Received: 21 November 2023 | Revised: 29 January 2024 | Accepted: 9 February 2024

DOI: 10.1111/apha.14118

ACTA PHYSIOLOGICA

RESEARCH PAPER

Sex differences in neuromuscular and biological determinants of isometric maximal force

Gaia Giuriato[1,2] | **Maria Grazia Romanelli**[1] | **Desirée Bartolini**[3] | **Gianluca Vernillo**[4,5] | **Anna Pedrinolla**[1,6] | **Tatiana Moro**[7] | **Martino Franchi**[7] | **Elena Locatelli**[1] | **Mehran Emadi Andani**[1] | **Fabio Giuseppe Laginestra**[1,8] | **Chiara Barbi**[1] | **Gloria Fiorini Aloisi**[1] | **Valentia Cavedon**[1] | **Chiara Milanese**[1] | **Elisa Orlandi**[1] | **Tonia De Simone**[1] | **Stefania Fochi**[1] | **Cristina Patuzzo**[1] | **Giovanni Malerba**[1] | **Paolo Fabene**[1] | **Massimo Donadelli**[1] | **Anna Maria Stabile**[9] | **Alessandra Pistilli**[9] | **Mario Rende**[9] | **Francesco Galli**[3] | **Federico Schena**[1] | **Massimo Venturelli**[1,10]

[1]Department of Neurosciences, Biomedicine and Movement Sciences, University of Verona, Verona, Italy

[2]Surgical, Medical and Dental Department of Morphological Sciences Related to Transplant, Oncology and Regenerative Medicine, University of Modena and Reggio Emilia, Modena, Italy

[3]Department of Pharmaceutical Sciences, University of Perugia, Perugia, Italy

[4]Department of Biomedical Sciences for Health, Università degli Studi di Milano, Milan, Italy

[5]Department of Social Sciences, University of Alberta – Augustana Campus, Camrose, Alberta, Canada

[6]Department of Cellular, Computational and Integrative Biology (CIBIO), University of Trento, Trento, Italy

[7]Department of Biomedical Sciences, University of Padova, Padova, Italy

[8]Department of Anesthesiology, University of Utah, Utah, USA

[9]Department of Medicine and Surgery, Section of Human Anatomy, Clinical and Forensic, School of Medicine, University of Perugia, Perugia, Italy

[10]Department of Internal Medicine, University of Utah, Utah, USA

Correspondence
Massimo Venturelli, Department of Neurosciences, Biomedicine and Movement Sciences, University of Verona, Via Casorati 43, Verona 37131, Italy.
Email: massimo.venturelli@univr.it; massimo.venturelli@utah.edu

Funding information
Italian Ministry of Education, University, and Research (MIUR) program's Departments of Excellence, Grant/Award Number: 2018–2022; Fondazione Perugia, Grant/Award Number: 2019.0326

Abstract

Aim: Force expression is characterized by an interplay of biological and molecular determinants that are expected to differentiate males and females in terms of maximal performance. These include muscle characteristics (muscle size, fiber type, contractility), neuromuscular regulation (central and peripheral factors of force expression), and individual genetic factors (miRNAs and gene/protein expression). This research aims to comprehensively assess these physiological variables and their role as determinants of maximal force difference between sexes.

Methods: Experimental evaluations include neuromuscular components of isometric contraction, intrinsic muscle characteristics (proteins and fiber type), and some biomarkers associated with muscle function (circulating miRNAs and gut microbiome) in 12 young and healthy males and 12 females.

Results: Male strength superiority appears to stem primarily from muscle size while muscle fiber-type distribution plays a crucial role in contractile properties. Moderate-to-strong pooled correlations between these muscle parameters

were established with specific circulating miRNAs, as well as muscle and plasma proteins.

Conclusion: Muscle size is crucial in explaining the differences in maximal voluntary isometric force generation between males and females with similar fiber type distribution. Potential physiological mechanisms are seen from associations between maximal force, skeletal muscle contractile properties, and biological markers.

KEYWORDS

gut microbiota, miRNA, muscle force, muscle proteins, neuromuscular determinants, sex differences

1 | INTRODUCTION

Despite the well-established fact that human physiology differs between males and females, much of the research in this area has been disproportionately focused on males.[1,2] Only recently, a renewed effort to examine the impact of sex on integrative physiological responses during exercise has been prompted.[3,4] While it is widely accepted that sex differences in muscular force account for sex disparities in exercise performance,[5] the physiological and molecular basis of these differences are still poorly understood and require further investigation.

Males present superior maximal force, usually 30%–75% greater than females, commonly attributable to the larger skeletal muscle mass.[1,2,6] However, several other factors can affect maximal force expression other than the cross-sectional area of the skeletal muscle.[7,8] Indeed, males usually display higher distribution and area of type II fibers,[9,10] leading to greater force and power production, quicker Ca^{2+} kinetics, and faster shortening-relaxation velocities. On the other hand, females recruit a higher number of motor units during force expression, with a greater neural drive and motor unit discharge rate at maximum contraction intensities.[11] All of these traits are corroborated by the magnitude of gene expression of various hormones,[5,12] such as testosterone and myostatin, which are linked to skeletal muscle mass growth,[13] hence exacerbating anatomical differences.

Moreover, post-transcriptional genetic regulators modulate and may be modulated by exercise performance.[14] Indeed, the circulating miRNAs, important for different cellular processes[15–18] like myogenesis,[19,20] are increasingly being studied as biomarkers in response to different types of exercise.[14,21] However, their role in the individuals' maximal force expression capacity has yet to be investigated. Recently, distinct miRNA expression patterns due to hormonal and genetic differences have been found in males and females.[22–24] Interestingly, 37% of genes have a sex bias in at least one tissue, with a preference for regional genome regulation.[25] Nowadays, sex-biased expression of circulating miRNAs is still poorly characterized.

The human gut microbiome (GM) is another functional biomarker recently linked to physical exercise (see, for review, Boytar, et al[26]). GM has been reported to have close communication with the central nervous system, regulating brain excitability and the related neurochemical pathways,[27] and the type of exercise and lifestyle influences its composition and abundance.[28,29] Indeed, because the gut-brain axis is a bi-directional pathway between the brain and the gastrointestinal system, it is reasonable to believe that it can affect central nervous system-regulated behaviors,[30] such as maximal force expression and muscle recruitment capacity. Furthermore, although conflicting data have been documented, gut bacteria could possibly influence the skeletal muscles directly. Indeed, the abundance of certain bacteria in the gut enhanced muscle growth/damage and strength/endurance performance in mice[31] but not in humans. Nonetheless, sex differences in the human gut microbiota are not well-documented,[32] nor is their possible influence on maximal force expression.

This study endeavored to investigate the impact of sex on force generation by integrating traditional and novel approaches, to provide a comprehensive and multifaceted understanding of this phenomenon. We hypothesized that sex may significantly impact muscle force, and that this effect could be mediated through various of muscle size. Therefore, the primary aim of this study was to explore the morpho-functional and molecular variables standing behind the sex-specific physical characteristics associated with lower limbs' maximal force. Identifying these variables could pave the way to better understand sex differences in maximal force. The secondary goals were to

investigate the possible connections between functional biomarkers and the expression of maximal force, and to determine whether these differences were due to sex or physical adaptations.

2 | RESULTS

2.1 | Anthropometry and muscle architecture

The main differences between males and females in anthropometric parameters were height (178 ± 5 vs. 163 ± 7 cm; Choen's $d = 0.8$), body weight (76 ± 10 vs. 59 ± 9 kg; $d = 1.8$), and total fat percentage (18 ± 5 vs. 26 ± 6%; $d = 1.4$, all $p < 0.001$). Males had a higher total lean mass of the dominant thigh (10 ± 1 vs. 7 ± 1 kg; $d = 3$), knee-extensors CSA (Figure 1E; 94 ± 15 vs. 68 ± 3 cm^2; $d = 2.4$), and *vastus lateralis* fascicle pennation angle (Figure 1F; 26 ± 3 vs. 20 ± 3 deg; $d = 2$, all $p < 0.001$).

2.2 | Muscle fiber type characteristics

The total number of fibers in the biopsies was not different ($p > 0.05$) between sexes. There were no differences in the distribution of type I (48 ± 14 vs. 59 ± 13%; $d = 0.8$, $p = 0.07$) and type II fibers (52 ± 14 vs. 41 ± 13%; $d = 0.8$, $p = 0.07$) in males and females. The Linear Mixed Model with a random intercept variable revealed that males had a higher type I fibers CSA (6689 ± 1748 vs. 5047 ± 1183 μm^2, $d = 1.1$, $p = 0.038$) and type II fibers CSA (6762 ± 2304 vs. 4610 ± 1499 μm^2, $d = 1.1$, $p = 0.036$) (Table S1, Figure 2B).

2.3 | Muscle torque and contractile properties

Males showed a higher absolute torque compared to females (Figure 3A; 196 ± 51 vs. 138 ± 34 Nm; $d = 1.3$, $p = 0.004$), but when expressed relative to the muscle extensor CSA, the difference was not statistically significant (Figure 3B; 2.2 ± 0.6 vs. 2.0 ± 0.5 Nm/cm^2; $d = 0.3$, $p = 0.57$). The RTD was faster for males at the 50, 100, and 200 ms time phases (Figure 4A; $d = 1.2$, $p = 0.004$; $d = 1.4$, $p = 0.002$; $d = 1.4$, $p = 0.002$; respectively), but when expressed for the individual MVIC peak torque, the differences were not statistically significant (Figure 4C; $d = 0.8$, $p = 0.07$; $d = 0.7$, $p = 0.10$; $d = 0.04$, $p = 0.92$, respectively). Both groups had similar voluntary muscle activation (92 ± 5 vs. 95 ± 5%; $d = 0.6$, $p = 0.68$). The absolute resting twitch showed significant differences (Figure 3A; 66 ± 14 vs. 43 ± 8 Nm; $d = 2.0$, $p < 0.001$), but when accounted for muscle CSA, males and females expressed the same torque (0.7 ± 0.2 vs. 0.6 ± 0.1 Nm/cm^2; $d = 0.6$, $p = 0.07$) (Figure 3B). The contractile properties highlighted by the different slopes of the resting twitch indicate faster-evoked contraction (3.2 ± 0.6 vs. 2.1 ± 0.4 Nm/ms; $d = 2.2$, $p < 0.001$) and relaxation (−1.9 ± 0.4 vs. −1.1 ± 0.3 Nm/ms; $d = 2.3$, $p < 0.001$) for the males (Figure 4B). However, when accounted for the muscle CSA, the results showed no difference in the contraction rate (Figure 4D; 0.04 ± 0.01 vs. 0.03 ± 0.01 Nm/cm^2/ms, $d = 1.0$, $p = 0.09$), which is in accordance with the lack of difference in the contraction time (65 ± 7 vs. 69 ± 9 ms; $d = 0.5$, $p = 0.09$). The normalized relaxation rate was still faster in males (Figure 4D; −0.021 ± 0.005 vs. −0.016 ± 0.004 Nm/cm^2/ms; $d = 1.1$, $p = 0.03$), similarly shown by the half relaxation time (69 ± 7 vs. 80 ± 16 ms; $d = 0.2$, $p = 0.05$).

2.4 | Corticospinal excitability

The Mmax of the *rectus femoris* and the *vastus lateralis* did not show differences ($d = 0.5$, $p = 0.18$ and $d = 0.7$, $p = 0.08$, respectively). During the MVICs, the MEP/Mmax was not different between the sexes for both the *vastus lateralis* (0.3 ± 0.1 vs. 0.3 ± 0.1 MEP/Mmax; $d = 0$, $p = 0.25$) and the *rectus femoris* (0.3 ± 0.2 vs. 0.3 ± 0.2 MEP/Mmax; $d = 0$, $p = 0.84$).

2.5 | Muscle and plasma proteins

Skeletal muscle proteomics showed no sex differences in the expression of mTOR (Figure S1; $d = 0.5$, $p = 0.139$). Females had lower Nrf2 levels ($d = 1.0$, $p = 0.017$) and increased levels of TNF-α ($d = 1.2$, $p = 0.010$) and pNFKB ($d = 1.3$, $p = 0.009$) in skeletal muscle (Figure 5). Other proteins are shown in Table 1 and Figure S1.

