear till the age of 9 years. The simple linear regression equations (except for RF volume) are summarized in Table 4.

[Table 3.]

[Table 4.]

Comparison of slopes

As linearity was confirmed in the pre-pubertal cohort for the muscle volumes of the MG, TA and ST and muscle belly length of all muscles, an ANCOVA was performed to detect differences in muscle morphology slopes between muscles and gender. The ANCOVA confirmed different slopes ($p<0.001$) for muscle volume between the MG, TA and ST, indicating muscle-specific volume profiles. Additionally, the slopes of muscle belly length in the pre-pubertal age were significantly different between the four muscles ($p<0.001$).

Muscle volume and belly length growth rates for all four individual muscles are enclosed in the supplementary data in Table S8.

No differences were found between boys and girls in the pre-pubertal cohort (<11 years) for the muscle volume development of the MG ($p=0.231$), the TA ($p=0.446$) and ST ($p=0.098$). Additionally, no differences between gender for muscle belly length development were reported for all four muscles in the pre-pubertal cohort (MG $p=0.940$, TA $p=0.593$, ST $p=0.577$, RF $p=0.446$). Differences in muscle volume and muscle belly length between gender and muscles could not be investigated in the pubertal cohort as the relation of this age group and muscle morphology parameters could not be described by a simple linear regression.

Exploration of normalization techniques

Different normalization techniques based on ratio and allometric scaling were explored in the total cohort. The slopes of the normalized data are presented in Table 5. A table with an overview of all individual normalization techniques and scatter plot overlays of all explorations are enclosed in the supplementary data (Figure S1-S2).

Overall, allometric scaling was found to be the most efficient technique in normalizing muscle volume, based on smallest slope. For MG and TA, the allometric scaling was the only scaling technique resulting in a non-significant correlation over age. No significant differences were found between ratio scaling to height^3 and allometric scaling for volume of the ST distal compartment ($p=0.509$). However, the

Steiger's Z-test revealed that the correlation coefficient of the allometric scaled RF volume was significantly smaller compared to the ratio scaling to body mass*height ($p<0.001$).
Muscle belly length was most thoroughly normalized by either allometric scaling (TA and RF) or ratio scaling to total leg length (ST, distal compartment) or lower leg length (MG). The Steiger's Z-test did not reveal any significant differences between the different scaling methods for muscle belly lengths.

[Table 5.]

Discussion

This investigation provided normative muscle morphology data which can serve as a reference of typical muscle development in studies on pediatric pathological conditions like cerebral palsy and Duchenne muscular dystrophy.
The current study showed that cross-sectional changes in muscle volume of the MG, TA and distal compartment of the ST can be described by a simple linear regression equation until the age of 11 years. From the age of 11 years onwards, the variation in volume of these three lower limb muscles increased, most likely as a result of puberty onset and accompanying hormonal and/or metabolic changes. The variability in growth of the RF seemed to increase already from a younger age onwards, i.e. from approximately 9 years of age (supplementary data (Table S8). Muscle belly lengths of all four muscles could be described by a simple linear regression in the pre-pubertal cohort (<11 years), as the distribution of variances remained equal for all muscles.
The results further suggest that cross-sectional observations in growth are muscle-specific. The MG showed the largest monthly growth rate in muscle volume and the distal compartment of the ST the smallest. The TA showed the largest monthly increase in muscle belly length, whereas the distal compartment of the ST had the smallest growth rate. Furthermore, muscle development of all four muscles was found to be similar for boys and girls until the age of 11 years. No muscle- or gender-specific differences were investigated in the pubertal cohort (≥11 years) as those growth curves could not be described by simple linear regressions.
The preliminary exploration of different normalization techniques by means of allometric and ratio scaling proved that muscle volume and muscle belly length can be corrected for differences in body dimensions, resulting in slopes near zero. We verified that normalized muscle morphology parameters became independent of age. This first exploration indicated that allometric scaling was the most efficient scaling method to normalize muscle volume over the full age range. For muscle belly length, allometric scaling or expressing muscle belly length as a ratio of total leg or bone length were the most promising methods in correcting for anthropometric variability in typically developing children.

Growth rates

To the best of our knowledge, this is the first cross-sectional 3DfUS study describing muscle morphology in typically developing children in a wide age range and for four different lower limb muscles. As muscle volume is an important determinant of muscular fitness and related to muscle strength and power, there is a growing interest in investigating muscle morphology and specifically muscle size measures. Unfortunately, previous studies included small samples that were insufficient to create a valid normative databases as reference for pathological data and these studies focused mainly on the MG (Barber *et al.*, 2013b; Vanmechelen, Shortland and Noble, 2018; Schless *et al.*, 2019; De Beukelaer *et al.*, 2021, 2022; S. Obst *et al.*, 2022).
Barber et al. (2013) and De Beukelaer at al. (2022) calculated cross-sectional growth rates as muscle volume divided by age (expressed in months), in order to express growth deficits in a population of children with spastic cerebral palsy, assuming muscle growth is linear (Barber *et al.*, 2013a; De Beukelaer *et al.*, 2022). The current study confirmed this underlying assumption for children of 3-11

years old and indicated that linear growth rates for muscle volume of the MG, TA and ST can be defined until the age of 11 years.

Previous investigations showed that estimations of growth rates can be a valuable tool to assay the degree of growth deficit or level of pathology in children with cerebral palsy (Barber *et al.*, 2013a; De Beukelaer *et al.*, 2022; S. J. Obst *et al.*, 2022). This methods was also applied to the current data and muscle-specific growth rates are enclosed in the supplementary data, Table S8. The MG volume growth rate calculated in this current study, i.e. 0.64 ml/month expressed as muscle volume per age, was in line with previously reported growth rates ranging between 0.52-0.71 ml/month (Barber *et al.*, 2013a; Herskind *et al.*, 2016; Willerslev-Olsen *et al.*, 2018; De Beukelaer *et al.*, 2022; S. J. Obst *et al.*, 2022; S. Obst *et al.*, 2022). Additionally, the slope of the regression equation of MG muscle volume with respect to age (Table 4), which takes into account the intercept, was approximately 0.57 ml/month. However, in longitudinal studies, where the growth rate is expressed as the ratio of change in muscle volume over the time of follow-up between repeated assessments, higher growth rates up to 1.14 ml/month have been reported (Barber *et al.*, 2013a; S. Obst *et al.*, 2022), suggesting that cross-sectional investigations underestimate muscle growth at certain ages. Therefore, the growth rate calculations seem most suitable for longitudinal designs with a least two assessments. For cross-sectional studies, the slope of the regression equation might serve as a better growth estimate, due to consideration of the intercept. However, the current data suggest that these slopes may still underestimate the amount of muscle growth at specific ages (Table 4). Obst et al. (2020) (S. J. Obst *et al.*, 2022) reported a muscle volume growth rate of the TA of 0.48 milliliters per month, which is in line with a growth rate of 0.41 milliliters per month in the current study. The current study reported an average muscle belly length growth of the MG of 1.75 millimeters per month over the full age range, which is much higher compared to studies in adolescents (10-19 years, 0.42 millimeters per month) and 5-12 year old children (0.83 millimeters per month)(Bénard *et al.*, 2011; Weide *et al.*, 2015). However, like muscle volume, a longitudinal study by Obst and colleagues (2022) showed that muscle belly length increases up to 2.53 millimeters per month (S. Obst *et al.*, 2022). Interestingly, the same study showed that age-predicted growth (based on age–volume or age–length simple linear regression equations), of the MG volume and muscle belly length were significantly underestimated compared to actual (longitudinal) growth over a 12-month period. These findings indicate that caution is needed when predicting muscle volume based solely on age. Therefore, the current study provided additional graphs (Figures 1 and 2) of muscle development over body mass and height, for which the assumptions of a simple linear regression and possible regression equations can also be defined on the available data. It is expected that growth cannot be explained by a change in just one parameter, but represents a combination of parameters, along with additional environmental, genetic and muscle-specific factors.

