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The MDM2 SNP309 differentially impacts cardiorespiratory fitness in young healthy women and men.

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ABSTRACT

PURPOSE: Maximal oxygen consumption (VO_2max), the predominant index of cardiorespiratory fitness (CRF), is a predictor of whole-body function and longevity in humans. Central cardiac function and the skeletal muscle's capacity to use oxygen are key determinants of VO_2max. Murine Double Minute 2 (MDM2), mainly known as an oncogene, could regulate myocardial hypertrophy, skeletal muscle angiogenesis and oxidative phosphorylation. A prevalent single nucleotide polymorphism in the MDM2 promoter (SNP309) substitutes a T for a G, supporting a greater transcriptional activity. We aim to assess whether SNP309 impacts intrinsic CRF.

METHODS: 82 young healthy nonathletic male and female adults aged 23±2 years performed cardiorespiratory exercise testing to determine their VO_2max ($mL \cdot kg^{-1} \cdot min^{-1}$). Genomic DNAs isolated from saliva were genotyped using Taqman-based qPCR.

RESULTS: A one-way ANOVA showed that SNP309 influenced relative VO_2max in the whole cohort (p=0.044) and in men (p=0.009), remaining non-significant in women (p=0.133). VO_2max was higher in TT homozygotes than in GT heterozygotes (whole cohort, 47±12 vs. 42±6 $mL \cdot kg^{-1} \cdot min^{-1}$, p=0.030; men, 53±8 vs. 45±6 $mL \cdot kg^{-1} \cdot min^{-1}$, p=0.011). A contingency analysis revealed a positive association between SNP309 in men in which the TT genotype was more frequent in the high VO_2max group (p=0.006). When considering G as the dominant allele, men bearing a G allele had lower relative VO_2max than TT homozygotes (47±7 vs. 53±8, GG/GT vs. TT, p=0.010). Conversely, women bearing a G allele had a higher relative VO_2max than TT homozygotes (39±5 vs. 34±7, GG/GT vs. TT, p=0.047).

CONCLUSION: SNP309 impacts VO_2max in a sex-dependent manner in our cohort.

KEY WORDS

Fitness, sex-differences, rs2279744 (MDM2-SNP309), single nucleotide polymorphism, VO_2max.

STATEMENTS AND DECLARATIONS

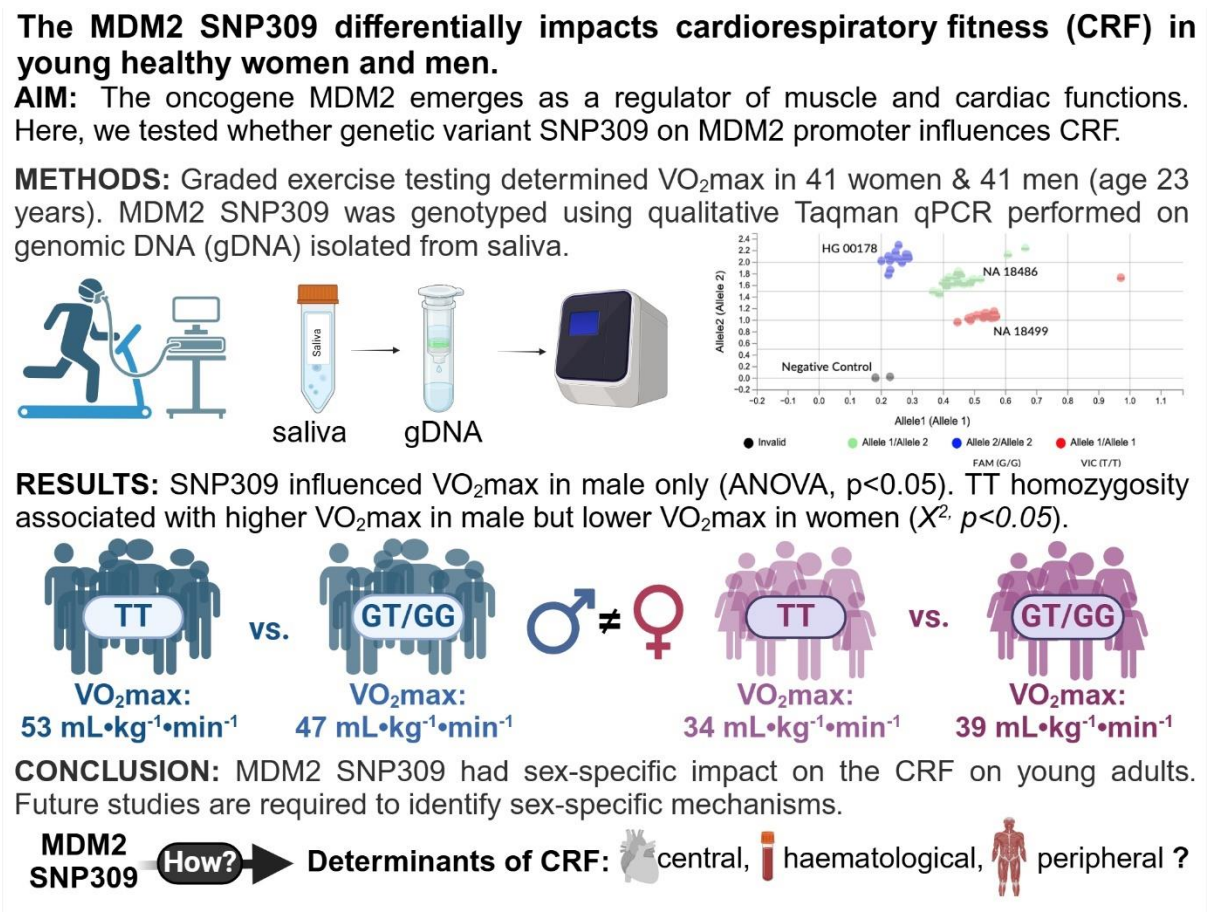
The authors declare no conflict of interest.

