THE GENETICS OF SARCOPENIA AND SKELETAL MUSCLE PHENOTYPES IN ELDERLY CAUCASIAN WOMEN

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Abstract

It is well documented that there is a loss of muscle mass and muscle strength with ageing, often termed sarcopenia. There remains inconsistency in identifying a definition of sarcopenia that can 1) provide a meaningful discrimination between non-sarcopenic and sarcopenic groups for muscle phenotypes, and 2) identify a sarcopenic population of sufficient numbers for subsequent genetic analysis. Of the factors that determine the prevalence of sarcopenia and the severity of impairments in muscle phenotypes, genetics remains unreported other than for a few single nucleotide polymorphisms (SNPs, e.g. *VDR*, *ACTN3* and *IL-6*). The aims of the present thesis were, in the first instance, to identify a meaningful definition of sarcopenia in a Caucasian elderly female population (n =307, 60-91 years), and thereafter to investigate the possible association of multiple SNPs (n=24) with sarcopenia and muscle phenotypes, and subsequently to assess the polygenic profile of muscle phenotypes in elderly women. A novel definition of sarcopenia was identified based on a Z-score approach using handgrip strength and skeletal muscle mass index. Thereafter, a novel association of *HIF1A* rs11549465 CC and *ACE* rs4341 CC as risk genotypes for sarcopenia was identified. Subsequently, skeletal muscle phenotypes differentiated by sarcopenia were assessed for further association with SNPs. In doing so, 12 out of 24 polymorphisms were identified as having an association with one or more of the investigated skeletal muscle phenotypes (e.g *PTK2*, *HIF1A* & *ACVR1B*). Adopting the polygenic data driven approach (GPS_{dd}), up to 8.2% and 5.0% of the variance of skeletal muscle size and strength were accounted for, this increased to 14.5% and 17.2% when age was included in the model. In conclusion, there appears to be a genetic influence on sarcopenia and skeletal muscle phenotypes; with novel skeletal muscle associations reported. The findings of this thesis have applications in a variety of areas, particularly within ageing populations, for whom completion of activities of daily living may be improved because of better understanding their individual-specific muscle mechanics, and genetic risk for physical impairment.

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Abbreviations

%	percentage
ACE	Angiotensin-Converting Enzyme
A	Adenine
ACSA	Anatomical Cross Sectional Area
ACTN	Alpha-actinin-3
ACVR1B	Activin A Receptor Type 1B
ADL	Activities of Daily Living
ALMI	Appendicular Lean Mass Index
ANCOVA	Analysis of Covariance
BIA	Bioelectrical Impedance Analysis
BMD	Bone Mineral Density
BMI	Body Mass Index
C	Cytosine
Cm	centimetre
CM-CSF	Granulocyte-macrophage colony-stimulating factor
CNTF	Ciliary Neurotrophic Factor
CNTFR	Ciliary neurotrophic factor receptor
COL1A1	Collagen type1 alpha 1
CSA	Cross sectional Area
CT	Computed Tomography
DEXA	Dual-Energy X-ray absorptiometry
DNA	Deoxy Nucleic Acid
EC	Eye Close
EDTA	Ethylenediaminetetraacetic Acid
EF	Elbow Flexion
EO	Eye Open
ESR1	Estrogen Receptor1
EWGSOP	European Working Group on Sarcopenia in Older Population
FAK	Focal Adhesion Kinase
FFM	Fat Free Mass
FT0	Fat-mass and obesity-associated protein
G	Guanosine
GPS	Genetic Predisposition Score
GS	Genetic Score
HGS	Handgrip Strength
HIF1A	Hypoxia-inducible factor 1-alpha
Ht	Height
HWE	Hardy Weinberg Equilibrium
ID3	Iterative Dichotomiser 3
IGF	Insulin-like growth factor-1
IL6	Interleukin-6
KE	Knee Extension
Kg	kilogram
kg/m^2	kilograms per square metre
kHz	kilo Hertz