Increased variation in muscle development during pubertal phase

The fact that muscle volume development over the full age-range (3-18 years) cannot be described by one simple linear regression indicates that changes in muscle volume do not change in direct proportion to changes in age (Smith, 2018). Therefore these parameters need a more refined prediction model than a simple linear regression. Visual inspection of the muscle morphology data with respect to age highlighted increasing heterogeneity in the muscle growth data after the onset of puberty. This was confirmed by lower R^2 values in the pubertal cohort suggesting that age explains less of the variation in muscle volume and length in children aged over 12 years and highlights that other factors co-influence this relation (Table 4). During the pubertal phase, children experience a growth spurt with gender-specific onset, influencing muscle development (Rauch *et al.*, 2004). Our data suggest that muscle development in boys and girls is similar up until the age of 11 years. Bénard et al. confirmed that the MG development in children between 5-12 years of age is not gender-specific (Bénard *et al.*, 2011). However, gender-specific muscle development may be expected from puberty onset onwards, due to hormonal changes, like increasing testosterone levels, that are associated with increased muscle mass and force and altered bone-muscle interaction (Round *et al.*, no date; Bhasin *et al.*, 1996; Braun and Gautel, 2011; Lang, 2011). Therefore, muscle morphology data of boys and girls can be pooled up until the age of 11 years. Additionally, it is likely that children in the pubertal stage

become more active and have different energy demands, resulting in increased muscle growth, compared to children in pre-pubertal stages (Shomaker *et al.*, 2010; Millward, 2021). In contrast to the findings in this current study, De Ste Croix et al. showed that muscle strength and body size, which are assumed to be strongly related to muscle size, were similar between boys and girls up until the age of 14 years in a four-year follow up (De Ste Croix *et al.*, 2002). However, Apibantaweesakul et al. showed that, during childhood, muscles first increase in size, before gaining strength, (Apibantaweesakul *et al.*, 2021). This might explain why there are gender-specific differences in muscle morphology before differences in strength. It should be noted that no strength evaluation was performed in the current study and these findings could therefore not be confirmed.

Alterations in muscle volume in comparison to changes in muscle belly length

Compared to volume, muscle belly length was found to grow more constant with age, indicated by higher R^2 values. Muscle belly length can increase by addition of sarcomeres in series. As some muscles are not parallel fibered, an addition of sarcomeres in parallel and increased fiber cross-sectional area can also result in an increase in muscle belly length (Kruse *et al.*, 2021). The complex combination of muscle growth in parallel and in series may explain the higher heterogeneity in muscle volume growth compared to increase in muscle belly length. Weide et al. showed that in the pennate MG muscle, longitudinal muscle growth was primarily mediated by increases in muscle fascicle diameter (Weide *et al.*, 2015). However, in parallel fibred muscles or muscles with a smaller pennation angle, the addition of sarcomeres in parallel or an increase in muscle fascicle diameter are expected to contribute less to alterations in length, but primarily to alterations in muscle volume.

Muscle belly length showed less variety and can be more easily described by a linear relationship than muscle volume. This suggests that the muscle belly length increases more homogeneous over age compared to muscle volume. However, in disorders affecting muscle belly length, like cerebral palsy, the relation between parallel and serial muscle growth is expected to be altered as a result of the reduced number of sarcomeres in series(Barber *et al.*, 2011; Lieber and Fridén, 2019).

Muscle-specific development

The differences in muscle development between different lower limb muscles may be caused by differences in biomechanical loading, mechanical stress, pennation angle, levels of stretch on the muscle belly and development of the nervous system like motor unit recruitment (Braun and Gautel, 2011; Radnor *et al.*, 2018; Apibantaweesakul *et al.*, 2021). Additionally, density and activity of satellite cell populations, precursors to skeletal muscle cells, is proven to be muscle-specific (Yin, Price and Rudnicki, 2013). Furthermore, Yin and colleagues summarized evidence according heterogeneity in potential of stem cell differentiation between muscles (Yin, Price and Rudnicki, 2013). The diversity in function, architecture and satellite cell characteristics could possibly explain the enlarged variation in development of the RF volume from a young age onward. However, future research will be required to further investigate those muscle-specific satellite cell characteristics. Additionally, Mogi et al. showed that RF muscle thickness was significantly correlated to maturity index, which was based on peak height velocity, in a cohort of 6-18 year old boys (Mogi and Wakahara, 2022). Even though the study by Mogi et al. was limited to boys and investigated muscle thickness by 2D-ultrasound, these results suggest that RF growth is closely related to height, and RF development may be described relative to height rather than age (Mogi and Wakahara, 2022). Furthermore, it should be taken into consideration that the RF is only one part of the quadriceps complex. Therefore, no conclusions can be drawn on the potential influence of the neighboring and synergistic muscles on RF muscle development. This should also be taken into consideration when investigating the isolated MG and ST. The fact that muscle development is muscle-specific might not be surprising due to the above mentioned muscle-specific differences including loading, stress and satellite cell populations. However, since most 3D-ultrasound study are limited to the MG, it is important to note that most previously reported results cannot be generalized to all muscles.

Scaling methods

The exploration of scaling methods showed that muscle volume can be most effectively normalized by allometric scaling, whereas the preferred normalization technique for muscle belly length seemed to be more muscle-specific. However, it should be mentioned that this was a first exploration based on visual inspection of scatter plots against age and description of slopes. Nuzzo and colleagues (2013) confirmed that allometric scaling was more appropriate for normalizing morphology (cross-sectional area and thickness) of the abdominal muscles and lumbar multifidus compared to ratio scaling (Nuzzo and Mayer, 2013). They concluded that ratio scaling was an inappropriate technique for normalizing muscle morphology parameters and allometric scaling is recommended in future studies.
Our aim was to normalize muscle growth over age, to eliminate growth, in order to compare children with different body sizes due to age differences or pathologies. Our results suggest that muscle-specific normalization techniques might be needed for muscle and tendon lengths. Furthermore, different scaling methods might be required to normalize pathological muscle morphology parameters. The fact that slopes are near but not equal to zero suggest that multiple factors relate to increases in muscle volume and belly length over age. Additionally, the fact that some correlation coefficients are still quite high or significant indicate that not all scaling techniques were sufficient to exclude age-induced alterations in muscle morphology.

Recommendations for future studies

In order to further investigate muscle development after the age of 11 years, the datasets for these pubertal (and potentially post-pubertal) ages should be expanded with extra cross-sectional and preferably longitudinal data, to allow explorations stratified per gender and based on different relationships, such as non-linear relations with break points. More complex statistical models and more detailed documentation of pubertal stage, body dimensions and skeletal growth might be required in order to describe the changes in muscle morphology during puberty. Confounding factors like physical fitness and activity, nutritional factors, genetics and endocrine and metabolic factors should also be considered.

Since this study only included healthy participants without any neurological disorders, no conclusions can be drawn on muscle development in pediatric pathologies, and caution is warranted when comparing pathological data with this normative database. Assumptions of simple linear regression equations should also be explored in pathological muscles before performing predictions or calculating growth rates. Especially in disorders where the endocrine or metabolic system are disrupted, skeletal and muscle growth development can be altered (Kao *et al.*, 2019). For example, Bajaj et al. confirmed that muscle volume is related to trabecular and cortical bone architecture in typically developing children, whereas it is shown that disorders affecting the central nervous system, like cerebral palsy, are associated with impaired trabecular bone architecture (Modlesky, Subramanian and Miller, 2008; Bajaj *et al.*, 2015). This highlights once more that the findings in this normative dataset cannot be generalized to pathological muscles.

Limitations

Some limitations have to be acknowledged. First, this study did not include any assessments or data on puberty stage. Therefore, the assumption that the increase in variation of muscle development is due to puberty onset needs to be confirmed. Additionally, the pubertal cohort might also include post-pubertal participants. Future studies that specifically investigate muscle development in the year preceding puberty onset and during pubertal growth spurt along with objective assessments of the onset of the pubertal phase are therefore recommended.
Additionally, participants who participated in more than six hours in sports per week were excluded. The limit of no more than six hours of sports performance a week was set arbitrarily. Yet, physical activity and the balance between active and sedentary time is an important aspect influencing muscle and skeletal growth (Gabel *et al.*, 2015; Meinhardt *et al.*, 2017; Tan *et al.*, 2018; Zymbal *et al.*, 2019; Wu *et al.*, 2021). No detailed documentation on sport participation or physical activity was collected in the included children. However, it should be noted that the majority of participants were physically

active and might therefore not be a valid representation of all children between 3 and 18 years. Since we recruited healthy volunteers, a bias towards more active children might have been introduced.
The majority of participants were Caucasian. No inclusion criteria based on ethnicity were applied. However, some studies suggested that muscle morphology can be different between ethnicities (Kunimasa *et al.*, 2022). We visualized the data of the non-Caucasian children and confirmed that their data were similar to the Caucasian participants (supplementary data Figure S3 and S4). Therefore, we assumed that the inclusion of different ethnicities did not influence the conclusions of this study.
In order to perform a first exploration of the different normalization techniques, it was assumed that the assumptions of a linear regression were met over the full age range and slopes were calculated following a simple linear regression model. Additionally, the allometric parameters were derived from a simple linear regression equation. While a log-transformation of the muscle morphology and body size parameters was performed to estimate the allometric parameter, the assumptions of a linear regression were assumed to be fulfilled for ratio scaling. If those techniques will be investigated in more detail in future studies, these assumptions should be checked.
Finally, in order to investigate muscle growth, a longitudinal design would be more appropriate, but would require a long follow-up period. A future longitudinal study would be able to detect within-individual changes in muscle morphology during growth and study the alterations in muscle morphology throughout the pubertal stages in more detail.