ABBREVIATIONS

a-$\tilde{v}O_2$diff. arteriovenous oxygen difference
BMI. Body mass index
CRF. Cardiorespiratory fitness
CVD. Cardiovascular disease
CaO_2 Arterial blood oxygen content
CO_2. Carbon Dioxide
CO. Cardiac output
CvO_2. Venous blood oxygen content
ER-α oestrogen receptor alpha

FoxO1. Forkhead box protein O1
FRIEND. Fitness Registry and Importance of Exercise National Database
GXT. Graded exercise test
HIF1-alpha. Hypoxia-inducible factor 1 subunit alpha
HR. Heart rate.
MDM2. Murine double minute 2
O_2. Oxygen
TP53. Tumour protein 53
SNP. Single nucleotide polymorphism
Sp-1. Specificity protein-1
VO_2max. Maximal oxygen consumption

GRAPHICAL ABSTRACT



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INTRODUCTION

Cardiorespiratory fitness (CRF), an index of the coordinated efficiency of the whole body to provide oxygen (O_2) at maximal exercise capacity (Kenney et al. 2012; Raghuveer et al. 2020), is a strong predictor of cardiovascular health (Kodama et al. 2009; Cahalin et al. 2013; Tabet et al. 2013; Nauman et al. 2017; Boulmpou et al. 2023). Multiple factors influence CRF, including an individual's genetic background, fitness level, level of habitual physical activity participation, which includes intensity-based exercise and general health (Schutte et al. 2016). Therefore, inter-individual differences in lifestyle are key modifiable determinants of CRF (Blair and Church 2004; Weston et al. 2014; Lin et al. 2015; Schindler et al. 2019). More individuals spend significant daily time in sedentary behaviours (Lavie et al. 2019). Not engaging in physical activities that are sufficient in frequency, intensity, and duration could impair individuals' capacity to use their genetic background at their full potential to develop high CRF or to counteract genetic predispositions to low CRF. Therefore, it will be important to identify more genetic determinants of intrinsic CRF in a sedentary state (Ghosh et al. 2019).

The genetic background of individuals is a non-modifiable determinant of CRF at baseline and influences CRF adaptability to training (Sundet et al. 1994; Bouchard et al. 1998, 1999, 2011; Williams et al. 2017). Confirming results of pioneering work performed in sedentary populations (Bouchard et al. 1998), twin studies have further validated that heritability could account for more than 50% of the variability in CRF in adolescents with various levels of physical activity (from sedentary to regular vigorous intensity exercise) (Zadro et al. 2017). Genetic variations influence both intrinsic VO_2max and VO_2max adaptability to training (Schutte et al. 2016; Williams et al. 2017). Single nucleotide polymorphisms (SNPs) are the most common form of genetic variants in the human genome, being the main contributors to inter-individual variations in the DNA sequence (Syvänen 2001). A SNP is a substitution of a single base pair within a gene. SNP's may have phenotypic consequences on a trait; however, most SNPs appear to not have a phenotypic effect, leading to no apparent changes in traits (Syvänen 2001). CRF is a considerably complex trait that could be influenced by multiple genes (Bouchard et al. 2011; Hanscombe et al. 2021). Yet, SNPs are found in genes coding for proteins that are key to the central cardiac function or to the peripheral capacity to extract oxygen (Fung et al. 2006; Simon-Sanchez et al. 2007). Some of those SNPs may relate as key genetic determinants of the CRF trait (Montgomery et al. 1997; Hagberg et al. 2002; Prior et al. 2006).

SNP309, a SNP with T-to-G substitution (SNP309) on the promoter of the MDM2 gene is highly frequent in human populations with allele frequency ranging from 0.11 to 0.49 (Millikan et al. 2006; Park et al. 2006; Paulin et al. 2008). The presence of SNP309 in cells promotes a greater expression of MDM2 protein, an E3 ubiquitin ligase involved in controlling the cellular stress response (Michael and Oren 2003; Bond et al. 2004; Wang et al. 2020). Through its capacity to interact with transcription factors (Fåhraeus and Olivares-Illana 2014; Lam and Roudier 2019) such as FoxO1(Fu et al. 2009; Milkiewicz et al. 2011), HIF1-alpha (Nieminen et al. 2005; Muthumani et al. 2014) or TP53 (Dameron et al. 1994; Chen et al. 2006), MDM2 regulates the expression of angiogenesis-related genes (Olfert and Birot 2011), reducing anti-angiogenic activity (Fu et al. 2009) and exerting pro-angiogenic effects (Aiken et al. 2016). In mice, MDM2 regulates skeletal muscle capillarization at basal level and in

response to endurance exercise (Roudier et al. 2012). Indeed, transgenic mice expressing low levels of MDM2 protein had reduced capillarization in their skeletal muscle (Roudier et al. 2012). Under oxidative and hypoxic stress, active translocation of MDM2 protein into the mitochondria represses mitochondrial gene transcription, negatively impacting respiration. (Arena et al. 2018). This mechanism could limit skeletal muscle function under reduced O_2 availability. Indeed, mice lacking striated muscle MDM2 present a reduced exercise capacity under mild hypoxia (15% O_2) (Arena et al. 2018). Additionally, cardiac expression of MDM2 could protect the heart from pathological myocardial hypertrophy to preserve left ventricular function and regulate cardiac contractility in response to adrenergic stimulation (Jean-Charles et al. 2017; Beyfuss et al. 2018; Gries et al. 2018). From these preclinical studies, MDM2 emerges as a gene that can have a functional impact on both the cardiac function and the peripheral capacity to extract O_2, the two key components of CRF. Naturally, the following question emerges: "Is SNP309 on MDM2 promoter a genetic determinant of one's VO_2max?"

Studies show that within the human population, the T allele supports lower transcription of MDM2, whereas the G allele demonstrates higher MDM2 expression (Bond et al. 2004). If SNP309 can enhance transcription, increasing cellular level of MDM2 protein and function, one might wonder whether this SNP309 is critically related to CRF. We hypothesized that individuals with the G phenotype have a greater CRF. To address this hypothesis, a cohort of female and male nonathletic university students had their VO_2max measured using a graded exercise test (GXT), anthropometric data were collected, and SNP309 genotypes were assessed by qPCR.