2.6 | Circulating miRNA

In this study, five out of 24 miRNAs analyzed by microarray analysis were differentially regulated between males and females (Figure 6 and Table 2). MiR-15b-5p and miR-326-3p were significantly upregulated in females compared to males, with log2 fold change values of 1.5 ($d = 1.8$, $p < 0.001$) and 1.4 ($d = 0.7$, $p = 0.02$), respectively. On the other hand, a significant

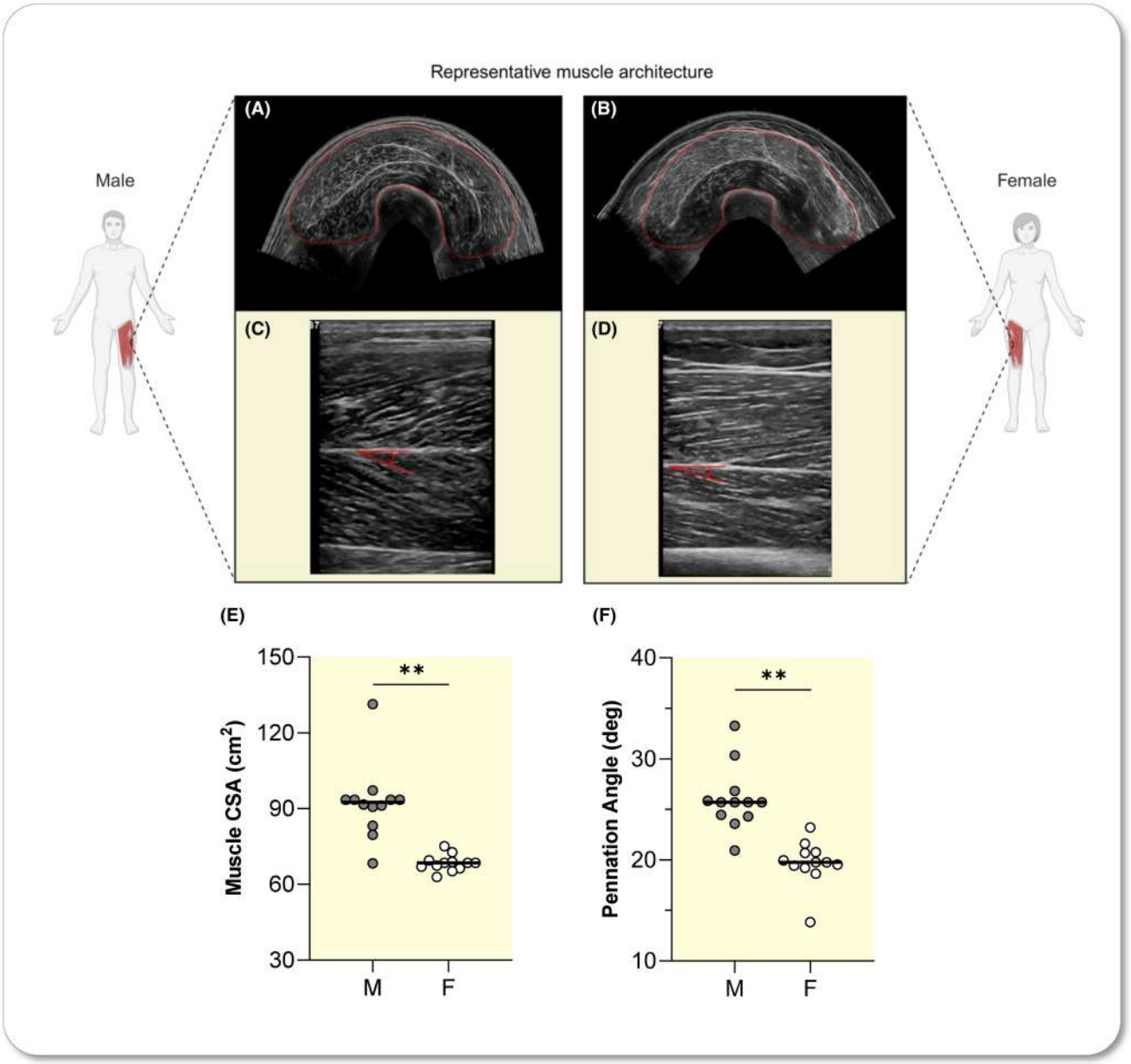

FIGURE 1 On the top, representative ultrasound images of muscle extensors cross-sectional area (CSA; A,B) and angle of pennation (C,D) for males and females. The CSA was acquired in an extended panoramic field of view at 50% of the tight length; the angle of pennation of the *vastus lateralis* was acquired at 50% of the tight length in the middle transversal portion of the muscle belly. On the bottom, muscle extensors' CSA (E) and *vastus lateralis* pennation angle (F) in males and females. Data are presented as mean ± SD. *$p < 0.05$ males (M) vs. females (F).

downregulation in females was observed for miR-27a-3p (log2 fold change of 0.4; $d = 1.1$, $p = 0.02$), miR-30b (log2 fold change of 1.7; $d = 1.8$, $p = 0.001$), and miR-128 (log2 fold change of 1.3; $d = 0.9$, $p = 0.03$).

2.7 | Gut microbiota

Analysis of bacterial abundance at the taxon phylum showed that *Firmicutes* and *Bacteroidota* were the most common phyla across all samples (Figure 7), accounting for more than 90% of total bacteria; *Firmicutes* showed a relative abundance of 58% in males and 60% in females ($p = 0.21$, p-adj $= 0.53$), whereas *Bacteroidota* were 40% and 36%, respectively ($p = 0.24$, p-adj $= 0.53$). The distribution of some families differed between males and females before adjustment for multiple comparisons, namely, *Streptococcaceae* ($p = 0.008$, p-adj $= 0.20$) and *Peptostreptococcaceae* ($p = 0.034$, p-adj $= 0.61$) that were more common in

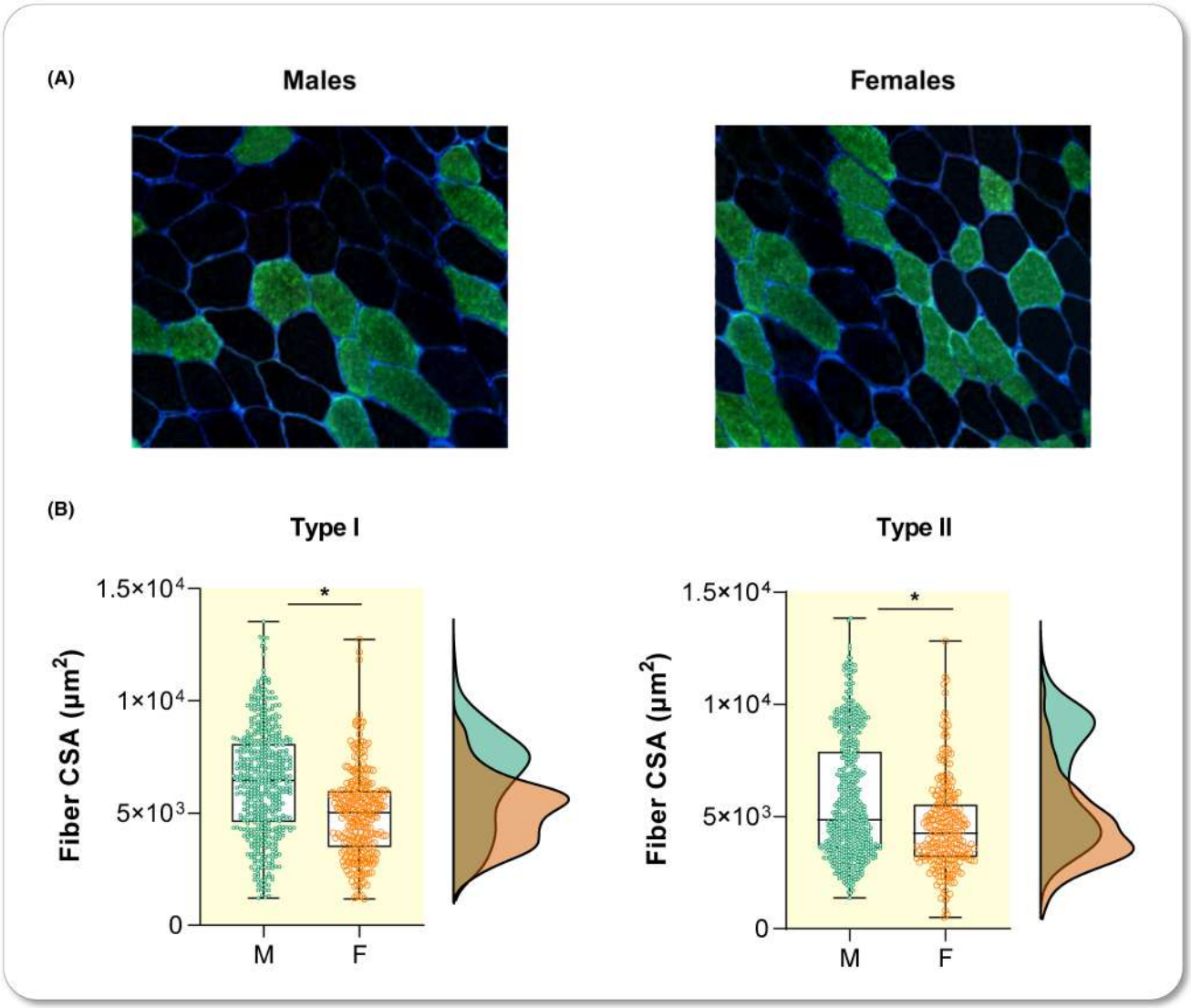

FIGURE 2 On the top (A), a representative immunohistochemical image demonstrating males' (left) and females (right) type I fibers (green) and laminin (blue). On the bottom (B), boxplots and raincloud plots as a result of the linear mixed model analysis performed for type I and type II fiber CSA data of M (green) and F (orange) participants. The fibers CSA analysis was conducted on at least 50 fibers of 10 males (M) and 10 females (F). *$p < 0.05$.

females, and *Tannerellaceae* ($p = 0.005$, p-adj $= 0.20$) that was more abundant in males. No sex-related difference was observed regarding gut microbiota richness ($p = 0.24$).

2.8 | Pooled data correlation matrix

Positive pooled correlations were observed on 24 subjects (12 males and 12 females) between the absolute MVIC and the knee CSA ($r = 0.59$, $p < 0.001$, adj-$p = 0.013$) and the resting twitch absolute values ($r = 0.78$, $p < 0.001$, adj-$p < 0.001$, Table 3). Moreover, people with higher MVIC had a faster contraction (Figure 8A; $r = 0.75$, $p < 0.001$, adj-$p < 0.001$) and relaxation rates (Figure 8B; $r = -0.64$, $p < 0.001$, adj-$p = 0.004$). From a histological point of view, higher expressed torque positively correlated (observed on 20 subjects; 10 males and 10 females) with the type I and II fibers' CSA (type I: $r = 0.64$, $p = 0.003$, adj-$p = 0.013$; type II: $r = 0.61$, $p = 0.004$, adj-$p = 0.021$, Table 3). Other correlations are presented in Table 3.

The exploratory matrix showed that the absolute MVIC was moderately negatively correlated with the miR-495-3p (Figure 8D; $r = -0.64$, $p = 0.002$) and the miR-15b-5p (Figure 8C; $r = -0.51$, $p = 0.037$). The increase in the MVIC was negatively associated with the abundance of certain enterotypes families of the gut microbiota: specifically, *UCG-010* ($r = -0.47$, $p = 0.035$) and *Anaerovoracaceae* (Figure 8E; $r = -0.59$, $p = 0.006$) families (*Firmicutes* phylum).