m	metre
mA	mili Ampere
MRI	Magnetic Resonance Imaging
MSTN	Myostatin
MTHFR	Methylene tetrahydrofolate reductase
MVC	Maximum Voluntary Contraction
N	Newton
NO	Nitric Oxide
NOS3	Nitric Oxide Synthase 3
OLST	One Leg Standing Test
PASE	Physical Activity Scale for Elderly
PCR	Polymerase Chain Reaction
PTK2	Protein Tyrosine Kinase 2
QoL	Quality of Life
R	Resistance
s	second
SD	Standard Deviation
SMI	Skeletal Muscle Mass Index
SMM	Skeletal Muscle Mass
SNP	Single Nucleotide Polymorphism
SPSS	Statistical Package for the Social Sciences
T	Thymine
TGS	Total Genotype Score
TRHR	Thyrotropin Releasing Hormone Receptor
TTN	Titin
VDR	Vitamin D Receptor
VI	Vastus Intermedius
VL	Vastus Lateralis
VO_2max	maximal oxygen consumption
µg	micrograms
µL	microlitres

1. Literature review

1.1 Introduction

1.1.1 Muscle Mass and strength performance over the adult life span

Physical independence of the elderly is determined by skeletal muscle phenotypes, specifically muscle mass and muscle strength (Hughes et al., 2001; Reid and Fielding, 2012; dos Santos et al., 2017; Wang et al., 2017b). There are changes in muscle mass and muscle strength during the adult lifespan. Regardless of sex, the period of peak musculoskeletal function is observed between 20-30 years and some slowing of rates of contraction between 40-50 years but changes in absolute muscle strength are minor until about the sixth decade of life, and are primarily determined by the loss of muscle mass with ageing (Vandervoort and McComas, 1986; Doherty et al., 1993; Porter et al., 1995). This loss of muscle mass becomes obvious and declines of around 40% in cross sectional area (CSA) of the vastus lateralis (VL) are reported from 20-80 years (Lexell et al., 1988).

Lower extremity muscle size and muscle strength are particularly important for daily activities (Kojima et al., 2014; Martien et al., 2015), and a reduction in vastus lateralis muscle size, with ageing is attributed to a decrease in both muscle fibre numbers and muscle size (Lexell et al., 1983; Lexell et al., 1988; Piasecki et al., 2016). Similarly, the upper limb muscle mass and muscle strength, are also reported to decline with ageing (Kallman et al., 1990; Metter et al., 1997; Lynch et al., 1999; Abe et al., 2016). This decline in muscle mass and muscle strength with increased age has adverse effects on elderly daily activities; resulting in reduced mobility, fall related injuries and impaired quality of life (Campbell et al., 1989; Reid and Fielding, 2012; Zengin et al., 2017; Yang et al., 2018). Since this reduction in quality of Life (QoL) and its management imposes a significant economic burden for healthcare, it is crucial to understand how ageing and its interaction with environmental and genetic factors affect skeletal muscle phenotypes and functional capacity in an elderly population.

Inter-individual variability exists between muscle mass and muscle strength; for muscle size population variance of 9-18% is reported (Maughan et al., 1983b;

Stebbings et al., 2014) and approximately 17% for specific force (Stebbings et al., 2014). The population variance of muscle phenotypes such as muscle mass and strength are due to the interaction of genetics, behaviour and molecular factors (Carmelli and Reed, 2000; Tiainen et al., 2009). The heritability of muscle mass and muscle strength have been reported, as high as 66% for muscle mass and 82% for muscle strength in large population studies (Arden and Spector, 1997; Thomis et al., 1998; Abney et al., 2001; Huygens et al., 2004). As peak strength in early adulthood is the starting point from which muscle declines with age, it is important to understand the changes with ageing and the potential genetic contributions to this process.