Conclusion

This study provides a unique normative database of four lower limb muscles in typically developing children between the age of 3 and 18 years. These data can be used as a reference database for pediatric populations and may also serve as a reference frame to better understand both physiological and pathological muscle development. Future studies investigating muscle development should consider muscle-specific development, the impact of puberty and potential pubertal differences in gender when investigating muscle morphology and comparing data to other cohorts with different age ranges or pathologies. Based on the results of this current investigation, it is considered appropriate to describe muscle volume (and until the age of 9 years for volume of the RF muscle) and muscle belly length alterations over age by a simple linear regression equation up until the age of 11 years in typically developing children. Normalizing muscle morphology data by allometric scaling is a promising tool for comparison muscle volumes of different pediatric populations. For muscle lengths, normalization can be achieved by both allometric and ratio scaling. However, other normalization techniques might be needed in different pathologies and should be carefully considered while matching data or (control) groups.

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CONFLICT OF INTREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

This study was designed by KD, HF, AVC, PC and CVdB. NP, BH, NDB, IVK, EH and TD were responsible for data collection. NP and KD conducted all presented analyses. All authors have had complete access to the study data throughout the study. NP, KD, PC, BH, NDB, IVK and FW contributed to the interpretation of the results. All authors were involved in the critical revision and editing of the manuscript that was written by NP and KD. All authors approve the final version of the manuscript and agree to be accountable for the content of the work.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Figshare repository DOI:……

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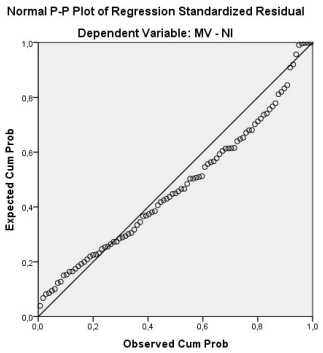
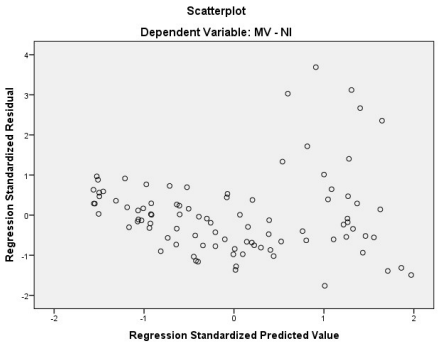
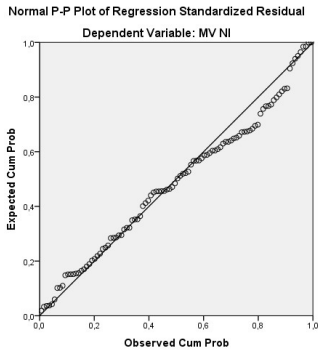
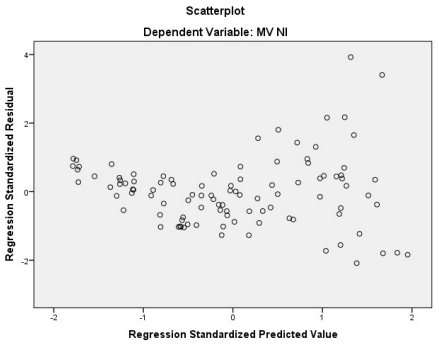
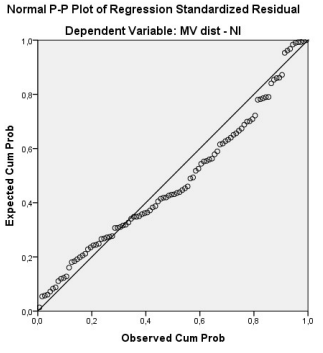
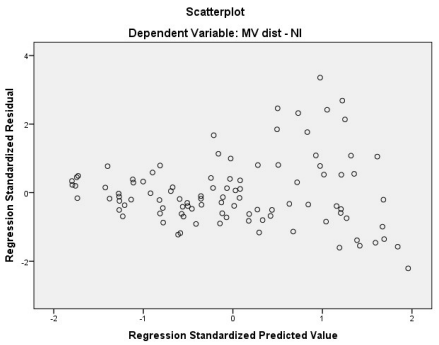
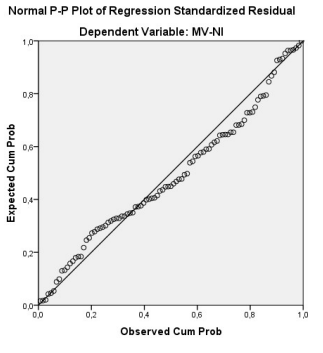
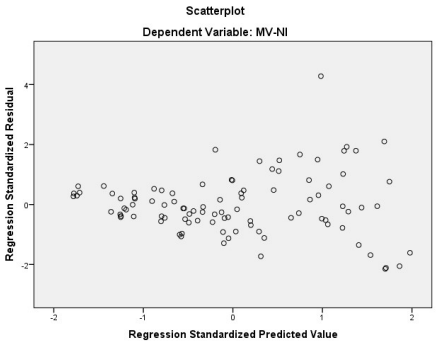
List of abbreviations

3DfUS = three-dimensional freehand ultrasound
ANCOVA = analysis of covariance
B-mode = brightness mode
cm = centimeters
dB = decibels
ICCs = intra-class correlation coefficients
kg = kilograms
MG = medial gastrocnemius
mHz = mega hertz
mL = milliliters
mm = milimeter
n = number
n.a. = not applicable
p = p-value
P-P = predicted probability plot
r = correlation coefficient
R^2= coefficient of determination
RF = rectus femoris
SEM = standard error of measurement
ST = semitendinosus
TA = tibialis anterior

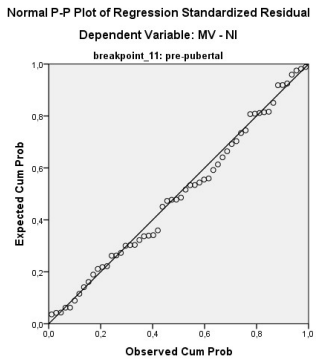
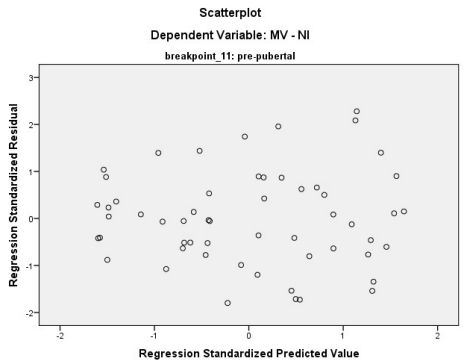
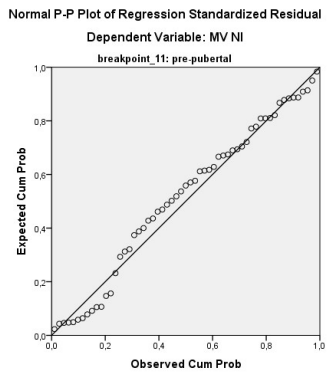
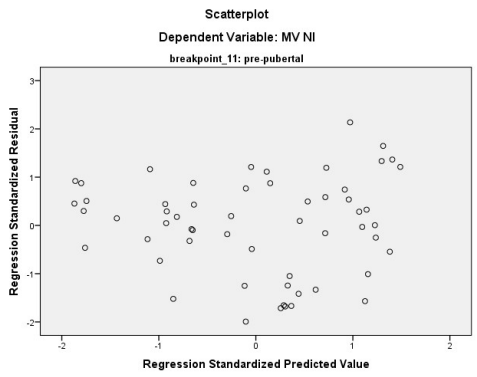
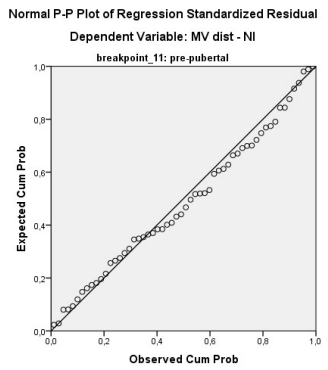
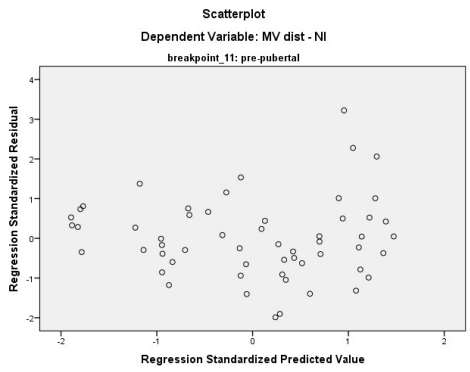
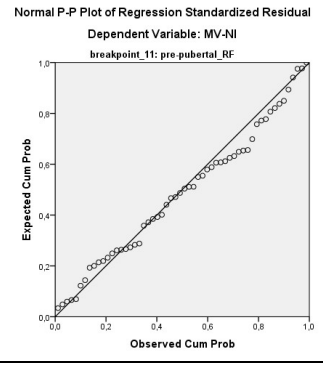
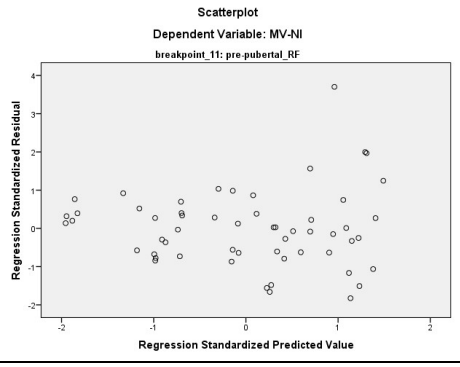
Supplementary material