METHODS

Human Studies

Healthy nonathletic university students who were not engaged in a regimented training program were recruited to the undergraduate research program to participate in the study. Each participant provided written informed consent. Eligibility criteria included participants to be healthy, aged 18 to 30 years and to have no contraindications to perform maximal exercise testing. Exclusion criteria included being diagnosed with hypertension, cardiovascular diseases, other chronic diseases and a BMI above or equal to 35 kg/m^2. This study was approved by the York University Human Participants Review Committee, whose research ethics guidelines are in accordance with the Canadian Tri-Council research ethics (Certificate #: E2019-235). Each participant completed the Physical Activity Readiness Questionnaire for Everyone (PAR-Q+) and, if necessary, the ePARmed-X+ (Bredin et al. 2013) to ensure they were cleared for unrestricted physical activity (e.g. moderate to maximal intensity exercise). Pre-exercise blood pressure and pulse or heart rate measurements were taken by qualified exercise physiologists to clear participants for unrestricted physical activity. Following the pre-exercise screening procedure, all participants self-reported their demographics (age and sex), and anthropometric (height, body mass and body mass index (BMI $kg \cdot m^2$)) data was measured.

Aerobic Fitness Test - VO_2max Determination

The modified Astrand protocol was used to determine participants' VO_2max via indirect calorimetry using discrete component open circuit spirometry (Gledhill et al. 1994; Hancock et al. 2023). While wearing a nose clip, participants inhaled air from the atmosphere and exhaled air through a rubber mouthpiece with a two-way y-valve and a 1.5-inch flexible corrugated hose. Exhaled air was collected during the final 30 seconds of each 2-minute workload and analysed by rapid response gas analysers for the fractional concentrations of O_2 and carbon dioxide (CO_2). The O_2 and CO_2 analysers were calibrated before and during each workload using gravimetrically analysed gases. Following a 5-minute walking warm-up on the treadmill at 3.5 mph and 2.0% incline, participants began the incremental to maximal GXT by jogging or running at 5.0 mph at an incline of 2.0% depending on their body mass, for 2 minutes. During each subsequent 2-minute workload, the incline was kept constant, and the treadmills speed was increased by 1.0 mph until a suitable running speed was achieved (~6.0-7.0 mph). Then, the speed of the treadmill was kept constant, and the incline was progressively increased by 2.0% during each subsequent 2-minute workload until participants either attained a plateau in the VO_2max using verification workloads or were no longer physically able to continue (i.e., volitional fatigue) (Hancock et al. 2023). Heart rate (HR) was measured throughout GXT using a Polar HR chest monitor (Polar Electro, Kempele, Finland).

DNA collection and genotyping of the SNP309

Saliva samples were collected from participants before the aerobic fitness test was performed. Subjects were instructed not to eat, drink, chew gum for 30 minutes prior to sample collection. Saliva samples were extracted and purified using the Purelink Genomic DNA Purification Kit by Invitrogen (Burlington, ON, Canada), following the manufacturer's instructions. Total DNA samples were quantified followed by genotyping using the Taqman

SNP probe (ThermoFisher, Burlington, ON, Canada, rs2279744, #15968533, probe sequence: GGGGGCCGGGGGCTGCGGGGCCGCT**[T/G]**CGGCGCGGGAGGTCCGGATGATCGC, [VIC/FAM]). 10 to 20ng of genomic DNA was used for real-time PCR. Positive controls obtained from the NINDS Human Genetics Resource Center DNA and Cell Line Repository were used for quality control check (HG00178, NA18486, NA18499, Corielle Institute for Medical Research Biobank, Philadelphia, NJ, United States). The original genotyping was performed in the laboratories of Drs. Singleton and Hardy (Fung et al. 2006; Simon-Sanchez et al. 2007).

Statistical analysis

All analyses were performed using the Prism GraphPad software 10 (Boston, MA, United States). Descriptive statistics are expressed as mean and standard deviation (mean ± SD). Participant characteristics were analysed using Fisher t-test. We used a X^2 (CHI Square - (Fisher Exact Test) analysis to test the influence of sex and ethnicity on the allele and genotype distribution. A required sample size of 140 alleles for the whole study was calculated based on the frequency data reported in the SNP NCBI database for SNP309, rs2279744 (G allele frequency in control individual 0.32), an α value of 0.05, and a power of 80% (1-β=0.8) (Slager and Schaid 2001; Jalilvand et al. 2020). Therefore, we recruited 82 subjects (164 alleles) including 41 males and 41 females.

When testing diverse populations, the common practise is to normalize VO_2max relative to body mass when examining association with cardiovascular risk (Salier Eriksson et al. 2021) or to test association between genetic variants and CRF (Lolli et al. 2017; Klevjer et al. 2022). In this study, we examined the association between SNP309 genotype and relative VO_2max ($mL \cdot kg^{-1} \cdot min^{-1}$).

The association between CRF and MDM2 SNP309 polymorphism was tested through three approaches. First, individuals were grouped based on genotype, GG, GT and TT to calculate mean maximum heart rate (max HR), relative VO_2max ($mL \cdot kg^{-1} \cdot min^{-1}$) and BMI. Using a two-way ANOVA approach, we observed a significant interaction between sex and genotype (e.g., p=0.030 for VO_2max), indicating that genotype induced an opposite effect depending on sex. Therefore, the impact of genotype was then tested using a one-way ANOVA, based on the hypothesis of a codominant model where these three genotypes represent a dose effect with the G allele generating more transcripts than the T allele (Bond et al. 2004). In our second approach, the cohort was divided into two groups of high or low relative VO_2max. Our thresholds for determining high and low VO2max values were based on the Fitness Registry and the Importance of Exercise National Database (FRIEND) as a reference (Kaminsky et al. 2015). This database contains measures from laboratories that demonstrate a robust expertise in performing cardiopulmonary testing; and is therefore employed by scholarly societies to determine normative values (e.g American Heart association, (Peterman et al. 2021)). The range of good VO_2max was used for both males and females, resulting in the threshold of <40 $mL \cdot kg^{-1} \cdot min^{-1}$ for females and <50 $mL \cdot kg^{-1} \cdot min^{-1}$ for males. We then used X^2 (CHI Square - (Fisher Exact Test)) analysis to assess whether VO_2max status influenced the genotype distribution on a codominant model. In a third and last approach, we tested both recessive (TT vs. GT+GG) and dominant (GG vs. GT+TT) models, assessing whether the allele frequency

249 influences the VO_2max using a X^2 (CHI Square - (Fisher Exact Test)) analysis. P values equal
250 or lower than 0.05 were considered significant for all tests.