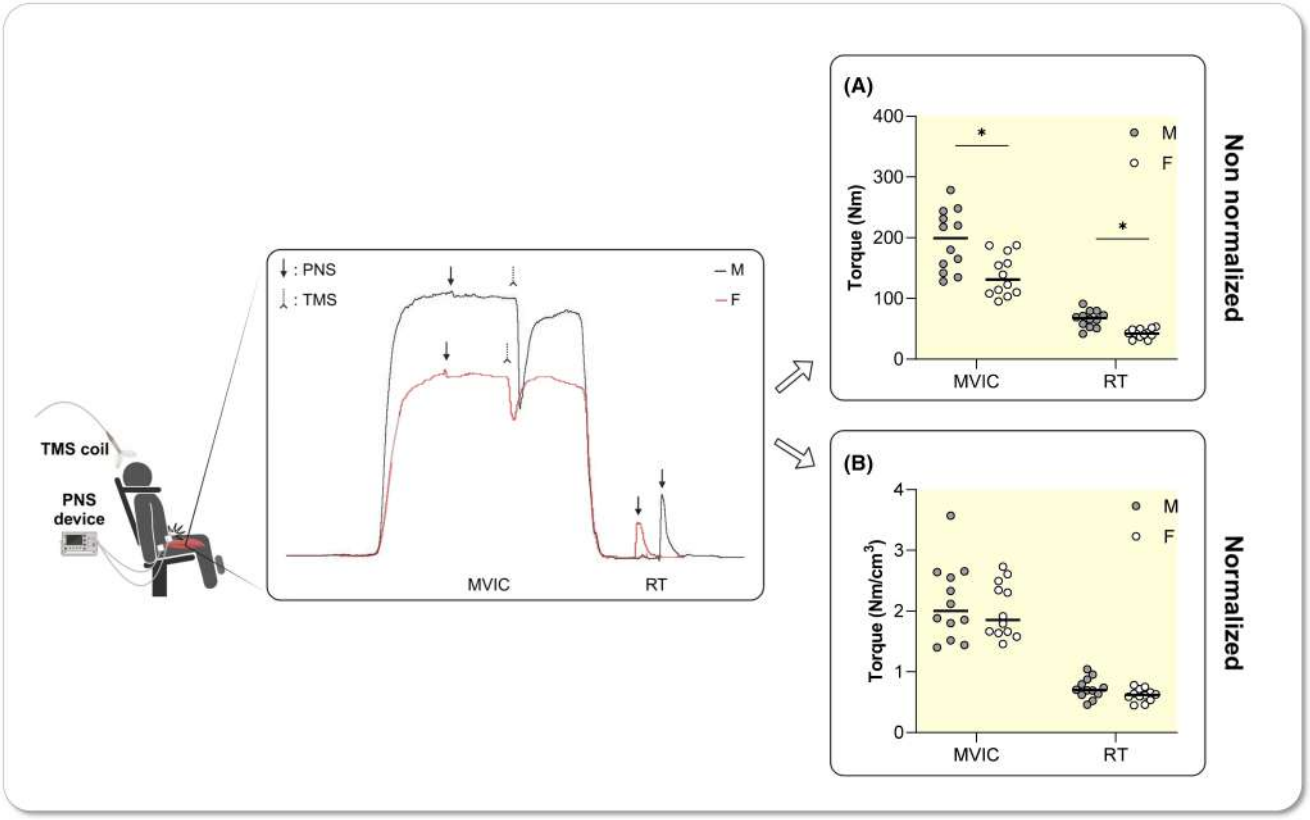

FIGURE 3 Representative resting twitch (RT) and maximal voluntary contraction (MVIC) with the interpolation of peripheral electrical stimulation (PNS, solid line) and transcranial magnetic stimulation (TMS, dashed line). It is appreciable the peak torque (MVIC) difference and the resting twitch (RT) between males (gray line) and females (red line). On the right, the representative trial is translated in individual participants of the two groups and median, expressed as Newton•lever length (A), and then normalized for the muscle cross-sectional area (B). *$p < 0.05$ males (M) vs. females (F).

3 | DISCUSSION

This study is the first to comprehensively explore the impact of sex on the neurophysiological and biological contributing factors of maximal isometric force expression. We confirm our hypothesis that sex differences in maximal isometric torque expression are mainly due to determinants of muscle size. Males' larger muscle mass is the main reason for the higher torque, which is moderately to strongly correlated with the evoked contractile properties of the muscle, indicating a predisposition in males for quicker Ca^{2+} kinetics and faster shortening-relaxation velocities. The biochemical analysis of the muscle tissue revealed that males present higher stress biomarkers, whereas females have a stronger inflammatory state. Interestingly, we are the first to show in humans that the capacity to generate maximal torque is moderately to strongly associated with some miRNAs, but is only partially related to sex differences. Moreover, the gut microbiota did not show any substantial differences at the phylum level but expressed some sex-biased enterotypes at the family level.

3.1 | Peripheral variables primarily regulate sex differences in maximal torque but not the ability to generate force rapidly

The expression of voluntary force is the outcome of a sequence of events that occur in the motor pathway,[33] a synergy between the central nervous system (central determinant of force expression) and the muscle (peripheral determinant of force expression). Our data reinforce previous reports of sex differences in absolute torque in the knee extensors,[2,34,35] and the association with the skeletal muscle CSA indicates that males' greater capacity to generate torque is primarily due to their larger muscles. Indeed, the number of fibers in a muscle and the amount of tension that can be generated influence the degree of force production. Furthermore, the increased size allows for the recruitment of a greater number of fibers, boosting the muscle's force-generating ability. The lack of sex difference in voluntary activation and corticospinal excitability suggests that the central nervous system does not contribute to the observed difference in torque expression between males and females. Accordingly, a substantial

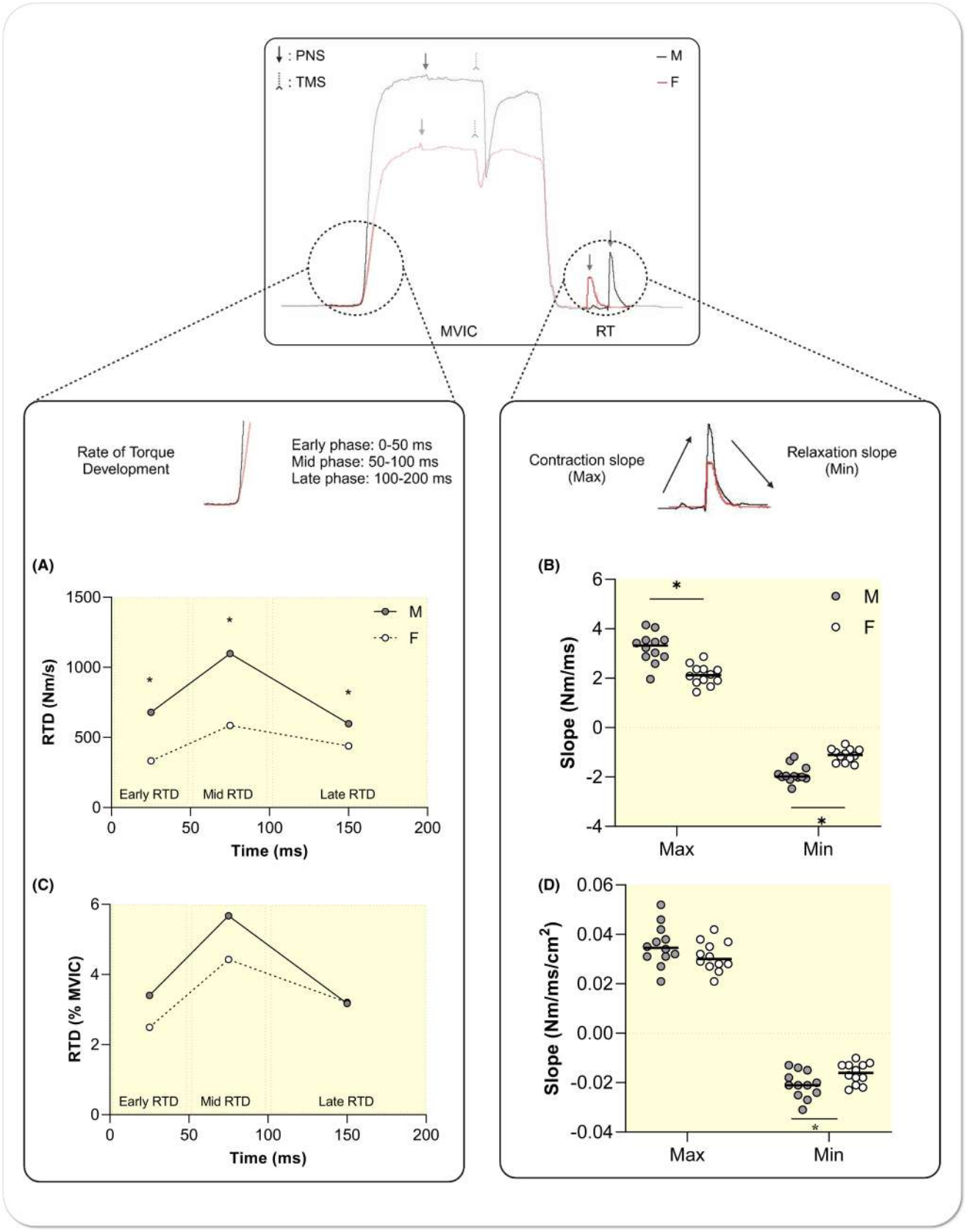

FIGURE 4 Maximal capacities of the voluntary rate of expressing force and muscle evoked contractile properties for males (gray) and females (white). (A) Absolute rate of torque development (RTD) and (C) RTD expressed relative to the individual peak torque during the 3 phases (early, mid and late) to highlight the different components of the ability to generate maximal torque, (B) the absolute and (D) normalized (for the muscle cross-sectional area (CSA)) contraction (Max) and relaxation (Min) slopes of the resting twitch (RT), as an index of muscle contractility. Data are presented as Mean (Panels A–C) or median ± SD (Panels B–D). *$p < 0.05$ males (M) vs. females (F).

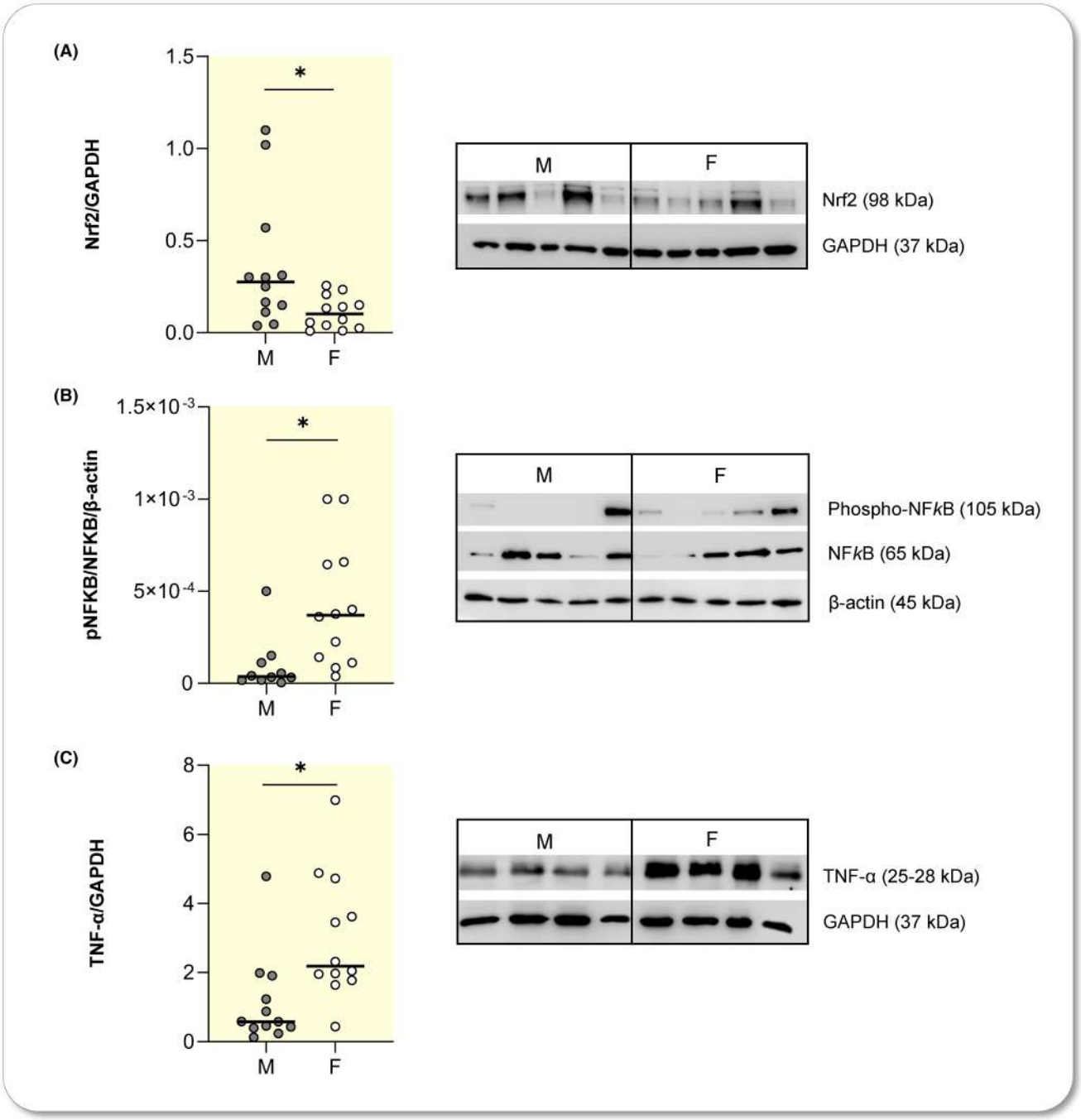

FIGURE 5 Protein levels in males (M, gray dot) and females (F, white dot), and corresponding western blot analysis. In detail, males presented higher expression of Nrf2 (A), and lower levels of Phospho-NF-*k*B (p-NF-*k*B) (males $n = 10$) (B) and TNF-α (C). The individual participants are represented as data points, while the median of the group is represented by horizontal bars. *$p < 0.05$ males (M) vs. females (F).

part of the difference in torque expression is likely due to peripheral mechanisms.

