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Original citation & hyperlink:
Brookes, C, Ribbans, WJ, El Khoury, LY & Raleigh, SM 2020, 'Variability within the human iNOS gene and Achilles tendon injuries: Evidence for a heterozygous advantage effect', Journal of Science and Medicine in Sport, vol. 23, no. 4, pp. 342-346.
https://dx.doi.org/10.1016/j.jsams.2019.11.001

DOI 10.1016/j.jsams.2019.11.001
ISSN 1440-2440

Publisher: Elsevier

Journal Pre-proof

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PII: S1440-2440(19)30782-0

DOI: https://doi.org/10.1016/j.jsams.2019.11.001

Reference: JSAMS 2197

To appear in: *Journal of Science and Medicine in Sport*

Received Date: 15 July 2019

Revised Date: 30 September 2019

Accepted Date: 4 November 2019

Please cite this article as: Brookes C, Ribbans WJ, El Khoury LY, Stuart MR, Variability within the human *iNOS* gene and Achilles tendon injuries: Evidence for a heterozygous advantage effect, *Journal of Science and Medicine in Sport* (2019), doi: https://doi.org/10.1016/j.jsams.2019.11.001

Paper Manuscript

Variability within the human *iNOS* gene and Achilles tendon injuries: Evidence for a heterozygous advantage effect

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Abstract

Objectives: The aim of this case control genetic association study was to explore whether two variants within the inducible nitric oxide synthase (*iNOS*) gene, rs2779249 (C/A) and rs2248814 (A/G), influenced the risk of Achilles tendinopathy in a British population. Design: Candidate gene, case control association study. Method: We recruited 145 individuals diagnosed with Achilles tendon pathology and 132 asymptomatic controls. All participants were genotyped for the *iNOS* variants using qPCR and significant associations were

discovered using a combination of Chi squared and ANOVA type analysis. Results: The CA genotype of the *iNOS* rs2779249 variant was protective and conformed to a heterozygous advantage model of inheritance as it was overrepresented in the control participants (p=0.009). In sex specific analysis the protective association persisted in male participants (p=0.016) but not in females. Unlike the rs2779249 variant, the rs2248814 variant was not associated with Achilles tendinopathy or Achilles tendon rupture. Conclusion: The rs2779249 CA genotype within the human *iNOS* gene appears to protect individuals from Achilles tendinopathy. This study further supports a genetic contribution to modifying the risk of Achilles tendon problems. The study also infers an important role for nitric oxide in tendon healing and/or degradation.

Keywords

Nitric Oxide; Genetics; Apoptosis; Inflammation; Achilles Tendon

Practical Implications

- The *iNOS* rs2779249 genotype could be used to assess risk of Achilles tendon problems in athletic populations.
- The *iNOS* rs2779249 genotype might have future application in personalised treatments for those with Achilles tendon pathologies.
- The inclusion of the *iNOS* rs2779249 genotype in current injury risk models might improve the predictive potential of such tests to assess risk.

Abbreviations

ANOVA, one way analysis of variance; ATP, Achilles tendon pathology; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; NOS, nitric oxide synthase; NO, nitric oxide; CON, control; TEN, tendinopathy; RUP, rupture; SNP, single nucleotide polymorphism; HWE, Hardy Weinberg equilibrium; GTN, glyceral Trinitrate.

Introduction

Achilles tendon pathology (ATP) is a complex, painful, degenerative condition, affecting both sporting and sedentary individuals[1]. 75% of Achilles tendon ruptures are observed in middle aged sport participating males[2]. Multiple intrinsic and extrinsic risk factors contribute to ATP aetiology, and the condition is known to have a genetic[3] and, more recently, an epigenetic basis[4,5].

The inducible nitric oxide synthase (*iNOS*) gene is one of three Nitric Oxide Synthase (NOS) genes (*eNOS, nNOS, iNOS*)[6] responsible for nitric oxide (NO) generation through L arginine oxidation and catalysed by the NOS enzymes[7]. NO is a free radical and is associated with multiple biological functions[8]. After tendon injury, NO is induced by all 3 NOS isoforms, with upregulated NOS activity reported in tendinopathy in both human and rat models[6]. Research suggests that *iNOS* expression is elevated 23 fold higher than controls four days after Achilles tendon injury[9]. Lin and colleagues[9] suggest that NO catalysed by the *iNOS* isoform of NOS induces apoptosis in inflammatory cells to eradicate the cells from the damaged area, preventing chronic inflammation and allowing remodelling to occur.