RESULTS

Eighty-two healthy nonathletic adults participated in the study, including males (n=41; age: 23±2 yr) and females (n= 41; age:23±2 yr; *Table 1*). Based on the BMI metric, no male participants were underweight, 23 males were normal weight, and 18 were overweight. One female participant was underweight, 29 females were normal weight, and 11 were overweight. The distribution of underweight, normal, overweight and obese was not different between male and female groups (p=0.165). There was no difference in age and BMI between male and female participants. Females had a statistically significant lower VO_2max (-11 $mL \cdot kg^{-1} \cdot min^{-1}$), body mass (-13 kg), height (-10 cm) and max HR (-5 bpm) relative to the male participants (p<0.0001).

Table 1 Morphometric and cardiorespiratory fitness characteristics of participants. Data shows mean ± SD, differences were tested using a Fisher T-test, * indicates significant differences (p<0.05)

	All subjects	Males	Females	Difference between male and female (p value)
VO_2max ($L \cdot min^{-1}$)	3.03 ± 0.81	3.67 ± 0.56	2.39 ± 0.42	<0.0001*
VO_2max ($mL \cdot kg^{-1} \cdot min^{-1}$)	44 ± 9	49 ± 8	38 ± 6	<0.0001*
Body Mass (kg)	69 ± 13	76 ± 12	63 ± 11	<0.0001*
Height (cm)	170 ± 8	175 ± 7	165 ± 6	<0.0001*
BMI ($kg \cdot m^{-2}$)	24 ± 4	25 ± 3	23 ± 4	0.297
Max HR (bpm)	197 ± 10	199 ± 8	194 ± 10	0.009*
Age	23 ± 2	23 ± 2	23 ± 2	0.845

No difference in the distribution of genotype and allele frequency was observed between male and female (*SI Table 1*). Ethnicity had an impact on allele frequency (p=0.024). In our cohort, per ethnicity, the T allele for MDM2 SNP309 from the highest to the lowest frequency was: 1) African American descendants (83% T, 17% G), 2) Middle Eastern descendants frequency (60% T, 40% G), 3) Caucasian (54% T, 46% G), 4) East Asian (44% T, 56% G), and 5) South Asian (39% T , 61% G).

Next, we used a co-dominant model. When analysing all subjects independently of sex, we found no significant effect of SNP309 genotype on height, body mass, max HR and BMI. The genotype significantly influenced the relative VO_2max (Table 2, One-way ANOVA, p=0.044). Post-hoc multiple comparisons did not find significant differences between genotypes (*Table 2*, Tukey's test, GG vs. GT, p=0.262, GG vs. TT p=0.842, and GT vs. TT, p=0.051). Based on the hypothesis that the TT genotype generates fewer MDM2 transcripts in tissues (Bond et al. 2004), we refined our analysis by defining this TT genotype as the control group. The VO_2max of TT individuals was significantly greater than in GT individuals (+5.4

mL·kg^{-1}·min^{-1}, Dunnett's post hoc test, p=0.036, *Fig. 1A*), but remained non-significantly different than the GG group.

Table 2 Interaction between the genotypes and the maximum HR, relative VO_2max and BMI. To test the impact of the genotype in a codominant model, we used a one-way ANOVA. The effect of SNP309 genotypes was considered significant when p-values were below 0.05. Post-hoc multiple comparisons were run comparing all groups (Dunnett's test). * indicates significant differences (p<0.05)

All subjects	**Genotype**			**P-value**
	GG	**GT**	**TT**	
N value	17	44	21	NA
Body mass (kg)	67 ± 11	69 ± 14	71 ± 12	0.617
Height (cm)	169 ± 8	168 ± 9	172 ± 8	0.644
Max HR (bpm)	199 ± 8	195 ± 11	198 ± 7	0.357
VO2max (mL·kg^{-1}·min^{-1})	45 ± 9	42 ± 6	47 ± 12	0.044*
BMI (kg·m^{-2})	23 ± 3	24 ± 4	24 ± 4	0.744
Males	**Genotype**			
	GG	**GT**	**TT**	
N value	9	18	14	NA
Body mass (kg)	72 ± 11	80 ± 11	73 ± 12	0.120
Height (cm)	174 ± 9	176 ± 7	175 ± 7	0.749
Max HR (bpm)	201 ± 7	199 ± 10	199 ± 6	0.898
VO2max (mL·kg^{-1}·min^{-1})	50 ± 7	45 ± 6	53 ± 8	0.009*
BMI (kg·m^{-2})	24 ± 2	26 ± 3	24 ± 3	0.064
Females	**Genotype**			
	GG	**GT**	**TT**	
N value	8	26	7	NA
Body mass (kg)	62 ± 8	62 ± 9	68 ± 12	0.339
Height (cm)	164 ± 3	164 ± 6	167 ± 6	0.419
Max HR (bpm)	197 ± 8	193 ± 11	195 ± 7	0.559
VO2max (mL·kg^{-1}·min^{-1})	40 ± 7	39 ± 5	34 ± 8	0.133
BMI (kg·m^{-2})	23 ± 3	23 ± 3	23 ± 7	0.982

The genotype did not impact the max HR and BMI for both male and female participants (*Table 2*). For male participants, the SNP309 genotype had a significant effect on the relative VO_2max (mL·kg-1·min-1, p=0.009). Multiple comparisons indicated that males with the TT genotype had greater relative VO_2max values than did the GT males (+8.08 mL·kg-1·min-1, p=0.003, Fig. 1B). No difference was observed in the female participants (*Fig. 1C*).