Our data show a pooled positive relationship between muscle CSA and the rate of evoked muscle contraction, meaning that individuals with greater muscle mass demonstrated a faster-evoked muscle contraction (Table 3). Muscle contraction speed is primarily determined by: (i) the number and type of muscle fibers and (ii) the nervous system's ability to recruit and coordinate those fibers. Indeed, in this study, the evoked contraction rate was initially correlated with the fiber distribution but not their CSA; however, upon adjustment for multiple comparisons, the observed significance was not sustained. On another note, during the aforementioned evoked contraction, females had slower relaxation rates and half relaxation times, usually linked to the modulation of SERCA pump activity and a slower re-uptake into the sarcoplasmic reticulum of the Ca^{2+}

TABLE 1 Densitometric analysis of proteins studied by immunoblot in 12 males and 12 females, unless otherwise specified.

		Relative IOD		
	M/F	Males	Females	*p*-value
Plasma				
IL-6 (pg/mL)	12/12	2.1 ± 2.3	2.6 ± 3.3	0.932
Nrf2/GAPDH	12/12	0.4 ± 0.4	0.1 ± 0.1	**0.017**
pNF-*k*B/NF-*k*B/β-actin	10/12	$9.6\times10^{-5}\pm1.5\times10^{-4}$	$4.2\times10^{-4}\pm3.4\times10^{-4}$	**0.009**
Muscle				
SIRT1/GAPDH	10/11	0.8 ± 1.1	0.8 ± 1.0	0.809
LC-3B/GAPDH	10/12	0.3 ± 0.3	0.1 ± 0.1	0.175
4-HNE/total protein	12/12	4.7 ± 7.3	7.7 ± 8.7	0.410
mTOR/GAPDH	12/12	0.5 ± 0.5	0.3 ± 0.3	0.443
TNF-α/GAPDH	12/12	1.1 ± 1.3	3.0 ± 1.8	**0.010**

Abbreviation: IOD, integrated optical density.

The values in bold are the one with a $p<0.05$, considered as statistical significant.

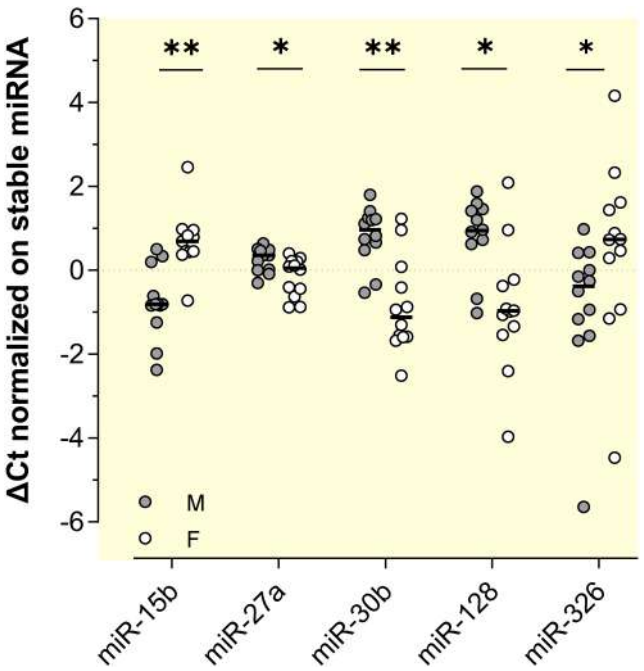

FIGURE 6 Significantly different circulating miRNAs analysis in males (gray) and females (white). The individual participants are represented as data points, while the median of the group is represented by horizontal bars. *$p<0.05$, **$p\leq0.001$ males (M) vs. females (F).

ions.[36] On this note, we also observed higher expression of miR-15b in females, which is described to downregulate target genes involved in regulating cellular ATP and SERCA pump activity.[37,38]

Other indices, such as the ability to increase force as quickly as possible, have gained interest due to their relationship with most performances of both sport-specific and functional daily tasks.[39] For this reason, we investigated the RTD characteristics and initially observed a sex difference; however, since RTD is affected by peak force capacity,[40] we further compared males and females using the RTD normalized for their peak MVIC torque. Accordingly, males and females presented similar values for the early phase of RTD, suggesting that the contribution of the neural factors, such as motor unit recruitment and firing rates,[41,42] was similar.[43–47] Interestingly, it has been reported that some physiological adaptations might mediate the association between the RTD and the maximal torque as a "confounder factor."[47] The logical thread of the first part of this study made us account for the knee extensors' CSA, resulting in muscle mass not influencing the different phases of RTD. This makes us suppose that the physiological mechanisms influencing the RTD, independently of the maximal torque generated, cannot be ascribed to the muscle size[48] and sex.

Because there exists an enhanced capacity to conduct explosive-type actions as type II fiber area increases,[49–51] we investigated a potential pooled fiber-RTD association. Neither the type II fiber distribution nor their area correlated with the normalized RTD phases. This implies that additional elements that affect the muscle-tendon unit and/or the nervous system may be important in determining maximal RTD.[39] Among these factors, sex hormones play a role in influencing the biomechanical properties of tendons. Indeed, connective tissues possess receptors for estrogen, progesterone, and testosterone. Testosterone reduces the elastic properties of the tendons[52] with increased dysfunction and fibrosis,[53] whereas estrogens have been associated with a higher relative number of smaller fibrils[54] and tend to decrease the diameter of tendons.[55] However, in this study, the biomechanical properties (i.e., electromechanical delay and force transmission to the tendon) have not been investigated.

TABLE 2 Differences in circulating miRNA expression between males and females.

miRNA	M/F	Log2 FC	*p*-value	Upregulated in
miR-15b-5p	12/11	1.47	**≤0.001**	Females
miR-27a-3p	12/12	−0.42	**0.02**	Males
miR-29a-3p	12/12	−0.35	0.27	Males
miR-30b-5p	12/12	−1.67	**≤0.001**	Males
miR-122-5p	11/7	−1.50	0.13	Males
miR-128-3p	12/12	−1.33	**0.03**	Males
miR-140-5p	12/12	0.18	0.97	Females
miR-146a-5p	12/12	−0.04	0.90	Males
miR-155-5p	12/8	−1.13	0.06	Males
miR-326-3p	12/12	1.35	**0.02**	Females
miR-495-3p	12/11	0.02	0.38	Females
miR-532-5p	12/12	0.67	0.33	Females
miR-941	11/6	−0.50	0.81	Females

Abbreviation: FC, fold change.

The values in bold are the one with a $p<0.05$, considered as statistical significant.

3.2 | Blood miRNAs in sex difference and as predictors of the maximal torque in humans

The sex-biased expression of circulating miRNAs in humans is poorly understood. Identifying the confounding element of sex in their expression has recently acquired relevance, primarily because several miRNAs are encoded on sex chromosomes. Of 24 miRNAs investigated, 4 showed different expression in males and females. In particular, hsa-miR-15b-5p was upregulated in females while hsa-miR-30b-5p, hsa-miR-27a-3p, and hsa-miR-155-5p were downregulated. Among those, Maillot et al[56] showed on a breast cancer cell line that miR-27a transcriptional regulation is estrogen-dependent, indicating and verifying the reduced expression we observed in males. The largest difference we observed was in hsa-miR-30b-5p, from the miR-30 family, which is important in adipocyte differentiation and fat synthesis.[57] In particular, its expression is elevated during adipose differentiation and greatly boosted thermogenic gene expression in primary adipocytes when forcedly overexpressed.[58]

Our findings did not confirm the increased expression of miR-155-5p in males, which has been linked to higher levels of neuroinflammation in cell lines and mice.[59,60] Moreover, males in our study did not present higher inflammatory cytokines, showing lower levels of TNF-α in the muscle tissue. On the other hand, other miRNAs and cytokines regulate muscle growth, thus, muscle force. In fact, miR-15b-5p has a fundamental role in the primary protein degradative pathways, including autophagy pathways and the ubiquitin-proteasome.[61] We observed a higher expression of circulating miR-15b in females, and equally important, we found a pooled significant negative association with the maximal torque expressed (Figure 8C; $r=-0.51$, $p=0.037$). Moreover, this miRNA has been described to target genes involved in fatty acid synthase[62] and the modulation of SERCA pump activity/expression in humans.[37,38] The latter catalyzes the hydrolysis of ATP in conjunction with the translocation of Ca^{2+} from the cytosol into the sarcoplasmic reticulum lumen and is involved in the control of the contraction/relaxation cycle. This finds confirmation in our analysis, with a negative association with the evoked contraction rate ($r=-0.61$, $p=0.009$) and a strong positive association with the relaxation rate ($r=0.74$, $p<0.001$) of the quadriceps. In addition, the maximal torque was negatively correlated with miR-495-3p (Figure 8D), which is predicted to target the TNF receptor superfamily member 1B[63] and encode the Ca^{2+}-dependent activator protein for secretion 2 (CADPS2).[64] The CADPS2 is a protein that affects the brain-derived neurotrophic factor (BDNF) secretion,[65] associated with developing myofibers which later express myosin heavy chain IIB,[66,67] and is more expressed in slow muscle fibers than in fast phenotype muscle fibers.[68–70] These findings indicate that individuals possessing higher levels of torque exhibit reduced expression of miR-495-3p, as seen in our male participants. This observation is consistent with prior predictions that miR-495-3p encodes the CADPS2, expressed at lower levels in type II muscle fibers.[68–70] Our findings support this mechanism and suggest that the maximum ability to produce force includes miRNA expression and appears to be partly independent of individual sex; however, a more focused investigation is required to demonstrate a conclusive connection.

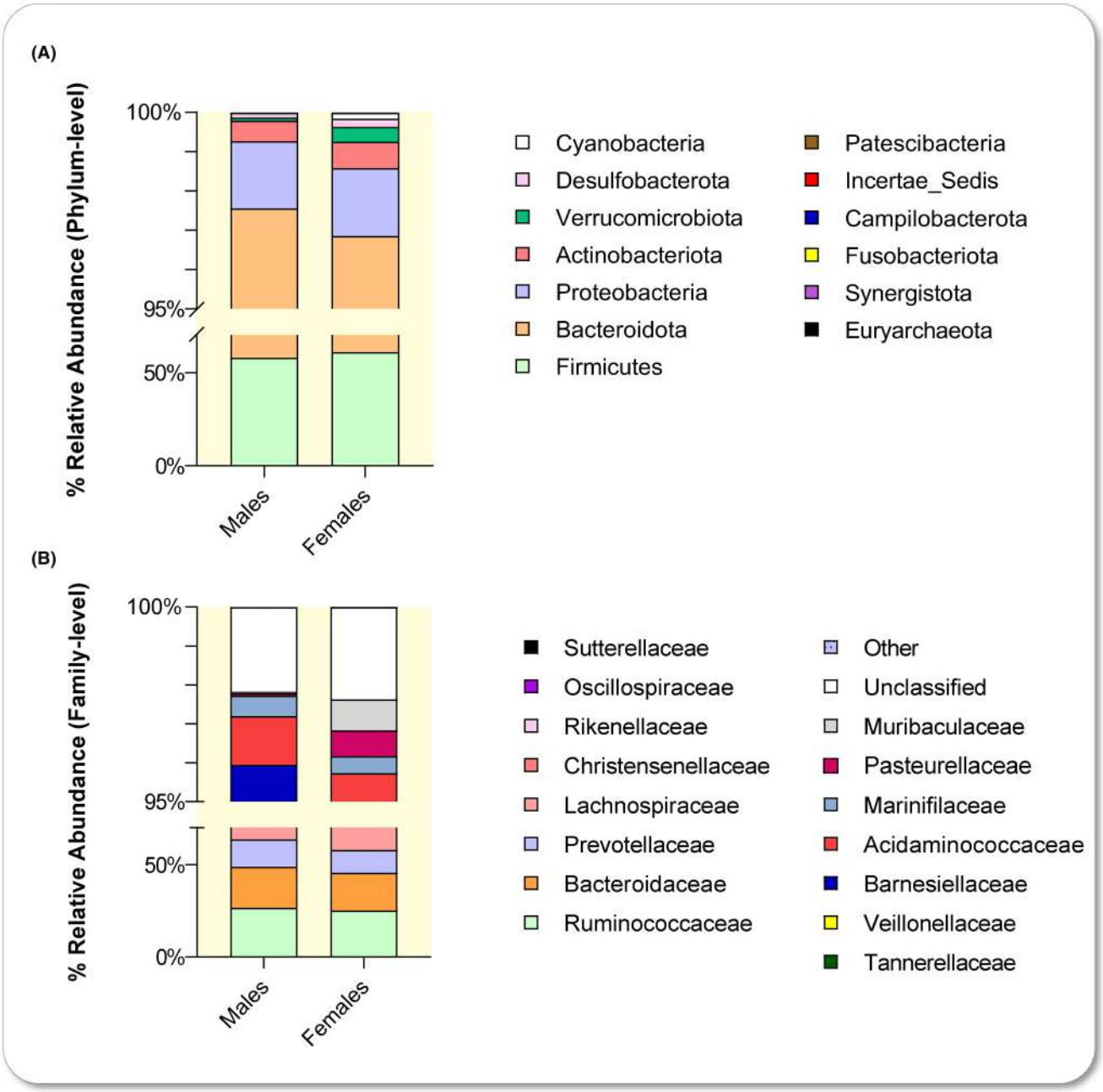

FIGURE 7 Gut microbiota composition in males and females at Phylum (A) and Family (B) level.