1.1.2 Loss of muscle mass with ageing

The generally quoted range for muscle atrophy with ageing varies between 0.5-2% per year (Frontera et al., 2000; Goodpaster et al., 2006; Mitchell et al., 2012). The difference in the rate of muscle mass loss is due to differences in the inclusive population in the studies (older adults Vs younger adults or entire age-span), study design (cross sectional or longitudinal) and the difference in measurement techniques used to estimate muscle mass (computed tomography (CT scan), magnetic resonance imaging (MRI), Bioelectrical Impedance Analysis (BIA), etc). Nonetheless, it is normally accepted that muscle mass peaks between 20-30 years of age (Evans and Lexell, 1995; Deschenes, 2004; Sayer et al., 2008). Sex differences in skeletal muscle mass persist throughout the life span. Interestingly however, the loss of muscle mass and size with ageing is often reported as being more evident in males (Janssen et al., 2000b); although the higher rate of muscle loss in males is not reported ubiquitously (Bassey and Harries, 1993; Doherty, 2001). Specific to body part, the lower limb is more prone to muscle loss than the upper limb (Janssen et al., 2000b). The change in psoas muscle mass is more noticeable in lower limb while least in soleus muscle with the ageing in elderly female (Ikezoe et al., 2011), a likely consequence of fibre type and patterns of recruitment. The muscle cross-sectional area is reduced by 12-35% between younger and older subjects in the lower limb muscle (Young et al., 1985; Overend et al., 1992; Morse et al., 2005). These patterns of muscle atrophy with old age are presented in Table 1.1.

Table 1.1 Muscle mass loss change with ageing between young and old population

Study	Techniques	Estimate	Sex	Young (years)	Aged (years)	N (Y,O)	% Difference	% Change/ year
Lexell et al.,1983	Cadaveric dissection	VL CSA	M	30±6	72±2	6,6	-17.6	-0.42
Young et al., 1985	US	Mid-thigh CSA	M	20-30	70-80	12,12	-25	-0.5
Lexell et al. ,1988	Cadaveric dissection	VL CSA	M	19±3	73±2	9,9	-26	-0.48
				19±3	82±1	9,8	-43	-0.63
Janssen et al., 2000	MRI	SMM	M	18-29	>70	66,11	-18	-0.36
Kyle et al., 2001	DEXA	ASMM	M	18-34	>80	68,26	-19.9	-3.3
			F	18-34	>80	40,30	-14.1	-2.3
Morse et al.,2005	MRI	GM Muscle Volume		25.3±4.4	73.8±3.5	12,19	-28	
Silva et al., 2009	DEXA	SMM	M	18-80	40±14.4	468	n/a	-0.46
			F	18-80	44.5±15.9	1280	n/a	-0.46
Wroblewski et al.,	Air displacement plethysmography	SMM	M	44.8±3.2	65.4±2.2	5,5	-6.7	-0.32
2011	and MRI			44.8±3.2	76.3±3.3	5,5	-12	-0.38
			F	47.0±2.8	65.0±3.0	5,5	-9.8	-0.54
				47.0±2.8	74.8±3.7	5,5	-16	-0.57

M, Male; F, Female; n, number of participant; Y, Young; O, old; SMM, Skeletal Muscle Mass; CSA, Cross Sectional Area; ASMM, Appendicular Skeletal Muscle Mass; GM, Gastrocnemius; n/a, not applicable;

MRI, Magnetic Resonance Imaging; KE, Knee extensor strength; EF, Elbow flexors; DEXA, Dual-energy X-ray absorptiometry; US, Ultrasound Scan