An *iNOS* gene transfection study demonstrated NO's ability to enhance collagen synthesis in tendon cells in vitro[10]. The increased collagen synthesis was inhibited when a NOS inhibitor was employed. The use of glyceryl trinitrate (GTN) patches containing NO, in conjunction with rehabilitation, has been studied in a randomised group of patients with Achilles tendinopathy in comparison to rehabilitation alone[11]. It was observed that the addition of the GTN patch significantly enhanced patient recovery, with 78% of tendons in the GTN group asymptomatic with daily activities as opposed to 49% in the placebo group (p=0.001). The addition of GTN patches to patient rehabilitation resulted in reduce pain with activity, and patients presented with lower pain scores after performing a hop test.

In a rat healing model, the administration of NO paracetamol vs two control groups (paracetamol group and a vehicle group) was investigated[12]. Paracetamol had no effect on healing when compared to the vehicle group. NO paracetamol vs paracetamol alone displayed similar failure loads, however, secondary endpoints of Achilles tendon healing differed between the two groups. At day 10 postoperatively, the NO paracetamol group displayed a smaller cross sectional area, a greater dry weight and lesser wet weight, a larger collagen content and better organization of collagen, suggesting the addition of NO increased collagen synthesis and encouraged collagen reorganisation[12].

Two *iNOS* single nucleotide polymorphisms (SNPs) rs2779249 (C/A) and rs2248814 (A/G) were of interest for this study. These variants are situated within the *iNOS* gene and its 5' flanking region on chromosome 17. The rs2779249 SNP is a functional variant -1026 bp upstream of the promoter region[13]. The C allele leads to a 4.73 fold lower transcriptional activity of the promoter compared to the A allele[14]. The rs2248814 SNP resides 28,260 bp downstream of the rs2779249 SNP within the intronic region flanked by exons 12 and 13. The rs2248814 SNP has been linked to impaired fracture healing[15]. Sathyendra and colleagues[15] looked at sixty two patients with long bone fractures, thirty three presenting with an atrophic nonunion (case group) and twenty nine showing uneventful fracture union (control group). The G allele was found to associate with more than twice the risk of non-union after fracture[15].

As NO plays an important role in collagen homeostasis within the tendon, we hypothesised that genetic variability in NO synthesising genes like *iNOS* could impact on the risk of tendon injuries. In order to test this hypothesis, we investigated the two SNPs in a British case control population.

Methods

145 Caucasian case (ATP) subjects (56 females, 89 males) were originally recruited from the County Clinic, Northampton. The subjects' pathologies were diagnosed using published criteria previously described for this cohort and assessed by the clinical author (WJR)[16]. This was based on clinical assessment and confirmed with the appropriate imaging modalities. Those patients considered for certain management techniques, such as high-volume stripping, or for confirmation of rupture underwent ultrasound. Others underwent MRI analysis. For the purpose of our study, the cohort was subdivided into a rupture subset and a chronic tendinopathy subset. For clinical management, the patients were further subdivided into complete ruptures, partial ruptures, tendinosis, and para-tendinopathy, however, these are outside the scope of this paper.

We used the abbreviation ATP to describe a set of combined Achilles tendon pathologies. Specifically, the ATP case participant group consisted of an Achilles tendinopathy subset (TEN) (n=103) and an Achilles rupture subset (RUP) (n=42). The RUP subset included subjects with partial or complete ruptures to the Achilles tendon. Full details of the specific pathological definitions for this cohort have previously been published[17]. In addition to the cases, 132 asymptomatic, healthy, Caucasian control (CON) participants (49 female, 83 male) were recruited from various sports clubs within the East Midlands region of the United Kingdom.

DNA from all participants was isolated as described in our previous study[16]. For this study we re analysed those same samples using TaqMan® SNP genotyping probes, along with specific forward and reverse primers, for the *iNOS* gene rs2779249 and rs2248814 SNPs. Each reaction contained 10 ng of DNA and each PCR plate contained both positive (repeated samples) and negative (non template) controls. Following a holding phase at 95°C for 10 minutes, PCR runs consisted of 40 cycles of a denaturing stage at 92 °C for 15 seconds and a final annealing stage at 60 °C for 1 minute. Genotypes for each sample were determined by end point fluorescence and called using StepOne software v2.1.