3.3 | Proteins in sex difference and as muscle regulators

The role of Nrf2 and pNF-*k*B in human skeletal muscle on exercise capacity has much to be investigated. Studies showed that Nrf2 is essential for normal muscle function, and when upregulated, muscle contractility is improved.[71,72] Moreover, NF-*k*B activity is one of the most critical signaling pathways associated with skeletal muscle mass loss in various physiological and pathological circumstances,[73] and its activation causes inflammation, fibrosis, and the breakdown of particular muscle proteins.[74] In this study, we observed positive associations of Nrf2 with the evoked muscle contraction (index of muscle contractility) and the muscle CSA, but no associations when the central nervous system was involved in the contraction (as during the MVIC). Similarly, negative correlations were observed between pNF-*k*B, the evoked muscle contraction, and its contraction and relaxation rates. The lower levels of Nrf2 in females with the higher expression of NF-*k*B support our results regarding the differences in muscle contractility and size.

TABLE 3 Pooled data correlation matrix between muscle parameters.

	M/F	Muscle CSA		MVIC		RT		Relaxation rate		Contraction rate	
		r	adj-*p*	*r*	adj-*p*	*r*	adj-*p*	*r*	adj-*p*	*r*	adj-*p*
Muscle CSA	12/12	–	–	0.59#	0.013	0.72*	<0.001	−0.56#	0.021	0.65#	0.003
MVIC	12/12	0.59#	0.013	–	–	0.78*	<0.001	−0.64#	0.004	0.75*	<0.001
RT	12/12	0.72*	<0.001	0.78*	<0.001	–	–	−0.88*	<0.001	0.96*	<0.001
Relaxation rate	12/12	−0.56#	0.021	−0.64#	0.004	−0.88*	<0.001	–	–	−0.94*	<0.001
Contraction rate	12/12	0.65#	0.003	0.75*	<0.001	−0.96*	<0.001	−0.94*	<0.001	–	–
Fiber I distribution	10/10	−0.23	1.000	−0.24	1.000	−0.38	0.516	0.46	0.203	−0.49	0.140
Fiber II distribution	10/10	0.23	1.000	0.24	1.000	0.38	0.516	−0.46	0.203	0.49	0.140
Fiber I CSA	10/10	0.54	0.074	0.64#	0.013	0.45	0.245	−0.45	0.240	0.45	0.237
Fibers II CSA	10/10	0.61#	0.022	0.61#	0.021	0.39	0.464	−0.35	0.646	0.37	0.530

Note: A correlation was considered strong (*) if the *r* value was larger than 0.7, and moderate (#) if between 0.5 and 0.7, with a adjusted $p < 0.05$.

Abbreviations: CSA, cross-sectional area; MVIC, muscle voluntary isometric contraction; RT, resting twitch.

The catabolic process is fundamental in the skeletal muscle. Among the most critical factors is the activity of LC-3B (an autophagy-promoting gene) and TNF-α (an inflammatory cytokine produced during acute inflammation). The TNF-α activity has been widely studied, and its presence has been related to diminished specific force in murine skeletal muscle.[75] The mechanism behind this phenomenon is believed to be mediated by decrements in the function of Ca^{2+}-activated myofibrillar proteins. If we translate these results into our human model, we identified a higher level of TNF-α in females, along with higher circulating miR-15b (negatively associated with the maximal torque) and a lower presence of LC-3B genes, corresponding to sex differences in voluntary torque, evoked contractility, and muscle size.

Overall, the data on the sex differences indicate that females have a higher inflammatory state in the muscle (with higher pNF-*k*B and TNF-α) while males seem to have higher cellular resistance regulators to oxidants (higher Nrf2 levels). Furthermore, we showed that higher Nrf2 presence (as in males) is associated with greater evoked torque, contraction, and relaxation rates. The opposite effect appears when higher pNF-kB levels are exhibited.

3.4 | Gut microbiota in sex difference and torque expression

Sex is considered an important biological variable affecting gut microbiota and the microbiota-dependent gut–brain pathway,[32] but contrasting findings on the sex difference appear from human[76,77] and animal studies.[78,79] Females have a more active hypothalamus-pituitary-adrenal axis than males, which is thought to be partly due to the action of estrogen.[80] In our investigation, males and females did not show substantial differences in the microbiota population at the phylum level, with a similar abundance of *Firmicutes* and *Bacteroidota*. Moreover, the ratio *Firmicutes*/*Bacteroidota*[81–83] was similar between males and females. After conducting a thorough analysis, it was discovered that there are sex-based differences in the composition of certain anaerobic bacteria families belonging to the *Firmicutes* phylum (*Streptococcaceae* and *Peptostreptococcaceae*), which were more prevalent in females. On the other hand, *Tannerellaceae*, a member of the *Bacteroidota* phylum, was more abundant in males.

The microbiota-dependent gut–brain pathway is a communication system operated directly via the vagus nerve, which links the central to the autonomic nervous system.[84] The vagus nerve connects with the gut bacteria in the enteric nervous system and conveys critical

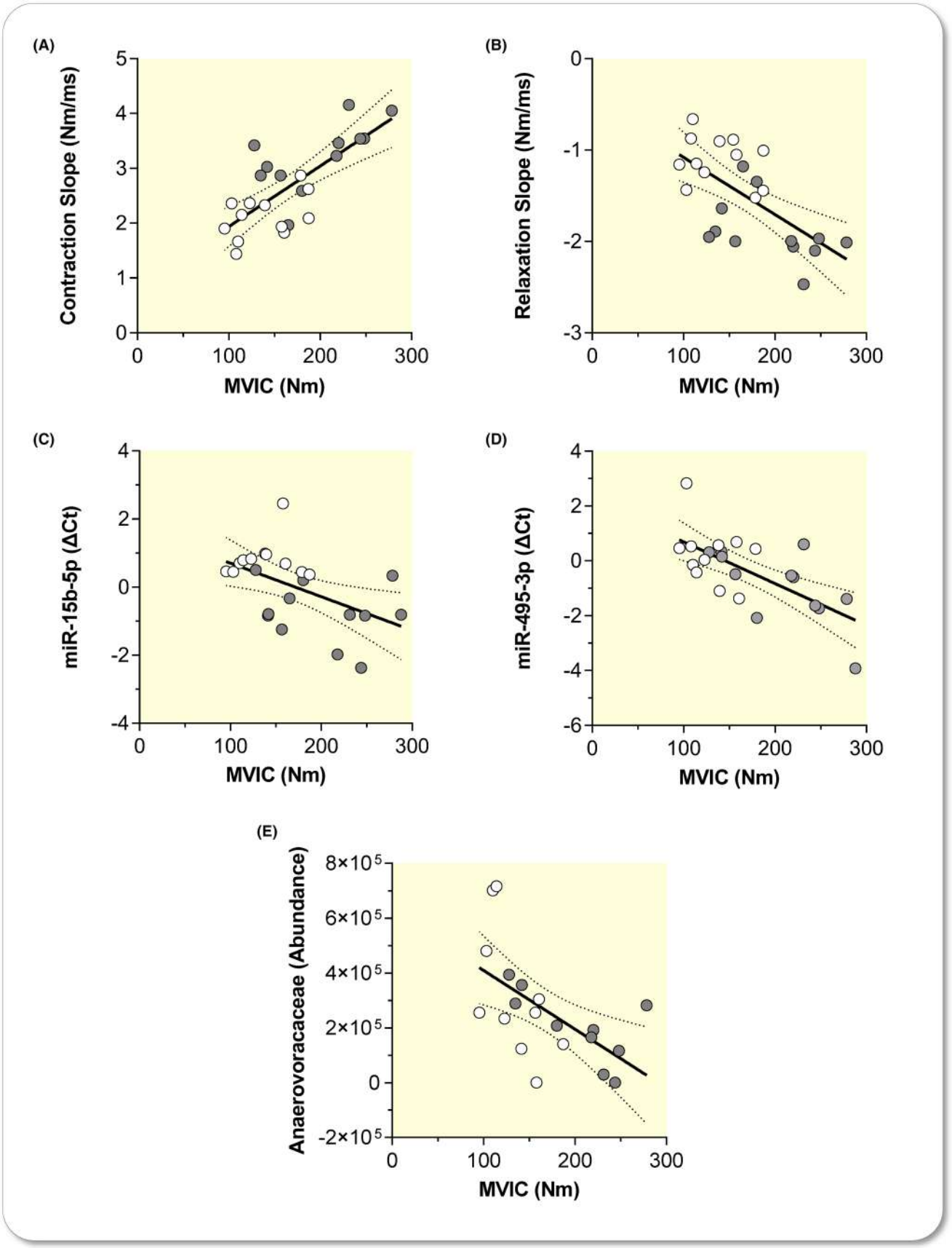

FIGURE 8 Correlation of pooled data between the maximal voluntary isometric contraction (MVIC) and the evoked contraction rate from the resting twitch (A), the evoked relaxation rate from the resting twitch (B), the expression of the miR-15b-5p (12 males, 11 females) (C), the expression of the miR-495-39 (12 males, 11 females) (D), the gut microbiota abundance of the *Anaerovoracaceae* family (10 males, 10 females) (E). The individual participants are represented as data points (Males in gray vs. Females in white), the 95% confidence bands are represented by the dotted lines.

information to the central nervous system, resulting in an appropriate central nervous system or various neurotransmitter responses.[85,86] In this study, we examined the corticospinal pathway's excitability and found a positive correlation with the presence of *Ruminococcaceae* bacteria ($r = 0.52$, $p = 0.02$; *Firmicutes* phylum). The *Firmicutes* phylum is known to be highly responsive to exercise-induced changes in the body.[84] Yet, previous studies have focused just on aerobic exercise,[83] which has a limited impact on muscle recruitment and neural adaptations compared to resistance-type exercise.[87,88] Acknowledging that the correlation between gut microbiota and the transmission of descending signals from higher brain areas may be spurious, further investigation is warranted to gain a comprehensive understanding of its implications.

Because fitness status, diet, and physical exercise interact with gut microbiota, altering its composition and promoting homeostasis and energy balance,[89–91] it should be acknowledged that our study participants consisted exclusively of active young individuals who were instructed to maintain their usual dietary habits, perhaps limiting the interpretation of our results on the microbiota. However, the potential impact of the gut microbiota on muscle function calls for additional research. This line of investigation holds promise for developing new interventions to optimize muscle health by selectively modulating the gut microbiome.

4 | MATERIALS AND METHODS

4.1 | Study participants

The sample size was computed using G-Power 3.1[92] and subjected to an independent sample t-test between-effects analysis of two groups (males and females). Assuming an expected effect size [Cohen's d[93]] of around 1.87 for the sex difference in knee extensors' maximum voluntary isometric contraction force (MVIC),[34] a p-value (type I error) of 0.05, and power of 0.95, the total sample size obtained was 18 (i.e., 9 participants per group). More participants were enrolled to account for possible dropouts and maximize statistical power. Therefore, 12 males (mean ± SD; age: 25 ± 3 years) and 12 females (age: 23 ± 2 years) completed the study. All participants were healthy and active, without any known cardiovascular, neuromuscular, or metabolic disease. They were also aware of the purpose and the risks associated with the study and gave informed written consent in accordance with the most recent revisions of the Declaration of Helsinki. The protocol (IRB #27111) was approved by the Institutional Review Board of the University of Verona. All the experiments were performed following the safety procedure for exercise testing in the scenario of COVID-19,[94] and all the material submitted is conform with good publishing practice in physiology.[95]

4.2 | Study design

The participants visited the laboratory on four different occasions. The first visit was dedicated to anthropometric measurements and a questionnaire to assess leg dominance (i.e., the Revised Waterloo Footedness Questionnaire[96]). During the second visit, participants performed a familiarization session involving submaximal and maximal voluntary isometric knee extensor contractions. During the third visit, the participants undertook a warm-up protocol followed by a series of MVICs of the dominant knee extensors (see *Neuromuscular testing protocol*). Knee extensors were chosen as target muscle group since they play a key role during ambulatory, functional, and sporting activities.[97,98] The first three visits were separated by 7 days, and each participant performed all tests at the same time of day. Because the neuromuscular function of the knee extensors can be influenced by the different phases of the menstrual cycle,[99] the first day of menstruation was considered as day 1 of the cycle and females visited the lab on day 14 ± 2 of their menstrual cycle. After ~10 days from the third visit, participants reported to the lab in a fasted state for the fourth and last session involving a blood draw, a muscle biopsy, and a fecal sample collection (Figure 9).