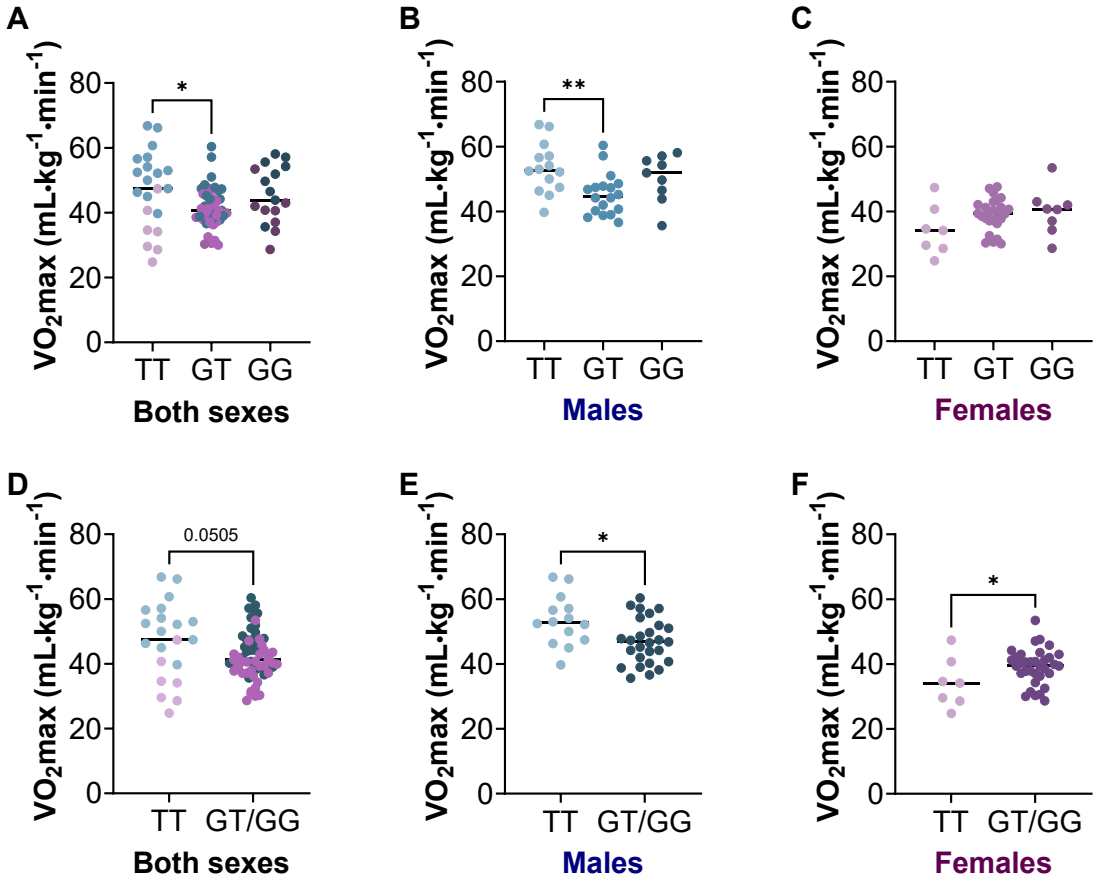

Fig. 1 Interaction between the SNP309 genotype and VO_2max ($mL \cdot kg^{-1} \cdot min^{-1}$) considering all participants (left), in females (middle) and males (right). Panels A to C show the VO_2max for all genotypes (TT, GT and GG) for both sexes, in males and in females. Data shows VO_2max values ($mL \cdot kg^{-1} \cdot min^{-1}$), and the line shows the mean VO_2max ($mL \cdot kg^{-1} \cdot min^{-1}$) for each genotype. Ordinary one-way ANOVA tested differences between the GG or GT groups and the reference TT group using Dunnett's post hoc test. * and ** indicate significant differences $p<0.05$ and $p<0.01$. Panels D to F show the impact of the presence of the G allele considering the dominant model (TT and GT/GG) on VO_2max for both sexes (D), males (E) and females (F). Data shows the VO_2max ($mL \cdot kg^{-1} \cdot min^{-1}$) in both sexes (D), in males (E) and in females (F). Differences were tested using a Fisher T-test, * indicates significant differences $p<0.05$. In panel D, the p value is 0.0505 when comparing the TT and GT/G groups.

Next, we tested the interaction between the VO_2max status (high or low) and the SNP309 genotypes, still considering a codominant model (*Table 3*). In males, the genotype distribution was different between the high and low VO_2max groups ($p=0.009$), with the TT genotype more frequently observed in the high VO_2max group. We found no significant difference in genotype distributions and in allele frequencies between the high and low VO_2max groups when analysing the whole cohort or the females.

Table 3 Distribution of genotype and allele frequency based on high and low VO_2max values using the cardiorespiratory fitness classification guidelines. "High" was defined as a relative VO_2max value above 40 $mL \cdot kg^{-1} \cdot min^{-1}$ for females and above 50 $mL \cdot kg^{-1} \cdot min^{-1}$ for males. One-way ANOVA and T-test were computed to determine if there was a significant difference between frequencies. * indicates significant differences ($p<0.05$)

	Genotype distribution			p-value	Allele frequency		p-value
Total	**GG**	**GT**	**TT**		**G**	**T**	
High VO_2max	10	14	12	0.064	34	38	>1.0
Low VO_2max	7	30	9		44	48	
	Genotype distribution			**p-value**	**Allele frequency**		**p-value**
Males	**GG**	**GT**	**TT**		**G**	**T**	
High VO_2max	5	3	10	0.006*	13	23	0.264
Low VO_2max	4	15	4		23	23	
	Genotype distribution			**p-value**	**Allele frequency**		**p-value**
Females	**GG**	**GT**	**TT**		**G**	**T**	
High VO_2max	5	11	2	0.394	21	15	0.275
Low VO_2max	3	15	5		21	25	

Finally, we compared the relative VO_2max in groups, using either a G-dominant or G-recessive models (*SI Table 2* and *Fig. 1D-F*). In the G-dominant model, the relative VO_2max of homozygous TT individuals was different compared to individuals carrying a G allele. This difference was statistically significant for both males and females, and almost approached significance when considering both sexes (p=0.051). Homozygous TT males had a statistically significant higher relative VO_2max than males carrying a G allele (p=0.011). Homozygous TT females had a statistically significant lower VO_2max than females carrying a G allele (p=0.048). In the model where the G allele is considered recessive, we observed no differences (*SI Table 2*, males, p= 0.627; females p=0.416; both sexes p=0.384).