1.1.3 Loss of muscle strength with ageing

Skeletal muscle strength is a function of muscle mass; studies have evidenced the moderate to high correlation between muscle mass and muscle strength (r = 0.38-0.79) (Maughan et al., 1983a; Reed et al., 1991; Hayashida et al., 2014), depending on the method of strength and mass incorporated in each study. Muscle strength reduces by about 15% per decade after 50 years (Proctor et al., 1998; Doherty, 2001), and has been reported as high as around 3% per year in the older populations studied (von Haehling et al., 2010). Consistent with these rates of decline, handgrip strength declined by 20% and 15% in a 5-year follow-up study in males and females aged over 75 years, respectively (Dey et al., 2009). Although not a direct contributing muscle to ambulation, a lower handgrip strength has been described as a good predictor of mortality (Ling et al., 2010; Volaklis et al., 2015). With ageing, there is a decline in muscle mass and muscle size, which contributes highly to the decline in muscle strength (Brooks and Faulkner, 1994; Kamel, 2003; Morse et al., 2005; Trombetti et al., 2016). It largely established that the age-related weakness is primarily by the loss of skeletal muscle mass. However, the declines in muscle strength are almost 2 to 5 times greater than the declines in muscle mass, with ageing (Mitchell et al., 2012), and this disproportionate loss of strength over size has been demonstrated in cross sectional and longitudinal studies (Frontera et al., 2000; Hughes et al., 2001).

Table 1.2 Muscle strength loss reported with ageing

Study	Population	Movement	muscle strength loss
(Goodpaster et al., 2006)	1880 M/F (70-79 years)	KE	2.6-4.1%/year
(Frontera et al., 2000)	12 M (65.4±4.2 years)	KE/EF	1.3-2.5%/year
(Doherty, 2001)			20-40% between 20th and 80th life year
(Marcell et al., 2014)	59 M,35F (58.6 years)	KE/KF	3.6-5%/year
(Proctor et al., 1998)	20-80 years		35-40%

(Keller and Engelhardt, 2013)	<40 years (n=14) >40 years (n=12)	KE	16.6-40.9% loss between 40-60 years

Abbreviations: KE, Knee extension; EF, Elbow Flexion; M, Male; F, Female

1.2 Sarcopenia

The decline in muscle mass and muscle strength with ageing described above and in Table 1.1 & Table 1.2, is defined as "sarcopenia". The term sarcopenia was first coined by Rosenberg et al., (1997) as a poverty of flesh, more recently however, low muscle strength/function has been included in the definition (Cruz-Jentoft et al., 2010). The rationale for the inclusion of strength into the definition is due to the disproportionate loss in muscle mass and muscle strength (Goodpaster et al., 2006). Although muscle weakness and skeletal muscle atrophy are obvious symptoms of this geriatric syndrome, there is ongoing debate on the operational definition, screening and diagnosis, and optimal management and treatment of the condition (Cruz-Jentoft et al., 2010; Fielding et al., 2011; Cederholm et al., 2013). Although the definitions all relate to a muscle atrophy with ageing, there is not a consensus on the definition of sarcopenia, and the exact definition is still under debate and is therefore not yet included in the International Classification of Diseases (Cruz-Jentoft et al., 2010; Fielding et al., 2011). The lack of consensus among the definitions can be attributed to the variability in the muscle mass measuring techniques, cut-offs to define sarcopenic thresholds, and the reference population and ethnicity of the studied population (Cruz-Jentoft et al., 2010). To date, there have been six major international efforts for getting the agreement (Table 1.3) while there remains less agreement in the definition.

Table 1.3 Sarcopenia definitions with different international efforts and consensus