4.3 | Anthropometrics

Body composition (height, weight, body fat percentage, and the lean mass of the dominant thigh) was measured using a dual-energy x-ray absorptiometry (DXA) total body scanner (QDR Horizon, Hologic MA, USA; fan-beam technology, software for Windows XP version 13.6). Our laboratory's detailed preparation and scanning procedure have been previously described.[100]

4.4 | Muscle architecture

The knee extensors muscle cross-sectional area (CSA) was determined from panoramic ultrasound scans (GE Logiq-7, General Electric Medical Systems, Milwaukee, WI, USA)[101] using a 12–14 MHz probe and manually post-processed directly on the ultrasound. Details on the protocol used can be found in Appendix S1.

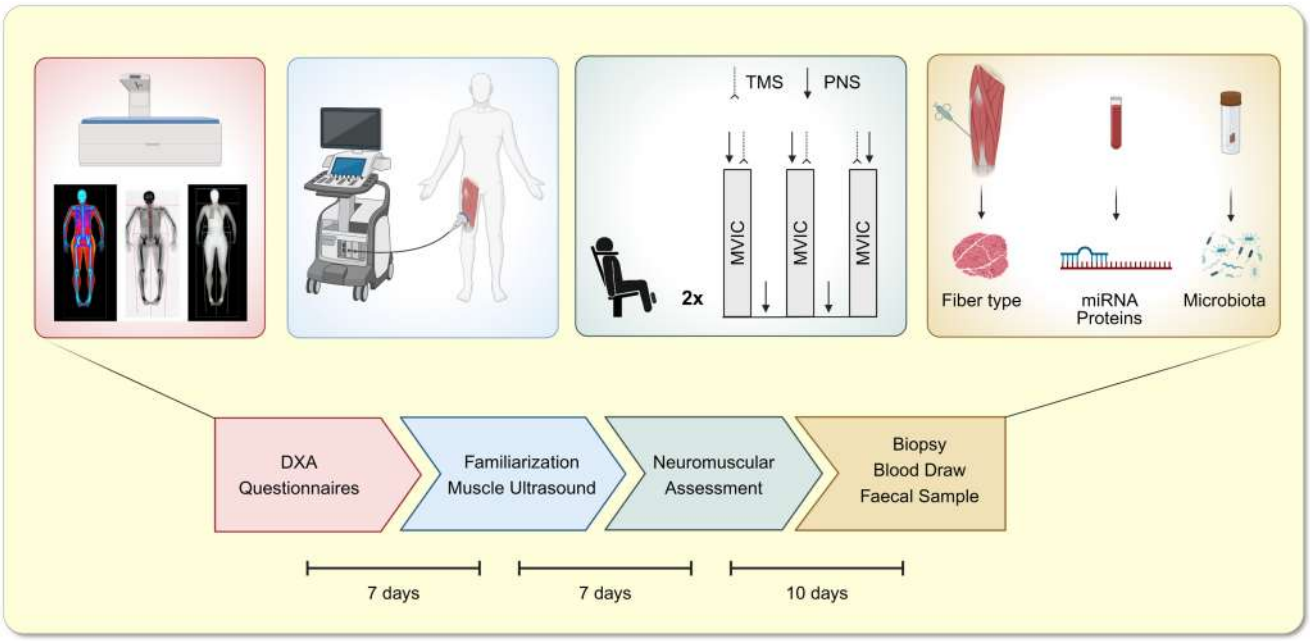

FIGURE 9 Study design. Participants arrived at the laboratory on four occasions. The force test consisted of two sets of three maximal voluntary contractions. DXA, dual-energy x-ray absorptiometry total body scanner; MVIC, maximal voluntary isometric contraction; PNS, peripheral nerve stimulation; TMS, transcranial magnetic stimulation.

4.5 | Neuromuscular testing protocol

The neuromuscular testing protocol consisted of two neuromuscular assessments (separated by 2 min; see *Neuromuscular assessment* section and Figure 9) with transcranial magnetic stimulation (TMS) and peripheral nerve stimulation (PNS). Before beginning the neuromuscular assessment, participants undertook a warm-up protocol involving three repetitions of knee extension at 10%, 30%, 50%, and one contraction at 70% of the maximum voluntary isometric contraction (MVIC) recorded during the familiarization session. Each warm-up contraction was 5 s long, with 5 s of rest in between contractions. The warm-up was followed by two 5-s MVICs, with 2 min of rest in between.

4.6 | Neuromuscular assessment

The neuromuscular assessment consisted of two series of three maximal voluntary isometric contractions of the dominant knee extensors (interspersed with 1 min of rest between the contractions and 2 min between sets) with and without TMS and PNS (Figure 9). Specifically, participants performed one ~4 s MVIC superimposed with a PNS and a TMS delivered at the force plateau and another PNS evoked 2 s after the end of the MVIC on the relaxed muscle in a potentiated state (i.e., potentiated resting twitch (RT); see Figure 3 for a representative MVIC and RT). One minute after the RT, participants performed another similar MVIC with the RT. The third MVIC was performed after another minute, and a TMS followed by a PNS were delivered at force plateau. No RT was performed. Participants were also requested to return to their pre-stimulus level of force as quickly as possible following the stimuli.[102] Once the participant returned to the maximal force, PNS or TMS was delivered. All the peak forces from the six MVIC trials were within 5% of each other. Details on the force, electromyography (EMG), PNS, and TMS are in Appendix S1.

4.7 | Biopsy procedures

Muscle tissue samples were obtained from the lateral portion of the *vastus lateralis* of the dominant leg using a 13G semi-automatic mini-invasive biopsy needle (Vantage B GSB 13-10, Zamar, Italy). The samples were obtained after proper aseptic preparation and local lidocaine injection. Participants were asked to restrain from physical activity the 2 days preceding the procedure.[103] Samples were immediately frozen in liquid nitrogen-cooled isopentane for 20–30 s and stored at −80°C for immunohistochemical and biochemical analysis.

4.8 | Muscle tissue immunohistochemistry

Well-oriented muscle tissue samples were cross-sectionally placed on a cork with Tissue Tek optimal cutting temperature (OCT; Thermo Fisher Scientific, Rockford, IL, USA) for immunohistochemical analysis, following the details of the previously described procedure.[104,105] Briefly, sections (7-μm thick) were cut in a cryostat, air-dried for 1 h, and repeatedly incubated at room temperature with anti-myosin heavy chain isoforms type I (DSHB; BA.D5, IgG2b) and laminin (L9393; Sigma Aldrich, St. Louis, MO, USA). The next day, slides were incubated with specific secondary antibodies, mounted with fluorescent mounting media, and analyzed. Additional information regarding the preparation is illustrated in Moro et al[104] fiber-type distribution and CSA were then calculated[105] on 129 ± 97 (mean ± SD) fibers per subject. Fiber CSA analysis was possible on 10 males and 10 females, on the samples without freezing damages. All immunofluorescence analyses were completed in a blinded fashion.

4.9 | Plasma IL-6 determination

IL-6 concentration was detected by enzyme-linked immuno-sorbent assay (ELISA) Enzyme Immunoassay Kit (#KHC0061 Thermo Fisher), according to the manufacturer's protocol. The amount of IL-6 was determined by measuring absorbance at 450 nm. The standard curve demonstrated a direct relationship between optical density and IL-6 concentration. Plasma IL-6 concentration was expressed in pg/mL.

4.10 | Protein extraction and immunoblots on muscle tissue

For immunoblots, samples were weighed and resuspended in ice-cold Cell Lytic MT Lysis Buffer (Sigma-Aldrich, C3228) with a cocktail of protease inhibitors (Roche) (300 μL every 10 mg of tissue). Then after preliminary homogenization in a 1-mL potter, the samples steadily maintained in ice were further processed 3 times (15 s each with intervals of 1 min) with a sonicator. Then, the samples left in ice for 1 h were centrifuged twice at 12 000 rpm for 20 min at 4°C to recover in the supernatant the total protein extract that was maintained at −80°C until use. BCA protein assay (Pierce Biotechnology, Rockford, IL, USA)[106] was used to measure total protein concentrations; bovine serum albumin (BSA) was used as an external standard. Proteins (20 μg) were resolved by 10%–12% SDS-PAGE and immobilized on a nitrocellulose membrane (Thermo Fisher Scientific). Five percent skim milk (Euroclone) in Tris-buffered saline (20 mM Tris, 150 mM NaCl, pH 7.4) and 0.1% Tween-20 (Sigma-Aldrich) were incubated with the relevant antibodies according to Bartolini et al[107] Further details are in Appendix S1.

4.11 | Circulating miRNAs

Five milliliter of blood samples were obtained from participants and collected into vacutest tubes. Serum was obtained from samples allowing blood to clot for 30 min at room temperature before centrifugation. Samples were then centrifuged at 1900 ***g*** for 15 min at 4°C, and serum samples were collected in cryovials and kept at −80°C for further analyses. Extracellular vesicles (EVs) were isolated from 500 μL of serum using exoRNeasy Midi Kit (Qiagen) according to the manufacturer's instructions.[108] RNA extraction was performed as described.[108] Briefly, the serum samples were loaded onto the exoEasy spin column and centrifuged for 1 min at 500 g. QIAzol solution was added to the column to collect the lysate by centrifugation. Then chloroform was added to the mixture and shaken vigorously for 15 s. After centrifugation, the upper aqueous phase was collected and mixed with two volumes of ethanol. The samples were then loaded onto an RNeasy MinElute spin column and centrifuge at 10 000 rpm for 15 s at room temperature. Finally, the column was washed with buffer ribulose-5-phosphate-3-epimeras three times, and the RNA was eluted from the column with RNase-free water. Further details are in Appendix S1.

4.12 | Gut Microbiota gene sequencing

Microbial genomic DNA was extracted using the Stool DNA Isolation kit (Norgen Biotek Corp., Canada). Quantity, purity, and integrity of extracted DNA were checked by Qubit 2.0 Fluorometer (Life Technologies, USA),[109] Nanodrop spectrophotometer (Thermo Fisher Scientific, UK), and Fragment Analyzer capillary electrophoresis system (Agilent, Santa Clara, CA, USA), respectively. 16S rRNA gene libraries were constructed based on six overlapping amplicons covering the 9 hypervariable regions of the 16S rRNA gene (QIAseq 16S/ITS Screening Panel kit – Qiagen, Hilden, Germany). Library sequencing was performed using a 276PE approach on an Illumina MiSeq NGS system (MiSeq Reagent Kit v3), as indicated in the customer instructions (QIAGEN protocol–QIAseq 16S/ITS panel handbook: www.qiagen.com). Further details are in Appendix S1.

4.13 | Statistics

The Gaussian distribution of variables was determined with the Shapiro–Wilks test, and when required, data were transformed logarithmically before further analysis and, if necessary, compared using a nonparametric Mann–Whitney test or a Parametric test with Welch's correction. The Bonferroni correction was applied when needed. Additionally, to specifically examine the CSA of muscle fibers, a Linear Mixed Model (LMM) with a random intercept variable was employed, to account for the variability within groups or clusters in the data.

For the microbiota analysis, a differential abundance analysis was performed at all six taxonomic levels (Phylum, Class, Order, Family, Genus, Species) and ASV level using the library DESeq2[110] of R that compared ASV counts between groups.[109] The FDR algorithm was applied to compute the adjusted *p*-value to control the false discovery rate.[110]

To evaluate the global association between variables, a pooled data correlation matrix was generated (Table 3). A full Pearson's *r* correlation coefficient for each pair was computed, and, where appropriate, a Bonferroni correction was applied. A second pooled data matrix intended for exploratory purposes was generated. Statistical significance was set at $\alpha \leq 0.05$. A correlation was considered strong if the r value was larger than 0.7, and moderate if between 0.5 and 0.7.[111] Before that, the identification of the outliers was automatically performed using the Grubbs' method, with an $\alpha = 0.010$.

All the statistic was performed using GraphPad Prism (V.6.0, GraphPad Software) and Rstudio version 1.4.1717. Results are shown as males vs. females and, unless otherwise stated, reported as mean ± SD.

5 | CONCLUSION

The integrative and comprehensive design of this study investigates sex differences in force generation under several spotlights, representing a significant opportunity in muscle physiology research. Our results suggest that the major "macroscopic" factor responsible for males' ability to generate higher and faster force with the knee extensors can be ascribed to their greater muscle mass rather than differences in the central nervous system's ability to generate force. However, our investigation into the "microscopic" world reveals that the ability to produce force is linked to the control of gene expression, with the expression of some miRNAs associated with varying degrees of maximal force generation, regardless of sex. Moreover, males balance their greater force with higher antioxidant proteins, essential for battling damage and inflammation in the muscle tissue, while females tend to have higher levels of pro-inflammatory cytokines, related to muscle injury/regeneration ability. Furthermore, the abundance of some gut microorganisms at the family level can play a role in the capacity to express maximal force independently of sex. By understanding the molecular mechanisms and the potential role of the gut microbiota in influencing muscle function, we may be able to develop targeted interventions to improve muscle force and function and to optimize muscle health through modulation of the gut microbiome in males and females; thus, further investigation is required to clarify the role of these factors in sex-related differences of muscle physiology.