Part I. P-P and scatter plot to check the assumptions of a simple linear regression

Table S1. P-P and scatter plots of muscle volume for the total cohort

Muscle volume, total cohort	**P-P plot**	**Scatterplot**
Medial gastrocnemius		
Tibialis anterior		
Semitendinosus, distal compartment		
Rectus femoris		

943 **Table S2.** P-P and scatter plots of muscle volume for the pre-pubertal cohort

Muscle volume, pre-pubertal	**P-P plot**	**Scatterplot**
Medial gastrocnemius		
Tibialis anterior		
Semitendinosus, distal compartment		
Rectus femoris		

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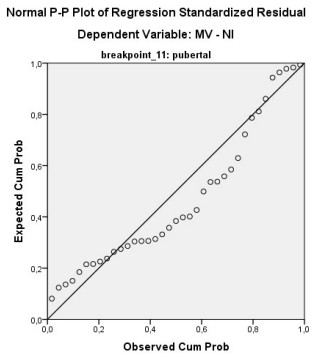
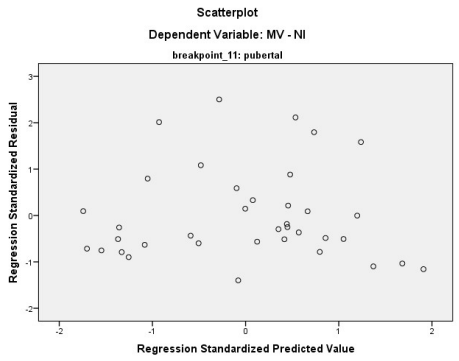
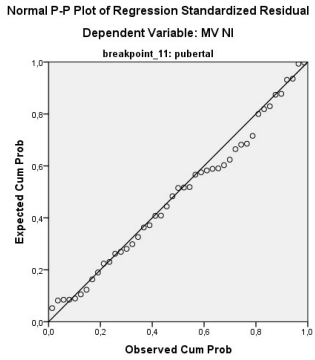
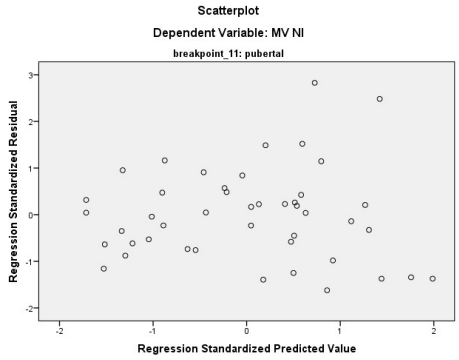
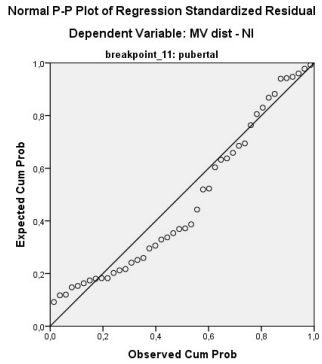
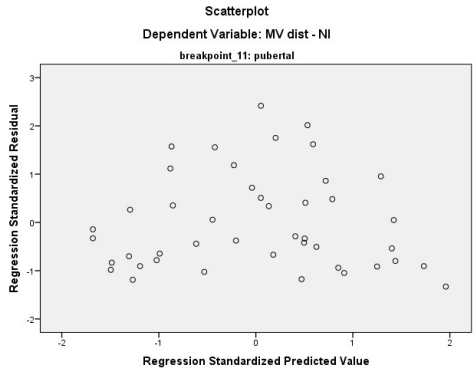
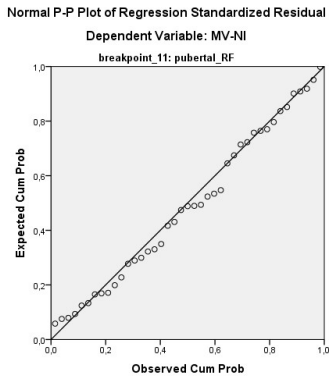
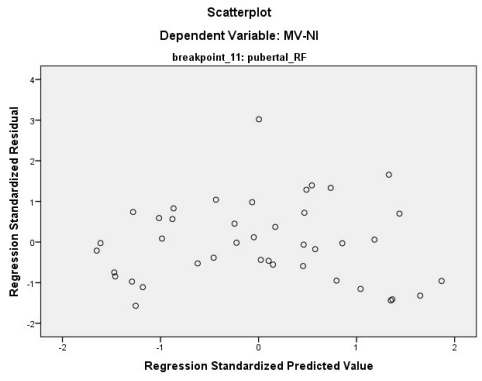
946 **Table S3.** P-P and scatter plots of muscle volume for the pubertal cohort

Muscle volume, pubertal	P-P plot	Scatterplot
Medial gastrocnemius		
Tibialis anterior		
Semitendinosus, distal compartment		
Rectus femoris		

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949 **Table S4.** P-P and scatter plots of muscle belly length for the total cohort

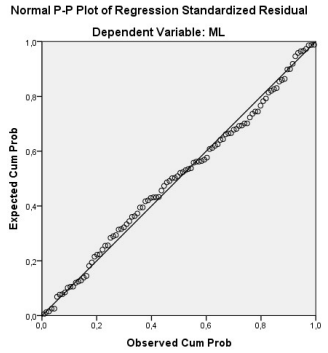
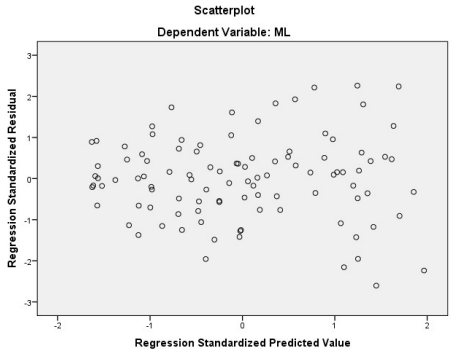
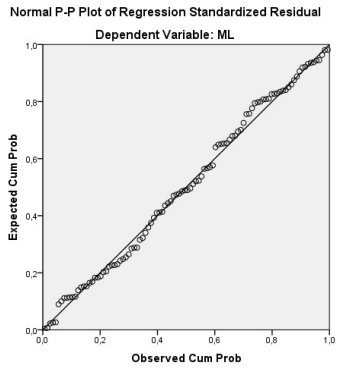
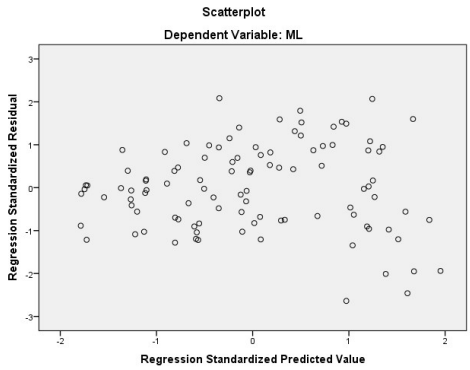
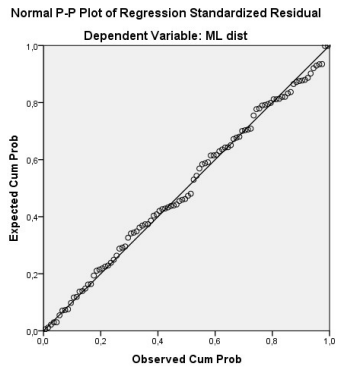
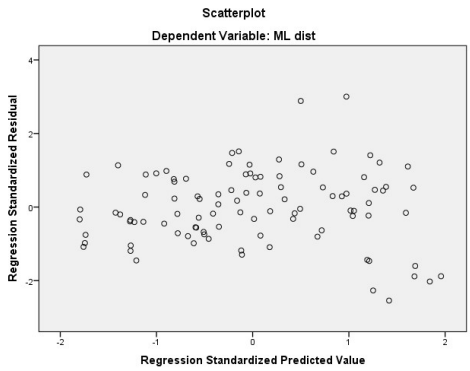
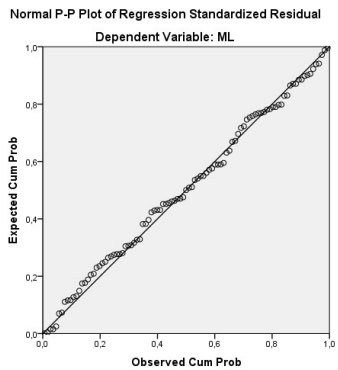
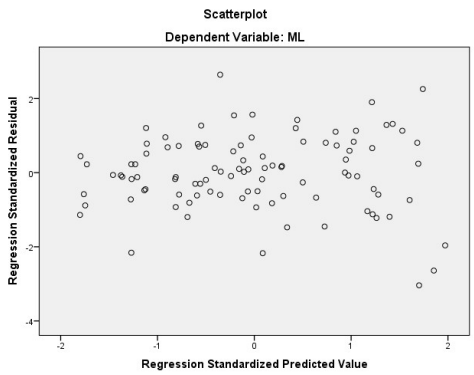
Muscle length, total cohort	**P-P plot**	**Scatterplot**
Medial gastrocnemius		
Tibialis anterior		
Semitendinosus, distal compartment		
Rectus femoris		