IBM SPSS Statistics version 22 (IBM Corporation, Armonk, New York) was used to carry out independent T tests on the participants characteristics (age, BMI, height and weight) between the ATP and CON groups, alongside analysis of variance (ANOVA) to determine whether anthropometric data interacted with genotype for both the ATP and CON groups. Pearson's Chi square analysis or Fisher's exact test were used to assess differences between allele and genotype distributions for both the rs2779249 and rs2248814 variants within the ATP and CON groups. A similar analysis was conducted to establish whether any associations were specific to just tendinopathy (TEN) and/or rupture (RUP) (as opposed to the collective pathology of ATP). We also tested whether sex interacted with genotype.

We used the SNPStats online analysis software (available at https://www.snpstats.net/start.htm) to establish whether alleles at either of the two loci investigated deviated from Hardy Weinberg equilibrium (HWE). Inferred haplotype analysis showing estimated relative frequency was also carried out. Haplotype frequencies were measured using an expectation maximisation algorithm. Further details on the software package and the type of analysis conducted has been published[18].

Using the Quanto power software (available at http://biostats.usc.edu/cgi-bin/DownloadQuanto.pl) we calculated that our study would have 81% power to detect associations at the 0.05 level of significance. All p values reported were unadjusted based on the rationale presented in our previous studies[19].

All experimental protocols received ethical approval from the Research Ethics Committee of the Faculty of Health and Society within the University of Northampton, UK. All subjects provided their written informed consent, in accordance with the Declaration of Helsinki, alongside the completion of an extensive medical history questionnaire.

Results

There were no statistical differences between the ATP and CON groups in relation to age, (p=0.264), height (p=0.067), weight (p= 0.370), and BMI (p=0.736). The ATP group ages were self reported age at onset of symptoms. There were no anthropometric interactions with the *iNOS* rs2779249 genotypes (table 1a); height, CON (p=0.183), ATP (p=0.129); weight, CON (p=0.175), ATP (p=0.886); age, CON (p=0.311), ATP (p=0.606); BMI, CON (p=0.330), ATP (p=0.843). The *iNOS* rs2248814 genotypes did not associate with weight (table 1b), CON (p=0.110), ATP (p=0.492); age, CON (p=0.085), ATP (p=0.713); BMI, CON (p=0.548), ATP; (p=0.440), however the *iNOS* rs2248814 AG genotype associated with height (p=0.029) in the CON group, but not the ATP group (p=0.915). This variant however, did not associate with ATP, tendinopathy or rupture.

We found that the *iNOS* rs2779249 CA genotype was overrepresented in the CON individuals (protective effect) (p=0.009; OR=0.46; CI 0.27-0.79) (figure 1a). Similarly, the CA genotype was significantly overrepresented in the control participants compared to the TEN subset (p=0.005; OR=0.40; CI 0.22-0.74) (figure 1b). However, no associations were noted when the Achilles RUP subset participants were investigated and compared to the control participants (p=0.600).

We observed the CA genotype of the same variant to be overrepresented in the male CON participants (p=0.016; OR=0.37; CI=0.18-0.76) (figure 1c), significantly overrepresented in the male CON participants in comparison to the male TEN subset (p=0.007; OR=0.30; CI= 0.13-0.69), but not significant in the male RUP subset (p=0.660). There were no significant differences in the genotype distribution for the *iNOS* variant rs2779249 when only females were compared between the CON and ATP participants (p=0.350), CON and TEN subset (p=0.330), and CON and RUP subset (p=0.720).

No significant differences were observed in the genotype distribution of the *iNOS* gene rs2248814 variant within all investigated participant subsets; all CON participants and all ATP participants (p=0.760); female CON and female ATP participants (p=0.500); and male CON and male ATP participants (p=0.910).

There were no deviations from HWE in the investigated participant groups, except where significant differences in the genotype distribution were observed (supplementary table 2). When this is the case, both the mixed participant sample and the CON participant sample remained in HWE, however the ATP participant sample violated HWE. Raw haplotype data between the CON and ATP participant group can be found in supplementary (supplementary figure 3). There were no significant haplotypic effects observed, global haplotype association p=0.077. There were no significant differences in allele distribution between the entire CON and entire ATP participant groups for both *iNOS* rs2779249 (C/A) (supplementary figure 2a) and rs2248814 (G/A) (supplementary figure 2b) variants.