DISCUSSION

The present work explores whether the SNP309 on the MDM2 promoter influences intrinsic CRF. When considering the whole cohort and male subjects, the SNP309 genotype significantly influenced the VO_2max, with the homozygous TT being associated with higher VO_2max than the GT genotype. The TT genotype was most frequent in the male population, having a high VO_2max (above 50 ml $mL \cdot kg^{-1} \cdot min^{-1}$). Indeed, 55% of males in the high VO_2max group had a TT genotype versus 17% in the low VO_2max group. These genotype effects of SNP309 were not observed in female subjects. Using a model where G is the dominant allele, we observed that homozygous TT men had a higher VO_2max than male subjects harbouring at least one G allele for SNP309 (GG and GT). Conversely, homozygous TT women had a

statistically significant lower VO_2max than women harbouring at least one G allele (GG and GT), suggesting that SNP309 might impact CRF in a sex-dependent manner.

In the present cohort, the genotype distribution was GG 19.5%, GT 54.9%, and TT 25.6%. The allele frequencies were 0.48 for the G allele and 0.52 for the T allele. We observed similar allele frequencies compared to previously published works (Millikan et al. 2006; Park et al. 2006; Paulin et al. 2008; Economopoulos and Sergentanis 2010; Jalilvand et al. 2020). We did not find sex-related differences in allelic and genotype frequencies, reinforcing the notion that differences observed between males and females do not result from differences in genotype or allele frequencies between the two sexes. The VO_2max in our cohort aged 23 years are in accordance with previous standards reported in the United States, more particularly the ones reported in the FRIEND database, that provides a reference for CRF measured in laboratories using cardiopulmonary exercise testing (Kaminsky et al. 2015; Peterman et al. 2020, 2021)). We report similar averages and sex–differences in males (48±11 vs. 49±9, FRIEND vs. present study) and in females (38±10 vs. 38±6, FRIEND vs. present study) in the age group 20 to 29 years (Kaminsky et al. 2015). These observations confirmed that our genotyping and cardiopulmonary approaches efficiently assessed the SNP309 genotype and CRF.

The G variant on SNP309 supports greater transcriptional activity of the MDM2 promoter and protein function (Bond et al. 2004; Post et al. 2010). Maintaining normal levels of MDM2 expression is essential for proper cardiac function and for tissues to achieve vascular homeostasis (Jones et al. 1998; Nieminen et al. 2005; Zhou et al. 2011; Muthumani et al. 2014; Toth et al. 2006; Hauck et al. 2017; Stanley-Hasnain et al. 2017). In the heart, MDM2 is required to prevent excessive and pathological cardiac hypertrophy (Hauck et al. 2017). In mouse skeletal muscle, MDM2 supports capillarization at basal level and in response to regular physical activity (Roudier et al. 2012; Aiken et al. 2016). Therefore, we hypothesize that the G allele would enhance cardiac function and skeletal muscle O_2 extraction due to higher capillarization, supporting a greater O_2 uptake during GXT. When analysing the whole cohort (males and females), the TT group tends to have a greater VO_2max than individuals harbouring at least one G allele ($p=0.0505$, Figure 1D). This result made us reconsider our research hypothesis. While cardiac output strongly correlates with oxygen uptake, skeletal muscle capillarization shows weak relation with VO_2max in healthy non-athletic individuals, unless exercise is performed under hypoxia (Cardús et al. 1998; Wagner 2017). In healthy non-athletic individuals, mitochondrial oxidative capacity was reported to be the predominant mechanism that limits muscle O_2 uptake (Cardús et al. 1998; Layec et al. 2015). Yet, higher capillarization might enhance endurance capacity by allowing individuals to continue developing power at high intensity of exercise despite no further increase in O_2 uptake (Coyle et al. 1988; Mitchell et al. 2018a). With endurance training, muscle capillarization increases (Ross et al, 2023) and muscle O_2 uptake relies more on O_2 transport at maximal exercise (Broxterman et al. 2024; Haseler et al. 1999; Haseler et al. 2024). Muscle capillarization might be a more important determinant of VO_2max in individuals with high training status, while mitochondrial capacity might limit O_2 uptake in untrained individuals (Richardson 2023; and Wagner 2017). Interestingly, MDM2 is a potent negative regulator of TP53. In addition to its role as the "Genome guardian", TP53 is a key regulator of cell metabolism, particularly in terms of mitochondrial oxidative capacity (Matoba et al. 2006). We initially did not consider MDM2 as

a key regulator of oxidative capacity when formulating our initial hypothesis. Indeed, a preclinical study reported that muscle-specific deletion of MDM2 was reported to influence endurance exercise capacity in mice, only under mild hypoxia (15% O_2). And, muscle-specific deletion of TP53 decreased basal content of mitochondria in the skeletal muscle without impairing mouse endurance exercise capacity (Beyfuss et al. 2018). The G allele on the SNP promoter was reported to increase the activity of MDM2, particularly toward its main target, TP53 (Bond et al. 2004; Arva et al. 2005). Since individuals recruited in our cohort were healthy but non-athletic, mitochondrial capacity might be the main limiting factor for muscle O_2 uptake (Broxterman et al. 2024). On the premise that the G allele negatively regulates TP53 mitochondrial function (Matoba et al. 2006), a TT homozygous might result in enhanced mitochondria oxidative capacity, potentially driving a greater exercise capacity.

When separating the cohort based on high and low VO_2max (above or below good 40 $mL \cdot kg^{-1} \cdot min^{-1}$ for females and <50 $mL \cdot kg^{-1} \cdot min^{-1}$ for males based on the FRIEND standard values), the distribution of alleles was different only in males ($p=0.006$), with a greater prevalence of TT in the high VO_2max group. This difference was not observed in the females. In this study, the young women bearing at least one G allele for SNP309 (GG and GT) had a greater VO_2max than their homozygous TT counterparts. Conversely, the males bearing at least one G allele for SNP309 (GG and GT) had a lower VO_2max than homozygous TT men. These contrasting sex-differences infer that SNP309 impacts CRF sex-dependently.