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Table S5. P-P and scatter plots of muscle belly length for the pre-pubertal cohort

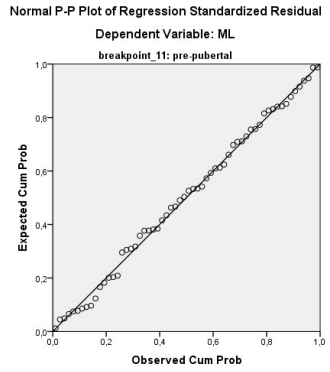
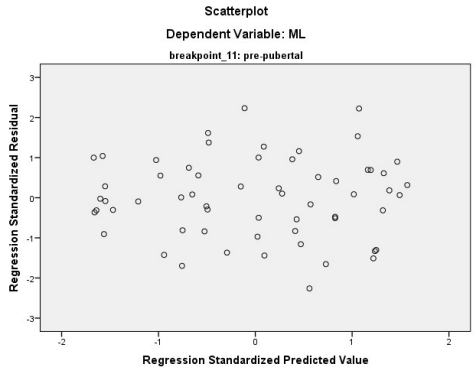
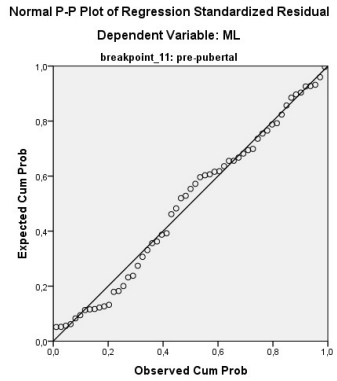
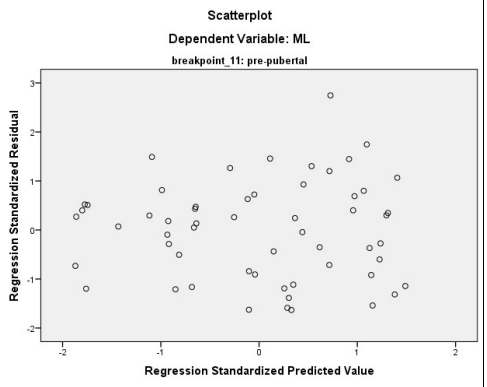
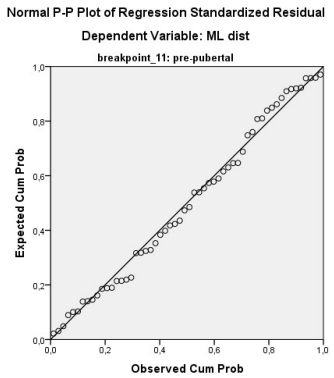
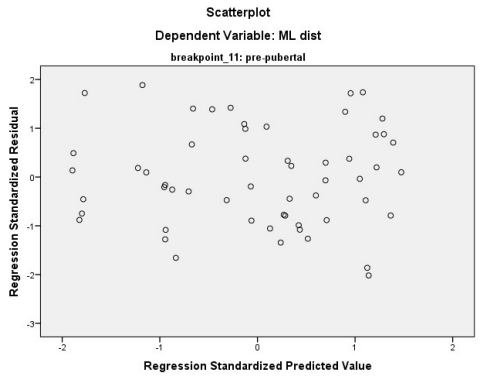
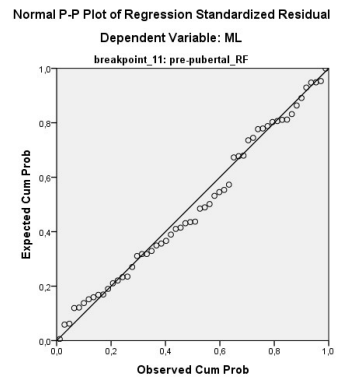
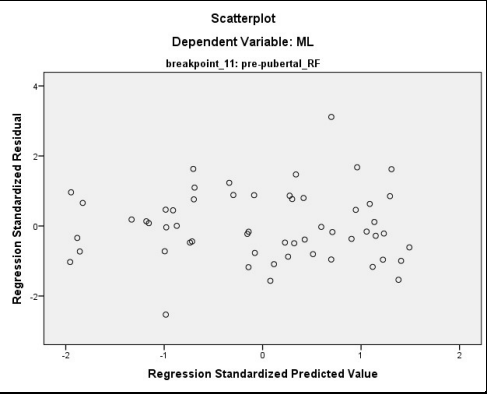
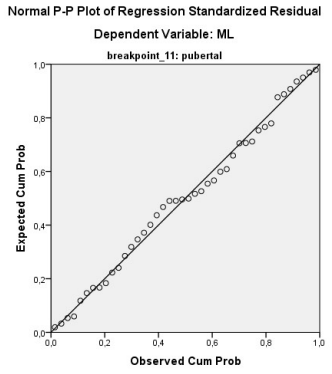
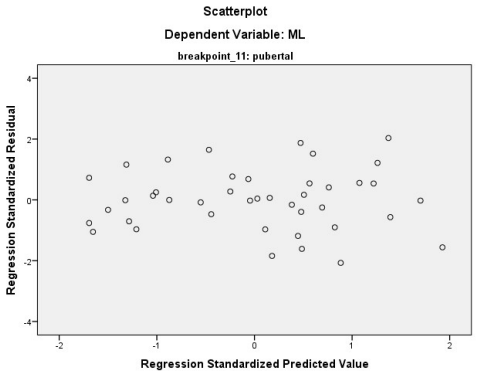
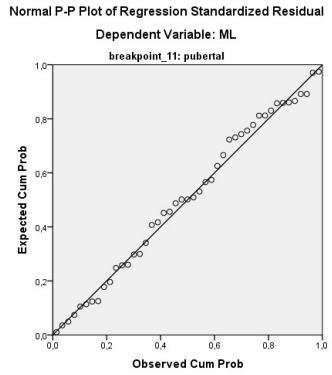
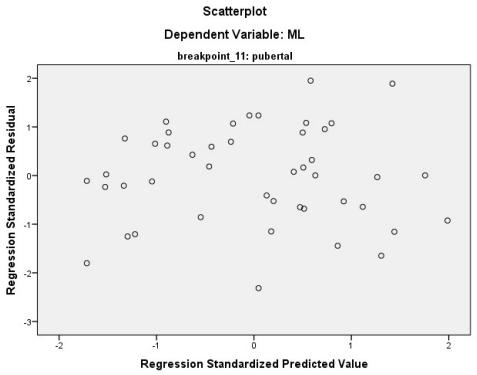
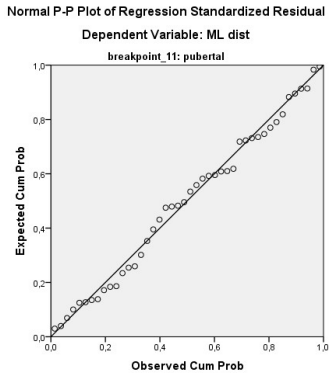
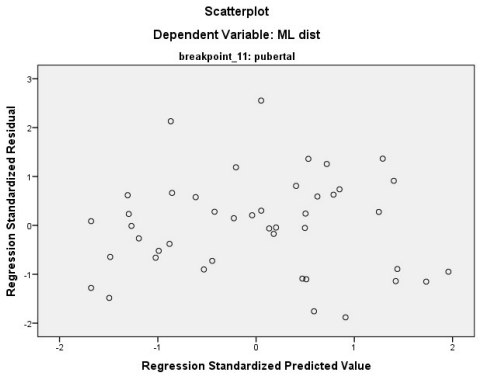
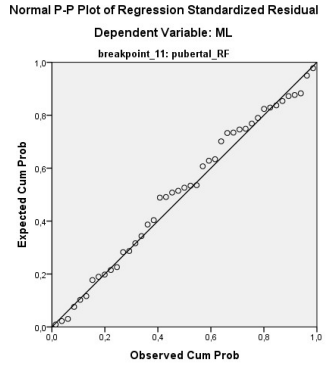
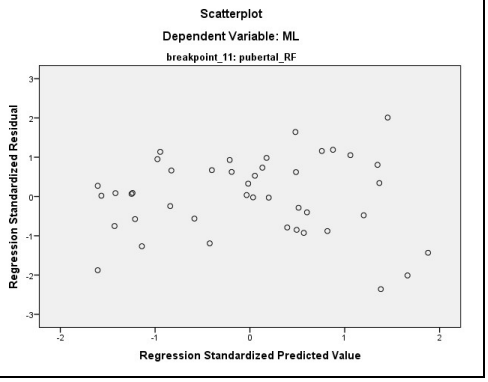
Muscle length, pre-pubertal	P-P plot	Scatterplot
Medial gastrocnemius		
Tibialis anterior		
Semitendinosus, distal compartment		
Rectus femoris		