Discussion

In this study we found the *iNOS* rs2779249 variant influenced the risk of ATP in a British cohort. Specifically, the CA genotype was enriched in the CON participant group, inferring the genotype as a contributing factor to reducing the risk of ATP. Our results are consistent with the preliminary findings of a study by Nell and colleagues in 2012[20]. In their study consisting of 78 individuals diagnosed with Achilles tendinopathy, they reported a marginally significant protective effect of the CA genotype (p=0.049). In our study we also assessed for sex differences and observed a significant overrepresentation of the CA genotype in the male CON participant group. However, this was not observed in females. It is important to note that genetic variants observed to associate with complex pathologies are contributing factors to the total risk and are not to be interpreted as directly responsible. Therefore, our data suggests the rs2779249 CA genotype contributes to a reduction in the risk of Achilles tendon injuries.

It is not fully understood why the *iNOS* rs2779249 CA variant exerts a protective effect against ATP, however NO is an important messenger molecule mediating both inflammatory and apoptotic pathways [21], and an appropriate balance of NO is essential for healthy cellular processes[22]. It is known that the C allele of the rs2779249 variant is associated with a 4.73 fold lower transcriptional activity than the A allele[14]. Possession of the CC genotype may infer a state in which NO levels are limited compared to those with the AA genotype. Therefore, it might be reasonable to assume that heterozygous CA individuals are producing an appropriate amount of NO, which may lower the risk of sustaining a tendon injury, compared to those with the CC or AA genotype who may be producing too little or too much NO respectively.

Dakin and colleagues investigated Achilles tendon biopsies from patients to determine whether an inflammatory process is a feature of Achilles tendinopathy and rupture and observed evidence of chronic inflammation[23]. Their investigation found complex macrophage activation protein signatures and observed target molecules expressed in patient tissue samples from interferon, NFκB, STAT6 and GCR activation pathways. NFκB's are known to control the expression of genes involved in cell adhesion, angiogenesis, apoptosis and the cell cycle[24], and the NFκB family are responsible for initiating expression of *iNOS*[25] and therefore are crucial for *iNOS* regulation[26].

iNOS is the predominant enzyme from the NOS family involved in inflammation[27] and has been described as a downstream inflammatory mediator in multiple cell types, including skeletal muscle cells[28]. Inducible NO has been highlighted as an important messenger in the regulation of myogenic precursor cell fate and the inflammatory response, indicating inflammation dependent muscle healing in a damage/regeneration mouse model[28]. Also evidencing that *iNOS* is scarcely expressed in healthy skeletal muscle[28]. Animal studies have demonstrated the importance of *iNOS* in promoting tendon healing following injury[29]. From a

clinical perspective, there has been interest in the epigenetic modification of *iNOS* activity in controlling symptoms of chronic Achilles tendinopathy. GTN skin patches liberate NO into the local tissues. It has been shown to be effective in several studies in the management of tendinopathy[30].

Although we have found that the *iNOS* rs2779249 CA genotype reduces the risk of ATP, our study does have some limitations. Firstly, although our study is adequately powered to detect associations in the entire cohort, stratifying by sex would have underpowered this analysis and therefore our sex specific data should be viewed with caution. Secondly, our study is limited to a single ethnic group and should be repeated in a non Caucasian cohort. Finally, it is now known that epigenetic factors play a significant part in the predisposition to musculoskeletal injury[4] and therefore further studies should examine the role of DNA methylation and histone modification in modifying the impact of genotype on the risk of these injuries.

Conclusion

In summary, we have identified the *iNOS* rs2779249 CA genotype as a protective factor contributing to reducing the risk of ATP. Although this genotype modifies risk, Achilles tendon injuries are complex and likely to arise from multiple interactions between genetic and environmental factors (epigenetics). This research enhances our current understanding of risk factors for ATP, and the *iNOS* gene remains a suitable candidate for further investigation regarding tendon pathology.

Funding

The University of Northampton.

Competing interests

The research team confirm that there are no competing interests.

Ethics approval

This research has been approved by the Research Ethics Committee of the Faculty of Health and Society within the University of Northampton, England.

Acknowledgements

The author would like to thank Dr Rebecca Rickaby for her involvement in the recruitment of the subjects and phenotypic data collection which has enabled this research.