AUTHOR CONTRIBUTIONS

Gaia Giuriato: Investigation; writing – original draft; methodology; validation; visualization; data curation; formal analysis. **Maria Grazia Romanelli:** Conceptualization; funding acquisition; writing – review and editing; methodology; visualization; supervision; resources; data curation. **Desirée Bartolini:** Investigation; writing – review and editing; methodology; validation; visualization; data curation; resources. **GIANLUCA VERNILLO:** Methodology; validation; visualization; writing – review and editing; supervision. **Anna Pedrinolla:** Investigation; validation; visualization; writing – review and editing. **Tatiana Moro:** Investigation; methodology; validation; visualization; writing – review and editing; data curation; formal analysis; resources. **Martino Franchi:** Methodology; validation; writing – review and editing. **Elena Locatelli:** Investigation; methodology; writing – review and editing; data curation. **Mehran Emadi Andani:** Writing – review and editing; methodology; software; validation; visualization. **Fabio Giuseppe Laginestra:** Investigation; validation; visualization; writing – review and editing. **Chiara Barbi:** Investigation; validation; visualization; writing – review and editing. **Gloria Fiorini Aloisi:** Investigation; validation; visualization. **Valentia Cavedon:** Investigation; methodology; resources; data curation; writing – review and editing; validation. **Chiara Milanese:** Resources; writing – review and editing; methodology; validation. **Elisa Orlandi:** Investigation; writing – review and editing; methodology; validation; visualization; data curation. **Tonia De Simone:** Investigation; methodology; validation; visualization; writing – review and editing; data curation. **Stefania Fochi:** Investigation; methodology; validation; visualization; writing – review and editing; data curation. **Cristina Patuzzo:** Data curation; methodology; visualization; validation; investigation. **Giovanni Malerba:** Writing – review and editing; validation; methodology; data curation; resources. **Paolo Fabene:** Writing – review and editing; visualization; validation; methodology. **Massimo Donadelli:** Writing – review and editing; visualization; validation; methodology. **Anna Maria Stabile:** Investigation; methodology;

validation; writing – review and editing; resources; data curation; funding acquisition. **Alessandra Pistilli:** Investigation; methodology; validation; visualization; writing – review and editing. **Mario Rende:** Resources; validation; methodology. **Francesco Galli:** Writing – review and editing; methodology; validation; visualization; resources; supervision. **Federico Schena:** Investigation; funding acquisition; writing – review and editing; methodology; supervision; resources; conceptualization; project administration. **Massimo Venturelli:** Conceptualization; funding acquisition; investigation; writing – review and editing; supervision; methodology; validation; visualization; project administration.

ACKNOWLEDGMENTS

The authors would like to express our sincere gratitude to all the individuals that have contributed in this study. In particular, we would like to thank all the participants for their time and willingness to complete this project. Last but not least, our heartfelt thanks go to Pietro Belloni for its valuable support.

FUNDING INFORMATION

This research was funded by the Italian Ministry of Research and University (MIUR; Rome, Italy) 5-year special funding (https://www.miur.gov.it/dipartimenti-di-eccellenza) and "Fondazione Perugia" (grant number 2019.0326).

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interest.

DATA AVAILABILITY STATEMENT

All study data are included in the article and/or SI Appendix. Any additional information is available from the corresponding author upon reasonable request.

ORCID

Gaia Giuriato https://orcid.org/0000-0002-8149-9450
Gianluca Vernillo https://orcid.org/0000-0003-2580-4470
Massimo Venturelli https://orcid.org/0000-0002-2469-8787

REFERENCES

1. Bishop P, Cureton K, Collins M. Sex difference in muscular strength in equally-trained men and women. *Ergonomics*. 1987;30(4):675-687.
2. Miller AE, MacDougall JD, Tarnopolsky MA, Sale DG. Gender differences in strength and muscle fiber characteristics. *Eur J Appl Physiol Occup Physiol*. 1993;66(3):254-262.
3. Hunter SK. Sex differences in human fatigability: mechanisms and insight to physiological responses. *Acta Physiol (Oxf)*. 2014;210(4):768-789.
4. Lulic-Kuryllo T, Inglis JG. Sex differences in motor unit behaviour: a review. *J Electromyogr Kinesiol*. 2022;66:102689.
5. Landen S, Hiam D, Voisin S, Jacques M, Lamon S, Eynon N. Physiological and molecular sex differences in human skeletal muscle in response to exercise training. *J Physiol*. 2023;601(3):419-434.
6. Misner JE, Massey BH, Going SB, Bemben MG, Ball TE. Sex differences in static strength and fatigability in three different muscle groups. *Res Q Exerc Sport*. 1990;61(3):238-242.
7. Herda TJ, Trevino MA, Sterczala AJ, et al. Muscular strength and power are correlated with motor unit action potential amplitudes, but not myosin heavy chain isoforms in sedentary males and females. *J Biomech*. 2019;86:251-255.
8. Trevino MA, Sterczala AJ, Miller JD, et al. Sex-related differences in muscle size explained by amplitudes of higher-threshold motor unit action potentials and muscle fibre typing. *Acta Physiol (Oxf)*. 2019;225(4):e13151.
9. Nuzzo JL. Sex differences in skeletal muscle fiber types: a meta-analysis. *Clin Anat*. 2023;37:81-91.
10. Staron RS, Hagerman FC, Hikida RS, et al. Fiber type composition of the vastus lateralis muscle of young men and women. *J Histochem Cytochem*. 2000;48(5):623-629.
11. Parra ME, Sterczala AJ, Miller JD, Trevino MA, Dimmick HL, Herda TJ. Sex-related differences in motor unit firing rates and action potential amplitudes of the first dorsal interosseous during high-, but not low-intensity contractions. *Exp Brain Res*. 2020;238(5):1133-1144.
12. Goldspink G, Scutt A, Loughna PT, Wells DJ, Jaenicke T, Gerlach GF. Gene expression in skeletal muscle in response to stretch and force generation. *Am J Phys*. 1992;262(3 Pt 2):R356-R363.
13. Hitachi K, Tsuchida K. Role of microRNAs in skeletal muscle hypertrophy. *Front Physiol*. 2013;4:408.
14. Fochi S, Giuriato G, De Simone T, et al. Regulation of microRNAs in satellite cell renewal, muscle function, sarcopenia and the role of exercise. *Int J Mol Sci*. 2020;21(18):6732.
15. Bartel DP. Metazoan microRNAs. *Cell*. 2018;173(1):20-51.
16. Gebert LFR, MacRae IJ. Regulation of microRNA function in animals. *Nat Rev Mol Cell Biol*. 2019;20(1):21-37.
17. Michlewski G, Caceres JF. Post-transcriptional control of miRNA biogenesis. *RNA*. 2019;25(1):1-16.
18. Saliminejad K, Khorram Khorshid HR, Soleymani Fard S, Ghaffari SH. An overview of microRNAs: biology, functions, therapeutics, and analysis methods. *J Cell Physiol*. 2019;234(5):5451-5465.
19. Yang W, Yang L, Wang J, et al. Msi2-mediated MiR7a-1 processing repression promotes myogenesis. *J Cachexia Sarcopenia Muscle*. 2022;13(1):728-742.
20. Dalle Carbonare L, Dorelli G, Li Vigni V, et al. Physical activity modulates miRNAs levels and enhances MYOD expression in myoblasts. *Stem Cell Rev Rep*. 2022;18(5):1865-1874.
21. Soplinska A, Zareba L, Wicik Z, et al. MicroRNAs as biomarkers of systemic changes in response to endurance exercise–a comprehensive review. *Diagnostics (Basel)*. 2020;10(10):813.
22. Dai R, Ahmed SA. Sexual dimorphism of miRNA expression: a new perspective in understanding the sex bias of autoimmune diseases. *Ther Clin Risk Manag*. 2014;10:151-163.
23. Cui C, Yang W, Shi J, et al. Identification and analysis of human sex-biased MicroRNAs. *Genomics Proteomics Bioinformatics*. 2018;16(3):200-211.

24. Meder B, Backes C, Haas J, et al. Influence of the confounding factors age and sex on microRNA profiles from peripheral blood. *Clin Chem*. 2014;60(9):1200-1208.
25. Oliva M, Munoz-Aguirre M, Kim-Hellmuth S, et al. The impact of sex on gene expression across human tissues. *Science*. 2020;369(6509):eaba3066.
26. Boytar AN, Skinner TL, Wallen RE, Jenkins DG, Dekker Nitert M. The effect of exercise prescription on the human gut microbiota and comparison between clinical and apparently healthy populations: a systematic review. *Nutrients*. 2023;15(6):1534.
27. Sherwin E, Sandhu KV, Dinan TG, Cryan JF. May the force Be with you: the light and dark sides of the microbiota-gut-brain Axis in neuropsychiatry. *CNS Drugs*. 2016;30(11):1019-1041.
28. Miranda-Comas G, Petering RC, Zaman N, Chang R. Implications of the gut microbiome in sports. *Sports Health*. 2022;14(6):894-898.
29. Chen H, Shen L, Liu Y, et al. Strength exercise confers protection in central nervous system autoimmunity by altering the gut microbiota. *Front Immunol*. 2021;12:628629.
30. Dohnalova L, Lundgren P, Carty JRE, et al. A microbiome-dependent gut-brain pathway regulates motivation for exercise. *Nature*. 2022;612(7941):739-747.
31. Chen YM, Wei L, Chiu YS, et al. *Lactobacillus plantarum* TWK10 supplementation improves exercise performance and increases muscle mass in mice. *Nutrients*. 2016;8(4):205.
32. Kim YS, Unno T, Kim BY, Park MS. Sex differences in gut microbiota. *World J Mens Health*. 2020;38(1):48-60.
33. Gandevia SC. Spinal and supraspinal factors in human muscle fatigue. *Physiol Rev*. 2001;81(4):1725-1789.
34. Ansdell P, Thomas K, Howatson G, Hunter S, Goodall S. Contraction intensity and sex differences in knee-extensor fatigability. *J Electromyogr Kinesiol*. 2017;37:68-74.
35. Wust RC, Morse CI, de Haan A, Jones DA, Degens H. Sex differences in contractile properties and fatigue resistance of human skeletal muscle. *Exp Physiol*. 2008;93(7):843-850.
36. Murray BE, Froemming GR, Maguire PB, Ohlendieck K. Excitation-contraction-relaxation cycle: role of Ca2+−regulatory membrane proteins in normal, stimulated and pathological skeletal muscle (review). *Int J Mol Med*. 1998;1(4):677-687.
37. Nishi H, Ono K, Iwanaga Y, et al. MicroRNA-15b modulates cellular ATP levels and degenerates mitochondria via Arl2 in neonatal rat cardiac myocytes. *J Biol Chem*. 2010;285(7):4920-4930.
38. Siengdee P, Trakooljul N, Murani E, Schwerin M, Wimmers K, Ponsuksili S. MicroRNAs regulate cellular ATP levels by targeting mitochondrial energy metabolism genes during C2C12 myoblast differentiation. *PLoS One*. 2015;10(5):e0127850.
39. Maffiuletti NA, Aagaard P, Blazevich AJ, Folland J, Tillin N, Duchateau J. Rate of force development: physiological and methodological considerations. *Eur J Appl Physiol*. 2016;116(6):1091-1116.
40. Andersen LL, Aagaard P. Influence of maximal muscle strength and intrinsic muscle contractile properties on contractile rate of force development. *Eur J Appl Physiol*. 2006;96(1):46-52.
41. D'Emanuele S, Maffiuletti NA, Tarperi C, Rainoldi A, Schena F, Boccia G. Rate of force development as an indicator of neuromuscular fatigue: a scoping review. *Front Hum Neurosci*. 2021;15:701916.
42. Del Vecchio A, Negro F, Holobar A, et al. You are as fast as your motor neurons: speed of recruitment and maximal discharge of motor neurons determine the maximal rate of force development in humans. *J Physiol*. 2019;597(9):2445-2456.
43. Hannah R, Minshull C, Buckthorpe MW, Folland JP. Explosive neuromuscular performance of males versus females. *Exp Physiol*. 2012;97(5):618-629.
44. Behm DG, Sale DG. Voluntary and evoked muscle contractile characteristics in active men and women. *Can J Appl Physiol*. 1994;19(3):253-265.
45. Bell DG, Jacobs I. Electro-mechanical response times and rate of force development in males and females. *Med Sci Sports Exerc*. 1986;18(1):31-36.
46. Inglis JG, Gabriel DA. Sex differences in motor unit discharge rates at maximal and submaximal levels of force output. *Appl Physiol Nutr Metab*. 2020;45(11):1197-1207.
47. Inglis JG, McIntosh K, Gabriel DA. Neural, biomechanical, and physiological factors involved in sex-related differences in the maximal rate of isometric torque development. *Eur J Appl Physiol*. 2017;117(1):17-26.
48. Holtermann A, Roeleveld K, Vereijken B, Ettema G. The effect of rate of force development on maximal force production: acute and training-related aspects. *Eur J Appl Physiol*. 2007;99(6):605-613.
49. Harridge SD, Bottinelli R, Canepari M, et al. Whole-muscle and single-fibre contractile properties and myosin heavy chain isoforms in humans. *Pflugers Arch*. 1996;432(5):913-920.
50. Klitgaard H, Mantoni M, Schiaffino S, et al. Function, morphology and protein expression of ageing skeletal muscle: a cross-sectional study of elderly men with different training backgrounds. *Acta Physiol Scand*. 1990;140(1):41-54.
51. Korhonen MT, Cristea A, Alen M, et al. Aging, muscle fiber type, and contractile function in sprint-trained athletes. *J Appl Physiol (1985)*. 2006;101(3):906-917.
52. Miller BF, Olesen JL, Hansen M, et al. Coordinated collagen and muscle protein synthesis in human patella tendon and quadriceps muscle after exercise. *J Physiol*. 2005;567(Pt 3):1021-1033.
53. Marqueti RC, Prestes J, Wang CC, et al. Biomechanical responses of different rat tendons to nandrolone decanoate and load exercise. *Scand J Med Sci Sports*. 2011;21(6):e91-e99.
54. Hansen M, Kongsgaard M, Holm L, et al. Effect of estrogen on tendon collagen synthesis, tendon structural characteristics, and biomechanical properties in postmenopausal women. *J Appl Physiol (1985)*. 2009;106(4):1385-1393.
55. Cook JL, Bass SL, Black JE. Hormone therapy is associated with smaller Achilles tendon diameter in active post-menopausal women. *Scand J Med Sci Sports*. 2007;17(2):128-132.
56. Maillot G, Lacroix-Triki M, Pierredon S, et al. Widespread estrogen-dependent repression of micrornas involved in breast tumor cell growth. *Cancer Res*. 2009;69(21):8332-8340.
57. Mao L, Liu S, Hu L, et al. miR-30 family: a promising regulator in development and disease. *Biomed Res Int*. 2018;2018:9623412.
58. Hu F, Wang M, Xiao T, et al. miR-30 promotes thermogenesis and the development of beige fat by targeting RIP140. *Diabetes*. 2015;64(6):2056-2068.
59. Huang Z, Huang H, Shen M, et al. MicroRNA-155-5p modulates the progression of acute respiratory distress syndrome by targeting interleukin receptors. *Bioengineered*. 2022;13(5):11732-11741.
60. Wen Q, Wang Y, Pan Q, et al. MicroRNA-155-5p promotes neuroinflammation and central sensitization via inhibiting SIRT1