Table S6. P-P and scatter plots of muscle belly length for the pubertal cohort

Muscle length, pubertal	P-P plot	Scatterplot
Medial gastrocnemius		
Tibialis anterior		
Semitendinosus, distal compartment		
Rectus femoris		

Part II – Allometric scaling parameters

Allometric parameters are the slopes of the regression equations between the log-transformed body size measure and log-transformed muscle morphology parameters. The muscle-specific parameters for both muscle volume and muscle belly length are presented in Table S7.

Table S7. Allometric derived parameters for different muscles and morphological parameters

	Muscle volume	Muscle belly length
Medial gastrocnemius	1.246	1.133
Tibialis anterior	1.229	1.364
Semitendinosus, distal compartment	1.139	1.187
Rectus femoris	1.262	1.233

Part III – muscle-specific growth rates

Muscle-specific growth rates for muscle volume and muscle belly length were calculated by the following formulas following the methods by Barber and De Beukelaer et al.(Barber *et al.*, 2013a; De Beukelaer *et al.*, 2022) :

$$\textbf{growth rate, muscle volume} = \frac{muscle\ volume\ (mL)}{age\ (months)}$$

$$\textbf{growth rate, muscle belly length} = \frac{muscle\ belly\ length\ (mm)}{age\ (months)}$$

Muscle-specific growth rates are presented in Table S8.

Table S8. Muscle-specific growth rates for muscle volume and muscle belly length

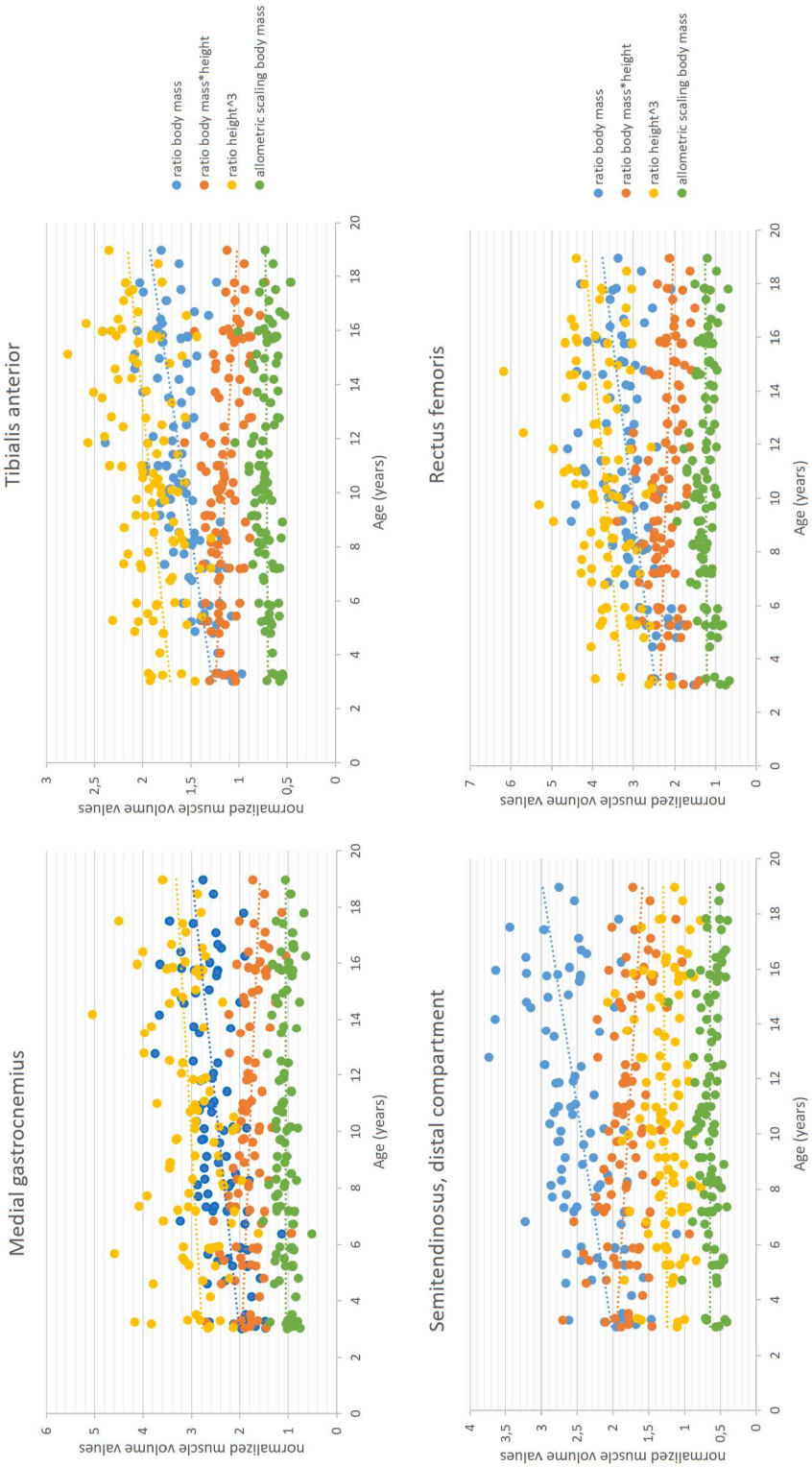
Growth rates MV (mL/month)	Medial gastrocnemius	Tibialis anterior	Semitendinosus, distal compartment	Rectus femoris
Total	0.71 ± 0.19	0.46 ± 0.10	0.30 ± 0.08	0.87 ± 0.22
Pre-pubertal (<11 years)	0.64 ± 0.15	0.41 ± 0.07	0.28 ± 0.07	0.78 ± 0.17
				*0.74 ± 0.14
Pubertal (≥11 years)	0.81 ± 0.19	0.53 ± 0.10	0.33 ± 0.09	0.99 ± 0.22

Growth rates ML (mm/month)	Medial gastrocnemius	Tibialis anterior	Semitendinosus, distal compartment	Rectus femoris
Total	1.75 ± 0.64	2.18 ± 0.60	1.58 ± 0.50	2.29 ± 0.66
Pre-pubertal (<11 years)	2.06 ± 0.66	2.50 ± 0.60	1.85 ± 0.50	2.67 ± 0.65
Pubertal (≥11 years)	1.30 ± 0.17	1.76 ± 0.22	1.23 ± 0.18	1.80 ± 0.19