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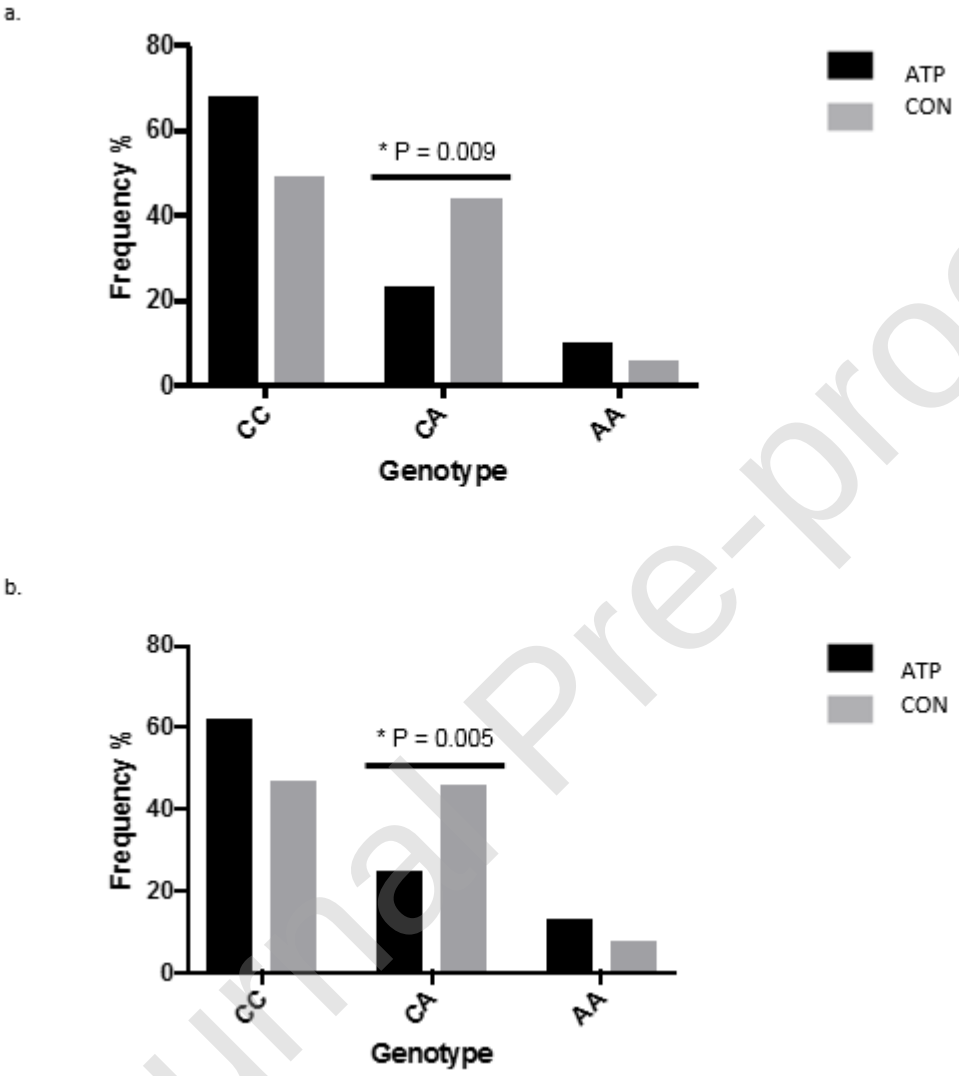
Figure and table legends:

Figure 1:



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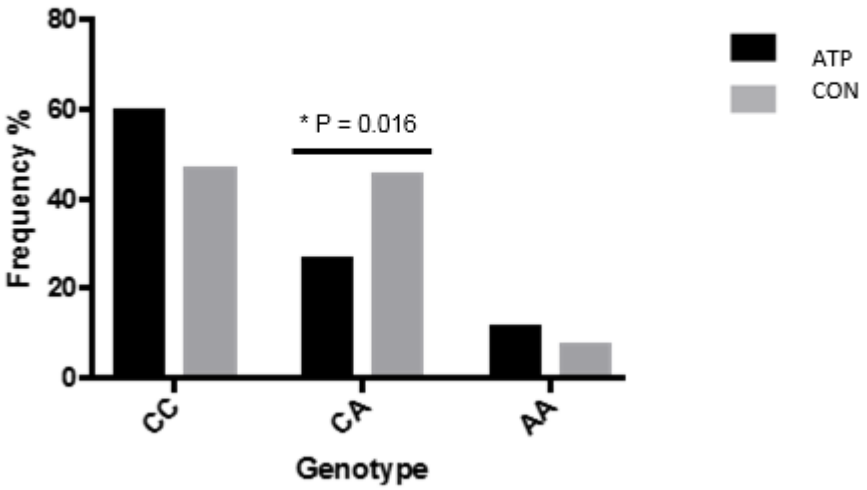