Interestingly, in transgenic mice expressing humanized T or G alleles (Ortiz et al. 2018), the G allele drives sex-differences in basal level of MDM2 in the cardiac and skeletal muscle tissues. In females, the genotype did not impact cardiac MDM2; yet TT males had lower cardiac expression of MDM2 than GG homozygotes (Ortiz et al. 2018). Conversely, the G allele increased MDM2 in the skeletal muscle of females (GG versus TT), having no impact of the skeletal muscle of male mice (Ortiz et al. 2018). If similar impacts of SNP309 were verified in human subjects, SNP309 might influence the cardiac determinants of CRF in men more than in women. And conversely, SNP309 could have a greater impact on skeletal muscle determinant of VO_2max in women than in men (e.g. O_2 extraction). These tissue- and sex-specific difference results from the change in transcriptional regulation of MDM2 (Arva et al. 2005; Bond et al. 2006; Zhang et al. 2015; Ortiz et al. 2018)

In men, the TT genotype could positively correlate with CRF, corroborating preclinical observations made in male mice (Toth et al. 2006; Park et al. 2009; Hauck et al. 2017; Stanley-Hasnain et al. 2017; Arena et al. 2018; Beyfuss et al. 2018). TP53 supports maximal exercise capacity, increasing gene expression, and promoting oxidative phosphorylation and mitochondria biogenesis in the skeletal muscle of male mice (Park et al. 2009). Independent of TP53, MDM2 negatively regulates mitochondrial respiration by reducing the expression of the mitochondrial gene coding for NADH-dehydrogenase 6 (Arena et al. 2018). If TT homozygous males were to express reduced levels of MDM2 transcript and protein, it might positively impact the intrinsic muscle oxidative metabolism. To validate this hypothesis, future studies will need to assess the impact of SNP309 variants on the expression of MDM2 and markers of the oxidative metabolism in skeletal muscle biopsies in larger cohorts.

Beyond the skeletal muscle, changes in MDM2 expression might also influence CRF by influencing central cardiac function. MDM2 expression was reported to impact the cardiac

structure and functions. MDM2 hypomorphic mice have larger cardiomyocytes and are more susceptible to pathological cardiac hypertrophy (Toth et al. 2006). In transgenic mice, full deletion of MDM2 in cardiomyocytes led to concentric hypertrophy in adult male mice (Hauck et al. 2017; Stanley-Hasnain et al. 2017). MDM2 might then have anti-hypertrophic functions, with more evidence available in male mice. No studies have investigated whether the TT genotype is associated with a pro-hypertrophic trait in a sex-dependent manner.

Our results suggests that the presence of G allele in the women genome might be associated with greater CRF. By substituting a T nucleotide with a G nucleotide, the SNP309 increases the binding of the transcription factor Sp-1 on MDM2 promoter (specificity protein-1) (Bond et al. 2004). Sp-1 and the oestrogen receptor (ER-α) interact to upregulate MDM2 transcription (Phelps et al. 2003; Stoner et al. 2004; Toth et al. 2006). In the presence of the SNP309, Sp-1 and ER-α support greater expression of MDM2 in women with an active oestrogen signalling pathway (Bond et al. 2006). This synergistic action of Sp-1 and ER-α might partly explain gender-based differences regarding the impact of SNP309 on tumorigenesis. Our findings question whether SNP309 supports sex-based differences in CRF through similar mechanisms.

Exposing transgenic mice to stressful conditions might bring insight regarding how SNP309 regulates MDM2 function (Zhang et al. 2015; Ortiz et al. 2018). In response to a stress that promotes ER-α activity (i.e. azoxymethane), mice bearing the GG genotype express more MDM2 (up to 50% more) in their colon (Zhang et al. 2015). Interestingly, mice bearing the TT genotype had a lower capacity to downregulate transcription factor FoxO1 in this tissue (Zhang et al. 2015). In the skeletal muscle, FoxO1 is a potent inhibitor of angiogenesis (Roudier et al. 2013; Slopack et al. 2014; Nwadozi et al. 2016). A single bout of exercise activates MDM2, increasing its capacity to downregulate FoxO1 transcriptional activity, promoting a pro-angiogenic environment in the muscle (Aiken et al. 2016). High levels of MDM2 correlate positively with a higher proportion in type 1 fibre and a greater capillary-to-fibre ratio in the muscle of female rats (Roudier et al. 2012). Together, this suggests that an upregulation of MDM2 driven by oestrogen receptor signalling could make muscles prone to angiogenesis, having a beneficial impact on the muscle capillarization. If the capacity of G allele to enhance MDM2 expression in the skeletal muscle observed by Ortiz and colleagues in transgenic female mice (Ortiz et al. 2018) was verified in human beings, this might have implications for women's muscle fatigability.

In human beings, sex-difference in skeletal muscle fatigability were reported to be task and muscle group specific (Hunter 2014; Besson et al. 2022). A greater proportion of type 1 fibre and greater capillary density (+23% in vastus lateralis in women compared to men) was proposed to be part of the mechanisms through which women tend to be resistant to muscle fatigue after repeated dynamic contractions (Roepstorff et al. 2006; Hunter 2014). At a high intensity of aerobic exercise, the respiratory muscles of the women appear to extract more O_2 than men (Mitchell et al. 2018b; Espinosa-Ramírez et al. 2021). Despite a potentially greater deoxygenation in the respiratory muscle, women were reported to experience less respiratory muscle fatigue (Mitchell et al. 2018b; Espinosa-Ramírez et al. 2021). If capillarization allows individuals to continue to develop power despite no further increase O_2 uptake (Cardús et al. 1998; Layec et al. 2015), these results question whether women have a greater capillarization

of their respiratory muscles. Because women were under represented in the studies that investigated the correlation between oxygen supply and peripheral O_2 extraction (Cardús et al. 1998; Layec et al. 2015; Broxterman et al. 2024), whether respiratory muscle capillarization plays a more important role in determining the endurance capacity in women than it does in men remains largely unknown. This interpretation requires caution as divergent results were reported regarding sex-difference in capillary equipment between women and men (Coggan et al. 1992; Ahmed et al. 1997; Hostler et al. 2001; Porter et al. 2002; Torres et al. 2011; Binet et al. 2023). Whether women who bear the G allele for SNP309 and have an active oestrogen pathway acquire a pro-angiogenic potential in their skeletal muscle associated with greater capillarization remains unstudied. Women exhibit unique physiological and biomechanical characteristics when exercising and might reach VO_2max through different regulatory processes than men (Hunter 2014; Santisteban et al. 2022). Therefore, it cannot be excluded that SNP309 might influence women's VO_2max through multiple processes (e.g., haematological changes or differences in cardio-respiratory and/or whole-body functions), not just changes in muscle capillarization. Yet, studying how SNP309 drives sex-difference goes beyond the scope of the present study.