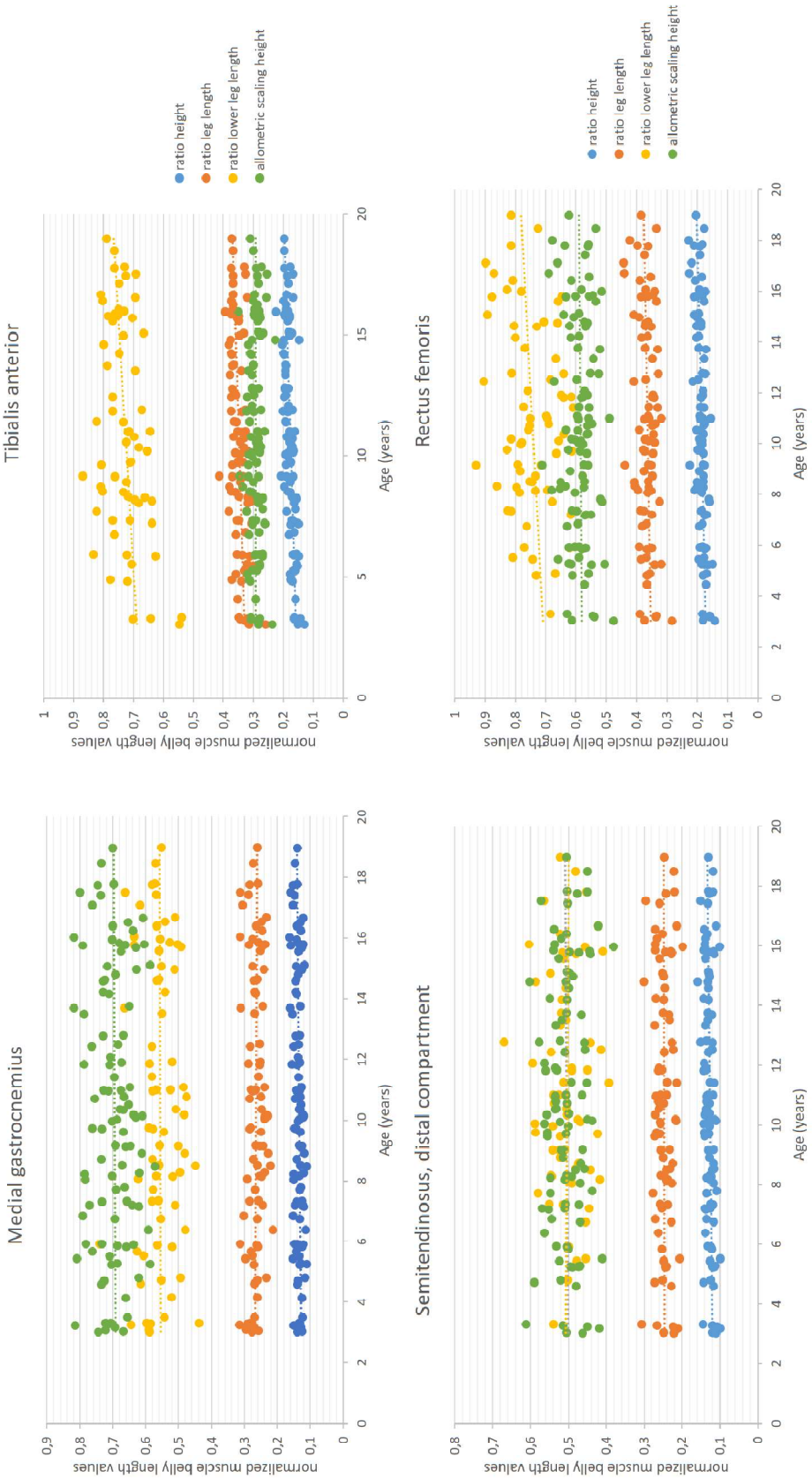
Growth rates are presented as mean ± standard deviation. Muscle volume growth rates are presented as milliliters per month, whereas muscle belly length growth is expressed as millimeter per month.
Highlighted in green = assumptions of a simple linear regression were met. Highlighted in red = assumptions of a simple linear regression were not met and therefore caution is warranted to use the growth rates.
**muscle growth rate <9 years of age.*
ML = muscle belly length, mL = milliliter, mm = millimeter, MV = muscle volume

Part IV. Comparison of different scaling techniques

Figure S1. Comparison of different scaling techniques for muscle volume of the four muscles



991 **Figure S2.** Comparison of different scaling techniques for muscle belly length of the four muscles



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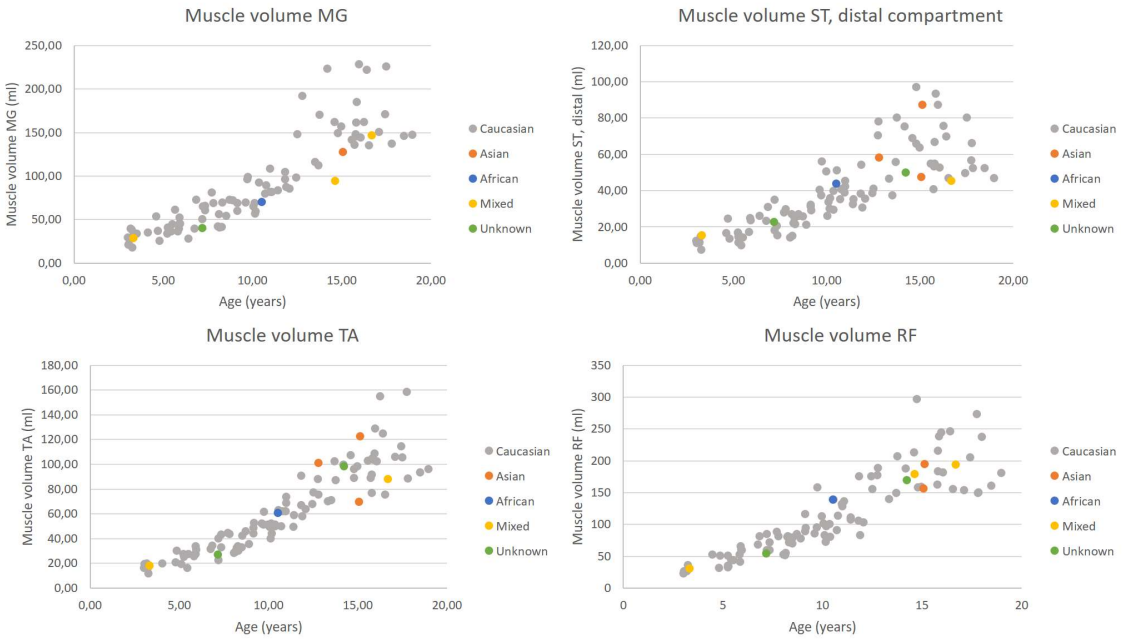
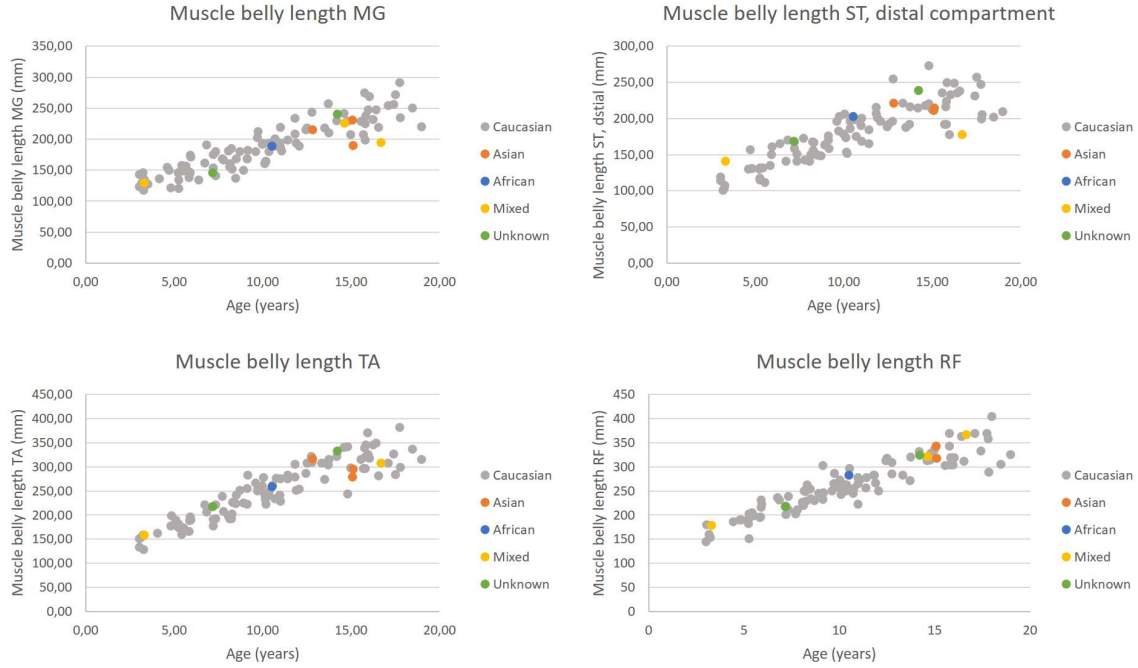
Part V. Presentation of different ethnicities