Study group	Definition	Criteria/Cut-off points
ESPEN group (Muscaritoli et al., 2010)	An age associated decline in muscle mass and strength	Criteria 1: Presence of low muscle mass >2SD below mean of the reference adult population Criteria 2: Low physical performance (gait speed) <0.8 m/s in 4m walk Diagnosis defined with the presence of Criteria 1 + Criteria 2
European Working Group on Sarcopenia in Older People (Cruz-Jentoft et al., 2010)	A continuous decline of skeletal muscle mass and strength with a risk of adverse outcomes such as mortality	Criteria 1: Presence of low muscle mass DXA measured ALMI >2SD below of the younger reference population (Baumgartner et al., 1998) Men <7.26 kg/m^2 Women <5.5 kg/m^2 Lowest quintile appendicular skeletal mass (ASM) of the distribution in a normative population (aged ≥ 65 years) (Newman et al., 2003): Men <2.29 Women <1.73 BIA >2SD below mean (SMI) of the reference younger adults (Chien et al., 2008): Men <8.87 kg/m^2 Women <6.42 kg/m^2
		Criteria 2: Presence of low handgrip strength (Lauretani et al., 2003) Men <30 kg Women <20 kg Criteria 3: Presence of low physical function Short Performance Battery (SPB)≤8(Guralnik et al., 2000) Gait speed <0.8 m/s (Lauretani et al.) Diagnosis based on presence of criteria 1 + 2 or 3
International Working Group on Sarcopenia (Fielding et al., 2011)	Loss of skeletal muscle function along with strength with increasing age	Patient unable to walk is screened for presence of low muscle mass by DXA to confirm low muscle mass
		Criteria 1: Presence of low physical function Gait Speed <1.0m/s, then DXA
		Criteria 2: Low muscle mass Low muscle mass: Appendicular fat free mass (AFFM) to height squared. Men <7.23 kg/m^2

		Women <5.67 kg/m^2
		Diagnosis based on the presence of both criteria
Society of Sarcopenia, Cachexia and Wasting Disorders (Morley et al., 2011)	Limited mobility-loss accompanied by loss in muscle mass	Criteria 1: Presence of low physical function Slow gait speed ≤1 m/s or <400m during 6 minutes' walk
		Criteria 2: Presence of low muscle mass >2SD below younger people between 20-30 years
		Diagnosis based on the presence of both criteria.
Asian Working Group for Sarcopenia (Chen et al., 2014)	Low muscle mass and low muscle strength or low gait speed.	Criteria 1: Presence of low muscle mass: appendicular skeletal muscle mass/height^2 >2SD below mean of the younger reference adults DXA Men <7.0 kg/m^2 Women <5.4 kg/m^2 BIA Men <7.0 kg/m^2 Women <5.7 kg/m^2
		Criteria 2: Presence of low grip strength: lowest quintile of the study population (Lauretani et al., 2003) Men <26 kg Women <18 kg
		Criteria 3: Low physical performance assessed with gait speed Gait speed ≤0.8 m/s
		Diagnosis based on presence of criteria 1 plus criteria 2 or 3
Foundation for the National Institutes of Health (FNIH) Sarcopenia Project (Studenski et al., 2014), (Dam et al., 2014)	Two probable FNIH definitions: muscle weakness and low lean mass (low handgrip strength + low ALM_{BMI}) or, clinically relevant low walking speed with muscle weakness and low lean mass (slow gait speed + low grip strength + low ALM_{BMI}).	Criteria 1: Appendicular lean body mass (ALM) Recommended: ALM_{BMI} Men <0.789 Women <0.512 Criteria 2: low handgrip strength Men <26 kg Women <16 kg Criteria 3: Low gait speed Gait speed≤0.8 m/s

DXA- dual energy X-ray absorptiometry; BIA- bioelectric impedance analysis; BMI- body mass index; ALM-appendicular lean body mass adjusted