This study has limitations. First, we did not control for our cohort's physical activity status, level of training and other lifestyle habits. The measures of VO_2max confirmed that our cohort is representative of a healthy nonathletic population for this age group (Gledhill et al. 1994). However, the participants likely had different physical activity levels, implying that our VO_2max measures might reflect a combination of both intrinsic and gained CRF. Secondly, equal numbers of men and women were enrolled, yet only 7 women had a TT genotype versus 14 men. This distribution of genotypes, inherent to our cohort, might have limited our capacity to detect changes in genotypic frequencies between the high and low VO_2max group in the women group (Table 3). However, using a model where G is the dominant allele, we gained sufficient statistical power to observe that women harbouring at least one G allele had a greater relative VO_2max than homozygous TT women. Finally, ethnicities were not evenly represented between genotypes. In the male cohort, while the GT and TT groups had similar representation of ethnicities, Asians appeared over-represented, and Europeans under-represented in the GG group. Because GG was the least frequent genotype in the male cohort, this uneven distribution of ethnicities might have limited our capacity to detect difference between the GG and TT males in the co-dominant model (Figure 1B). Additionally, the GG genotype was not observed in African American participants. The lack of diversity did not allow to test whether SNP309 could act ethnicity-dependently.

To conclude, our results suggest that MDM2 SNP309 influences CRF in young nonathletic adults. Surprisingly, when analysing both sexes, we did not observe difference between GG and TT genotypes. Yet, homozygous individuals with a TT genotype had a higher VO_2max than heterozygous GT individuals ($p<0.05$). A sex-dichotomous effect might partly explain these observations. Indeed, when using a model where the G allele is dominant, we observed a sex-dependent effect of MDM2 SNP309. The presence of the G allele is associated with a lower VO_2max in men. In women, the presence of the G allele was associated with a higher VO_2max. Our results question whether the presence of G allele on SNP309 could impact

differently the CRF trait in women compared to men. To delineate how MDM2 SNP309 might exert its sex-dichotomous effect, future studies will need to study how this SNP influences key determinants of CRF on a larger and more diverse cohort.

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AUTHORS CONTRIBUTIONS

ER, GH, VJ conceived and designed the research. ER, GH and VJ analyzed data. BL, ER, GH, SA performed experiments to validate the genomic DNA purification and interpreted results from the Taqman qPCR approach. GH performed genomic DNA collection, genotyping of the whole cohort and final validation of genotypes. LY and VJ performed and interpreted results from experiments related to exercise testing and collection of anthropometric data. ER and GH prepared figures and drafted the first version of the manuscript. GH and ER gathered all data and perform most of the statistical analyzes. All authors edited and revised the manuscript supporting further interpretation of data. All authors approved the final version of the manuscript.

DATA AVAILABILITY

Data will be made available upon request made directly to Dr. Roudier.

SUPPLEMENTAL INFORMATION

Supplemental figures and tables are available at the end of the manuscript.
Supplemental information will be made available upon publication using the repository indicated below.
SI – Haddadi et al. https://doi.org/10.5683/SP3/6IYRG7 (Private URL for reviewer-use only: https://borealisdata.ca/privateurl.xhtml?token=08a02039-e2a8-4bdd-8a0b-bb11ffd04d58).

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Supplementary information (SI)

SI Table 1: Genotype distribution and frequency of alleles in the population per sex and ethnicity. X^2 (CHI Square - (Fisher Exact Test)) analysis to test the influence of sex and ethnicity on the allele and genotype distribution. Ethnicity impacted the allele frequency (p=0.0241).

	Genotype distribution			Allele frequency	
	TT	**GT**	**GG**	**G**	**T**
All subjects	21 (25.6%)	44 (54.9%)	17 (19.5%)	78 (0.48)	86 (0.52)
Females	7	26	8	42	40
Males	14	18	9	36	46
Ethnicity:					
Middle eastern	2	2	1	4	6
South Asian	2	7	5	17	11
East Asian	2	12	5	20	16
African American	6	3	0	3	15
European	9	20	6	32	38

SI Table 2: Impact of the presence of the G allele on the VO_2max ($mL \cdot kg^{-1} \cdot min^{-1}$) of individuals, considering the dominant vs recessive model (G-dominant or G-recessive). Data shows mean ± SD for the VO_2max expressed in $mL \cdot kg^{-1} \cdot min^{-1}$ in the whole cohort, in males and females only. Differences were tested using a Fisher T-test, * indicates significant differences ($p<0.05$).

	Total (n=82)	**P value**	**Male (n= 41)**	**P value**	**Female (n=41)**	**P value**
Dominant	**VO_2max ($mL \cdot kg^{-1} \cdot min^{-1}$)**		**VO_2max ($mL \cdot kg^{-1} \cdot min^{-1}$)**		**VO_2max ($mL \cdot kg^{-1} \cdot min^{-1}$)**	
TT	47 ± 12 (21)	0.051	53 ± 8 (14)	0.011*	34 ± 7 (7)	0.048*
GT + GG	43 ± 7 (61)		47 ± 7 (27)		39 ± 5 (34)	
Recessive						
GG	45 ± 9 (17)	0.384	50 ± 7 (9)	0.627	40 ± 7 (8)	0.416
GT + TT	43 ± 9 (65)		49 ± 8 (32)		38 ± 6 (33)	