Figure S3. Presentation of different ethnicities within the full cohort for muscle volume data



MG = medial gastrocnemius, mL = milliliters, RF = rectus femoris, ST = semitendinosus, TA = tibialis anterior

Figure S4. Presentation of different ethnicities within the full cohort for muscle belly length data



MG = medial gastrocnemius, mm = millimeters, RF = rectus femoris, ST = semitendinosus, TA = tibialis anterior

TABLES

Table 1. Anatomical landmarks for calculating muscle and tendon lengths

Muscle	Origin	Insertion
Tibialis Anterior	Fronto-lateral surface of tibia	n.a.
Medial Gastrocnemius	Medial femoral condyle	Most proximal part of the calcaneus
Rectus Femoris	Spina iliaca anterior inferior	Insertion to the proximal edge of the patella
Semitendinosus, distal compartment	Origin distal compartment	n.a.

n.a. = not applicable

Table 2. Participant characteristics

	Total n = 118	MG n = 103	TA n = 102	ST n = 100	RF n = 99
Age (years)	10.35 ± 4.49	10.24 ± 4.42	10.65 ± 4.27	10.66 ± 4.25	10.64 ± 4.23
Gender	Male n=54 Female n=64	Male n=48 Female n=55	Male n=44 Female n=58	Male n=44 Female n=56	Male n=42 Female n=57
Ethnicity	Caucasian n=108 Asian n=3 African n=2 Mixed n=3[i] Missing n=2	Caucasian n=94 Asian n=3 African n=1 Mixed n=3 Missing n=2	Caucasian n=94 Asian n=3 African n=1 Mixed n=2 Missing n=2	Caucasian n=92 Asian n=3 African n=1 Mixed n=2 Missing n=2	Caucasian n=91 Asian n=2 African n=1 Mixed n=3 Missing n=2
Body mass (kg)	35.78 ± 17.24[ii]	35.40 ± 17.23	36.70 ± 17.22	36.64 ± 17.12	36.42 ± 16.45
Height (cm)	139.91 ± 25.15[iii]	139.32 ± 24.73	141.80 ± 24.17	141.76 ± 24.03	141.65 ± 23.51

cm = centimeter, kg = kilogram, MG = medial gastrocnemius, n = number, RF = rectus femoris, ST = semitendinosus, TA = tibialis anterior

[i]Caucasian/Asian, Caucasian/African, Caucasian/Indian, [ii] missing values n=3, [iii] missing values n=1

Table 3. Assumptions of linear regression for muscle volume

		Medial Gastrocnemius		Tibialis Anterior		Semitendinosus distal compartment		Rectus Femoris	
		Normal distribution of residuals	Homo-scedasticity of residuals	Normal distribution of residuals	Homo-scedasticity of residuals	Normal distribution of residuals	Homo-scedasticity of residuals	Normal distribution of residuals	Homo-scedasticity of residuals
Muscle volume	**Total**	X	X	X	X	X	X	X	X
	Pre-pubertal (<11 years)	√	√	√	√	√	√	X	X
	Pubertal (≥11 years)	X	X	√	√	X	√	X	√
Muscle belly length	**Total**	√	√	√	X	√	√	√	X
	Pre-pubertal (<11 years)	√	√	√	√	√	√	√	√
	Pubertal (≥11 years)	√	√	√	√	√	√	X	X

The green checkmark indicates that the assumption was met, whereas a red cross indicates that the assumption was not fulfilled. If both assumptions were met, the muscle- and cohort-specific box are highlighted in green.

Table 4. Simple linear regression equations describing muscle development of the four muscles

Muscle volume					
Muscle	**cohort**	**Simple linear regression equation**	**r**	**R^2**	**p-value**
Medial gastrocnemius	pre-pubertal	5.497 + 6.788 * age in years	0.819	0.671	<0.001
Tibialis anterior	pre-pubertal	-2.432 + 5.172 * age in years	0.890	0.792	<0.001
	pubertal	-6.261 + 6.713 * age in years	0.625	0.391	<0.001
Semitendinosus, distal compartment	pre-pubertal	-2.685 + 3.754 * age in years	0.794	0.630	<0.001
Muscle belly length					
Muscle	**cohort**	**Simple linear regression equation**	**r**	**R^2**	**p-value**
Medial gastrocnemius	pre-pubertal	104.766 + 7.666 * age in years	0.792	0.628	<0.001
	pubertal	132.180 + 6.416 * age in years	0.547	0.299	<0.001
Tibialis anterior	pre-pubertal	101.659 + 7.704 * age in years	0.892	0.796	<0.001
	pubertal	192.315 + 7.760 * age in years	0.525	0.276	<0.001
Semitendinosus, distal compartment	pre-pubertal	82.181 + 9.709 * age in years	0.840	0.706	<0.001
	pubertal	151.679 + 4.165 * age in years	0.369	0.136	0.014
Rectus femoris	pre-pubertal	119.667 + 14.104 * age in years	0.887	0.788	<0.001

r=correlation coefficient, R^2= Coefficient of Determination; proportion of the variance for a dependent variable that is explained by an independent variable. Pre-pubertal (0-10 years), pubertal (11-18 years)

Table 5. Slopes of different normalization techniques for muscle volume and muscle belly length in the total cohort

Normalization of muscle volume				
	ratio scaling			**allometric scaling**
	body mass	**body mass*height**	**height^3**	**body mass**
Medial Gastrocnemius	0.061 *(n=91, r=0.545, p=<0.001)*	-0.023 *(n=90, r=0.336, p=0.001)*	0.033 *(n=92, r=0.244, p=0.019)*	0.001 *(n=91, r=0.016, p=0.880*
Tibialis Anterior	0.040 *(n=99, r=0.612, p=<0.001)*	-0.014 *(n=98, r=0.381, p=<0.001)*	0.027 *(n=101, r=0.396, p=<0.001)*	0.002 *(n=99, r=0.073, p=0.472)*
Semitendinosus, distal compartment	0.014 *(n=97, r=0.227, p=0.025)*	-0.017 *(n=96, r=0.415, p=<0.001)*	0.003 *(n=99, r=0.046, p=0.648)*	0.000 *(n=97, r=0.007, p=0.943)*
Rectus Femoris	0.081 *(n=95, r= 0.512 p=<0.001)*	-0.019 *(n=94, r=0.201, p=0.052)*	0.057 *(n=96, r=0.344, p=<0.001)*	0.002 *(n=95, r=0.042, p=0.689)*
Normalization of muscle belly length				
	ratio scaling			**allometric scaling**
	height	**leg length**	**lower/ upper leg length**	**height**
Medial Gastrocnemius	0.0008 *(n=101, r=0.302, p=0.002)*	-0.0003 *(n=86, r=0.057, p=0.603)*	0.0003 *(n=70, r=0.026, p=0.832)*	0.0004 *(n=101, r=0.028, p=0.782)*
Tibialis Anterior	0.002 *(n=101, r=0.644, p=<0.001)*	0.002 *(n=86, r=0.441, p=<0.001)*	0.005 *(n=64, r=0.342, p=0.006)*	$-5.136*10^{-6}$ *(n=101, r=0.001, p=0.991)*

Semitendinosus, distal compartment	0.001 *(n=99, r=0.300, p=0.003)*	$9.349*10^{-5}$ *(n=83, r=0.019, p=0.862)*	0.0005 *(n=64, r=0.032, p=0.800)*	-0.0004 *(n=99, r=0.041, p=0.686)*
Rectus Femoris	0.002 *(n=98, r=0.503, p=<0.001)*	0.002 *(n=84, r=0.235, p=0.032)*	0.004 *(n=67, r=0.203, p=0.099)*	0.001 *(n=98, r=0.061, p=0.551)*

n = number of cases, n.a. = not applicable, r = correlation coefficient, p = p-value
The most appropriate scaling method per muscle are highlighted in blue.

FIGURE LEGENDS

Figure 1. Flowchart of included muscle morphology data
ML = muscle length, MV = muscle volume, n = number, TL = tendon length

Figure 2. Scatter plots of cross-sectional data of the muscle volume with respect to age, body mass and body height of all four lower limb muscles for the total participant population

Figure 3. Scatter plots of cross-sectional data of the muscle belly length with respect to age, body mass and body height of all four lower limb muscles for the total participant population