1.2.1 Defining sarcopenic thresholds using muscle size and muscle strength measures

As can be seen in Table 1.1 low muscle mass is the primary defining characteristic of sarcopenia. What is obvious is that there are numerous thresholds and cut-off levels adopted to describe the elderly as sarcopenic or not; each of which adopts a range of techniques to assess muscle size. Body imaging techniques such as CT, MRI and DXA scanning, are very accurate imaging systems, and are considered as the gold standard for estimating the amount of muscle mass (Cruz-Jentoft et al., 2010). These imaging techniques offer high levels of validity and reliability, they are however, expensive, non-portable and can produce small doses of radiation, or are offer lower adherence for the most impaired elderly. Bioelectrical impedance (BIA) is preferred in several sarcopenia studies as an alternative to these imaging techniques (Legrand et al., 2013; Volpato et al., 2013; Bianchi et al., 2017). BIA is inexpensive, easy to use, readily reproducible and accessible to the most impaired; in addition, it has been found to be highly correlate with MRI predictions (Janssen et al., 2000a; Chien et al., 2008). For defining the cut-offs for low muscle mass, most of the studies have compared with that of a matched young healthy population for sex and ethnicity, however there is not complete references datasets available for both sexes in all ethnic groups, with one group recommending a minimum of 100 participants per group (Morley et al., 2011).

There are several strength measures suggested for the assessment of sarcopenia; for instance, knee extension, knee flexion, handgrip strength and peak expiratory flow. However, handgrip strength is commonly used in clinical and research studies (Cruz-Jentoft et al., 2010). Isometric handgrip strength has been linked with the measures of overall strength and muscle mass of the body; specifically with lower extremity muscle torque and power; and calf cross-sectional area (Lauretani et al., 2003; Edwards et al., 2013; Alonso et al., 2018) and is an early predictor of mortality (Newman et al., 2006).

Like muscle mass, there is no consensus in defining the cut-off for low muscle strength in sarcopenia. Some studies use the cut-off from a young reference

population performing a gait analysis test, while some use the strength equal to the lowest quintile/quartile of the tested elderly population. Clearly, a research priority is to secure a globally accepted definition of sarcopenia that could discriminate the characteristics of sarcopenic elderly so that clinicians can make a correct diagnosis and future researches can base the studies on the standardised methods.

1.2.2 Prevalence of Sarcopenia

Considering the number of definitions available there is unsurprisingly considerable heterogeneity in the prevalence of sarcopenia (Table 1.4). This variation is explained by the difference in age group, study settings, reference population and definitions or cut-offs used (Bijlsma et al., 2013; Beaudart et al., 2015b). Majorities of the studies have investigated the prevalence of sarcopenia in Asia, European and American community settings, with the definition incorporating low muscle mass and low muscle function (Lee et al., 2013; Patel et al., 2013; Patil et al., 2013; Scott et al., 2014a; Yoshida et al., 2014). Studies have shown that sarcopenia prevalence increases with increasing age and frailty (Lee et al., 2013; Legrand et al., 2013; Lin et al., 2013). The prevalence of sarcopenia is lower in studies that include healthier subjects, or more stringent exclusion criteria; such as excluding more frequent fallers, participants aged over 81 years, or show evidence of cognitive or functional impairment (Patil et al., 2013). These stringent criteria resulted in less frail people being investigated, which probable resulted a very low prevalence of sarcopenia compared to others (2.7% in these Finnish women). The highest prevalence of sarcopenia has been reported in residential care settings with the inclusion of older and frailer participants, who exhibit multiple co-morbidities (Bahat et al., 2010; Landi et al., 2011; Landi et al., 2012b; Smoliner et al., 2014; Senior et al., 2015).