Figure 1a: Genotype Frequency distribution of the *iNOS* variant rs2779249 between the entire control participant cohort (CON) (N=129) and the entire Achilles tendon pathology cases (ATP) (N=121).

Figure 1b: Genotype Frequency distribution of the *iNOS* variant rs2779249 between the entire control participant cohort (CON) (N=129) and the entire tendinopathy participant subset (TEN) (N=93).

Figure 1c: Genotype Frequency distribution of the *iNOS* variant rs2779249 between the male control participants (male CON) (N=81) and the male Achilles tendon pathology cases (male ATP) (N=71).

Figure 1: *iNOS* rs2779249 variant genotype distribution in a British cohort. Significance was accepted when $p<0.05$. Frequency in percentage (%) of cohort analysed and N is the total number of participants genotypes in each group.

Table 1:

Table 1a: Characteristics of the entire CON and ATP participant groups for the *iNOS* variant rs2779249 using one-way ANOVA analysis stratified according to genotype.

rs2779249		**CON**			**ATP**		
		AA	**AC**	**CC**	**AA**	**AC**	**CC**
Age (years)	**N**	9	54	58	15	31	72
	Mean	41.67	43.30	39.91	40.20	44.61	43.19
	SD±	± 11.72	± 13.48	± 9.62	± 14.56	± 12.90	± 14.33
	P Value	0.311			0.606		
BMI (kg/m2)	**N**	9	55	57	14	24	49
	Mean	24.59	25.44	26.47	26.34	26.40	25.85
	SD±	± 3.65	± 4.90	± 4.18	± 5.22	± 4.53	± 3.72
	P Value	0.330			0.843		
Height (cm)	**N**	9	55	57	15	33	71
	Mean	170.89	174.07	176.74	171.93	169.76	173.86
	SD±	± 11.96	± 10.04	± 10.45	± 10.31	± 9.96	± 9.26
	P Value	0.183			0.129		
Weight (kg)	**N**	9	55	58	14	24	49
	Mean	73.00	78.04	83.43	79.14	76.58	78.04
	SD±	± 19.16	± 20.38	± 18.41	± 18.22	± 17.87	± 14.62
	P Value	0.175			0.886		

Table 1b: Characteristics of the entire CON and ATP participant groups for the *iNOS* variant rs2248814 using one-way ANOVA analysis stratified according to genotype.

rs2248814		**CON**			**ATP**		
		AA	**AG**	**GG**	**AA**	**AG**	**GG**
Age (years)	**N**	21	70	32	23	59	32
	Mean	38.14	41.10	45.09	41.39	44.19	43.19
	SD±	± 9.49	± 11.82	± 11.73	± 16.20	± 13.37	± 12.97
	P Value	0.085			0.713		
BMI (kg/m2)	**N**	21	70	32	20	36	27
	Mean	26.72	25.49	25.87	25.63	26.79	25.52
	SD±	± 4.71	± 4.56	± 4.24	± 2.98	± 4.58	± 4.62
	P Value	0.548			0.440		
Height (cm)	**N**	21	70	32	23	62	31
	Mean	179.67	173.04	176.03	172.52	172.68	171.77
	SD±	± 7.90	± 10.37	± 11.05	± 11.79	± 8.65	± 10.48
	P Value	**0.029**			0.915		
Weight (kg)[b]	**N**	21	70	33	20	36	27
	Mean	87.14	77.17	81.15	75.25	80.17	76.41
	SD±	± 20.29	± 19.24	± 18.86	± 13.88	± 16.56	± 17.72
	P Value	0.110			0.492		

Table 1 represents the participant characteristic ANOVA results for the *iNOS* rs2779249 variant and *iNOS* rs2248814 variant, stratified according to genotype. Significance was accepted when $p<0.05$. cm: centimetres; kg: kilogrammes; m: metres.