in a nitroglycerin-induced chronic migraine mouse model. *J Neuroinflammation*. 2021;18(1):287.
61. Sandri M. Protein breakdown in muscle wasting: role of autophagy-lysosome and ubiquitin-proteasome. *Int J Biochem Cell Biol*. 2013;45(10):2121-2129.
62. Ghafouri-Fard S, Khoshbakht T, Hussen BM, Jamal HH, Taheri M, Hajiesmaeili M. A comprehensive review on function of miR-15b-5p in malignant and non-malignant disorders. *Front Oncol*. 2022;12:870996.
63. Chen Y, Wang X. miRDB: an online database for prediction of functional microRNA targets. *Nucleic Acids Res*. 2019;48(D1):D 127-D131.
64. Welten SM, Bastiaansen AJ, de Jong RC, et al. Inhibition of 14q32 MicroRNAs miR-329, miR-487b, miR-494, and miR-495 increases neovascularization and blood flow recovery after ischemia. *Circ Res*. 2014;115(8):696-708.
65. Renteria I, Garcia-Suarez PC, Fry AC, et al. The molecular effects of BDNF synthesis on skeletal muscle: a mini-review. *Front Physiol*. 2022;13:934714.
66. Griesbeck O, Parsadanian AS, Sendtner M, Thoenen H. Expression of neurotrophins in skeletal muscle: quantitative comparison and significance for motoneuron survival and maintenance of function. *J Neurosci Res*. 1995;42(1):21-33.
67. Mousavi K, Parry DJ, Jasmin BJ. BDNF rescues myosin heavy chain IIB muscle fibers after neonatal nerve injury. *Am J Physiol Cell Physiol*. 2004;287(1):C22-C29.
68. Cuppini R, Sartini S, Agostini D, et al. Bdnf expression in rat skeletal muscle after acute or repeated exercise. *Arch Ital Biol*. 2007;145(2):99-110.
69. Ogborn DI, Gardiner PF. Effects of exercise and muscle type on BDNF, NT-4/5, and TrKB expression in skeletal muscle. *Muscle Nerve*. 2010;41(3):385-391.
70. Jimenez-Maldonado A, Cerna-Cortes J, Castro-Rodriguez EM, et al. Effects of moderate- and high-intensity chronic exercise on brain-derived neurotrophic factor expression in fast and slow muscles. *Muscle Nerve*. 2016;53(3):446-451.
71. Onoki T, Izumi Y, Takahashi M, et al. Skeletal muscle-specific Keap1 disruption modulates fatty acid utilization and enhances exercise capacity in female mice. *Redox Biol*. 2021;43:101966.
72. Gao L, Kumar V, Vellichirammal NN, et al. Functional, proteomic and bioinformatic analyses of Nrf2- and Keap1- null skeletal muscle. *J Physiol*. 2020;598(23):5427-5451.
73. Mourkioti F, Rosenthal N. NF-kappaB signaling in skeletal muscle: prospects for intervention in muscle diseases. *J Mol Med (Berl)*. 2008;86(7):747-759.
74. Li H, Malhotra S, Kumar A. Nuclear factor-kappa B signaling in skeletal muscle atrophy. *J Mol Med (Berl)*. 2008;86(10): 1113-1126.
75. Hardin BJ, Campbell KS, Smith JD, et al. TNF-alpha acts via TNFR1 and muscle-derived oxidants to depress myofibrillar force in murine skeletal muscle. *J Appl Physiol (1985)*. 2008;104(3):694-699.
76. Lay C, Rigottier-Gois L, Holmstrom K, et al. Colonic microbiota signatures across five northern European countries. *Appl Environ Microbiol*. 2005;71(7):4153-4155.
77. Yuan X, Chen R, Zhang Y, Lin X, Yang X. Sexual dimorphism of gut microbiota at different pubertal status. *Microb Cell Factories*. 2020;19(1):152.
78. Kovacs A, Ben-Jacob N, Tayem H, Halperin E, Iraqi FA, Gophna U. Genotype is a stronger determinant than sex of the mouse gut microbiota. *Microb Ecol*. 2011;61(2):423-428.
79. Peng C, Xu X, Li Y, et al. Sex-specific association between the gut microbiome and high-fat diet-induced metabolic disorders in mice. *Biol Sex Differ*. 2020;11(1):5.
80. Verma R, Balhara YP, Gupta CS. Gender differences in stress response: role of developmental and biological determinants. *Ind Psychiatry J*. 2011;20(1):4-10.
81. Magne F, Gotteland M, Gauthier L, et al. The firmicutes/bacteroidetes ratio: a relevant marker of gut dysbiosis in obese patients? *Nutrients*. 2020;12(5):1474.
82. Mariat D, Firmesse O, Levenez F, et al. The firmicutes/bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiol*. 2009;9:123.
83. Hughes RL. A review of the role of the gut microbiome in personalized sports nutrition. *Front Nutr*. 2019;6:191.
84. Dalton A, Mermier C, Zuhl M. Exercise influence on the microbiome-gut-brain axis. *Gut Microbes*. 2019;10(5):555-568.
85. Forsythe P, Kunze WA, Bienenstock J. On communication between gut microbes and the brain. *Curr Opin Gastroenterol*. 2012;28(6):557-562.
86. Forsythe P, Bienenstock J, Kunze WJAEMB. Microbial endocrinology and the microbiota-gut-brain axis. *Adv Exp Med Biol*. 2014;17(817):3-24.
87. Fimland MS, Helgerud J, Gruber M, Leivseth G, Hoff J. Functional maximal strength training induces neural transfer to single-joint tasks. *Eur J Appl Physiol*. 2009;107(1):21-29.
88. Tøien T, Unhjem R, Øren TS, Kvellestad ACG, Hoff J, Wang E. Neural plasticity with age: unilateral maximal strength training augments efferent neural drive to the contralateral limb in older adults. *J Gerontol A Biol Sci Med Sci*. 2017;73(5):596-602.
89. Monda V, Villano I, Messina A, et al. Exercise modifies the gut microbiota with positive health effects. *Oxidative Med Cell Longev*. 2017;2017:3831972.
90. Cook MD, Allen JM, Pence BD, et al. Exercise and gut immune function: evidence of alterations in colon immune cell homeostasis and microbiome characteristics with exercise training. *Immunol Cell Biol*. 2016;94(2):158-163.
91. Clauss M, Gerard P, Mosca A, Leclerc M. Interplay between exercise and gut microbiome in the context of human health and performance. *Front Nutr*. 2021;8:637010.
92. Faul F, Erdfelder E, Lang AG, Buchner A. G*power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods*. 2007;39(2):175-191.
93. Cohen J. *Statistical Power Analysis for the Behavioral Sciences*. 2nd ed. Routledge; 1988.
94. Venturelli M, Ce E, Paneroni M, et al. Safety procedures for exercise testing in the scenario of COVID-19: a position statement of the Societa Italiana Scienze Motorie e Sportive. *Sport Sci Health*. 2020;16(4):601-607.
95. Jensen BL, Persson PB. Good publication practice in physiology 2021. *Acta Physiol*. 2022;234(1):e13741.
96. Elias LJ, Bryden MP. Footedness is a better predictor of language lateralisation than handedness. *Laterality*. 1998;3(1):41-51.
97. Vernillo G, Barbi C, Temesi J, et al. Reliability of relaxation properties of knee-extensor muscles induced by transcranial magnetic stimulation. *Neurosci Lett*. 2022;782:136694.

98. Vernillo G, Khassetarash A, Millet GY, Temesi J. Use of transcranial magnetic stimulation to assess relaxation rates in unfatigued and fatigued knee-extensor muscles. *Exp Brain Res*. 2021;239(1):205-216.
99. Ansdell P, Brownstein CG, Skarabot J, et al. Menstrual cycle-associated modulations in neuromuscular function and fatigability of the knee extensors in eumenorrheic women. *J Appl Physiol (1985)*. 2019;126(6):1701-1712.
100. Cavedon V, Milanese C, Laginestra FG, et al. Bone and skeletal muscle changes in oldest-old women: the role of physical inactivity. *Aging Clin Exp Res*. 2020;32(2):207-214.
101. Sarto F, Sporri J, Fitze DP, Quinlan JI, Narici MV, Franchi MV. Implementing ultrasound imaging for the assessment of muscle and tendon properties in elite sports: practical aspects, methodological considerations and future directions. *Sports Med*. 2021;51(6):1151-1170.
102. Mathis J, de Quervain D, Hess CW. Dependence of the transcranially induced silent period on the 'instruction set' and the individual reaction time. *Electroencephalogr Clin Neurophysiol*. 1998;109(5):426-435.
103. Meola G, Bugiardini E, Cardani R. Muscle biopsy. *J Neurol*. 2012;259(4):601-610.
104. Moro T, Brightwell CR, Phalen DE, et al. Low skeletal muscle capillarization limits muscle adaptation to resistance exercise training in older adults. *Exp Gerontol*. 2019;127:110723.
105. Moro T, Brightwell CR, Volpi E, Rasmussen BB, Fry CS. Resistance exercise training promotes fiber type-specific myonuclear adaptations in older adults. *J Appl Physiol (1985)*. 2020;128(4):795-804.
106. Walker JM. The bicinchoninic acid (BCA) assay for protein quantitation. *Methods Mol Biol*. 1994;32:5-8.
107. Bartolini D, Commodi J, Piroddi M, et al. Glutathione S-transferase pi expression regulates the Nrf2-dependent response to hormetic diselenides. *Free Radic Biol Med*. 2015;88(Pt B):466-480.
108. Chen H, Zhou Y, Wang ZY, et al. Exosomal microRNA profiles from serum and cerebrospinal fluid in neurosyphilis. *Sex Transm Infect*. 2019;95(4):246-250.
109. Li Q, Chang Y, Zhang K, Chen H, Tao S, Zhang Z. Implication of the gut microbiome composition of type 2 diabetic patients from northern China. *Sci Rep*. 2020;10(1):5450.
110. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15(12):550.
111. Hopkins WG, Marshall SW, Batterham AM, Hanin J. Progressive statistics for studies in sports medicine and exercise science. *Med Sci Sports Exerc*. 2009;41(1):3-13.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Giuriato G, Romanelli MG, Bartolini D, et al. Sex differences in neuromuscular and biological determinants of isometric maximal force. *Acta Physiol*. 2024;00:e14118. doi:10.1111/apha.14118