Table 1.4 Prevalence of sarcopenia in different populations

Study	Sarcopenia definition and cut-offs	Subject characteristics n, age (years)	Setting	Prevalence
(Scott et al., 2014a)	Definition: low muscle mass and muscle strength Method: BIA Cut-offs: lowest 20% of the predictive population Men: <7.09kg/m^2 Women: <5.91kg/m^2 HGS cut-offs Male<28.8kg Female<18.2kg	Age: 50-79 years Men: 352, 61.7(7.1) years Women: 329, 61.0(6.8) years	Community dwelling Australia	Overall: 5%
(Legrand et al., 2013)	Definition: low muscle mass and muscle weakness Method: BIA Cut-offs: lowest 20% of the predictive population Men: <8.87kg/m^2 Women: <6.42kg/m^2 HGS cut-offs Male <30.0kg Female<20.0 Walking speed: 0.8m/s	Age≥80 years Men:103, 84.6(3.4) years Women: 185, 85.0(3.8) years	Community Dwelling Belgium	Overall: 12.5%
(Volpato et al., 2013)	Definition: low muscle mass plus either low HGS or muscle function Method: BIA Men: <8.87kg/m^2 Women: <6.42kg/m^2 HGS: based on BMI suggested in EWGSOP Walking speed: 0.8m/s	Age≥65 years Mean age: 77.1(5.5) Men: 250 Women: 288	Community dwelling Italy	Overall: 10.2% Male: 4.9% Female: 9.4%
(Yoshida et al., 2014)	Definition: low muscle mass and low muscle strength Method: BIA Cut-offs: lowest 20% of the predictive population Men: <7.09kg/m^2 Women: <5.91kg/m^2 HGS cut-offs Male <28.8kg Female<18.2 kg Walking speed: <=0.8m/s	Age≥65 years Men: 2343, 72.2 (5.5) years Women: 2468, 72.1(5.7) years	Community dwelling Japan	Overall: 7.5% Men:8.8% Women:7.4%
(Landi et al., 2012a)	Definition: low muscle mass and low muscle strength or physical performance Method: BIA Cut-offs: lowest 20% of the predictive population Men: <8.87kg/m^2	Age≥70 years Mean age: 84.1(6.9) Men: 31 Women: 91	Nursing home Italy	Overall: 32.8% Men: 15.6% Women: 17.2%

	Women: <6.42kg/m^2 HGS cut-offs Male <30.0kg Female<20 kg Walking speed: <=0.8m/s			
(Bianchi et al., 2015)	Definition: EWGSOP low muscle mass and low muscle strength or physical performance Method: BIA Men: <8.87kg/m^2 Women: <6.42kg/m^2 HGS: based on BMI suggested in EWGSOP Walking speed: <=0.8m/s	Age:65-94 years Mean 77.1(5.5) years Men: 250 Women: 288	Community dwelling Italy	Overall: 10.2%
(ter Borg et al., 2016)	Definition: EWGSOP low muscle mass and low muscle strength or physical performance Method: BIA Men: <10.75kg/m^2 Women: <6.75kg/m^2 Male <30.0kg Female<20 kg Walking speed: <=0.8m/s	Age≥65 Median Age: 74 years Men: 110 Women: 117	community dwelling Netherlands	Overall: 23%
(Brown et al., 2016)	Definition: EWGSOP low muscle mass and gait speed Method: BIA Men: <10.76kg/m^2 Women: <6.75kg/m^2 Walking speed: <=0.8m/s	Age ≥60 years 70.1(0.14) Male: 1925 Female: 2500	community dwelling US	Overall: 36.5%
(Bianchi et al., 2017)	Definition: EWGSOP low muscle mass and gait speed Method: BIA Men: <8.87kg/m^2 Women: <6.42kg/m^2 HGS: based on BMI suggested in EWGSOP Walking speed: <=0.8m/s	81.0(6.8) years Men: 315 Women: 340	Hospital Italy	Overall: 34.7%

1.3 Pathophysiology of sarcopenia

Ageing accompanied by a decline in skeletal muscle mass and muscle strength is directed by several factors; however, the multifaceted mechanisms driving sarcopenia are still to be elucidated. Some of the major contributing factors that causes sarcopenia are explained below.

1.3.1 Muscle morphology and denervation

Sarcopenia is characterized by a reduction in muscle fibre number and fibre size with type II muscle fibres showing the greatest prevalence of atrophy at a myocellular level (Dreyer et al., 2006; Kosek et al., 2006), such that the quantity of MHC2A and 2X mRNA decreases with age (Balagopal et al., 2001). The contribution of fibre atrophy and loss was described in the vastus lateralis, whereby 18% smaller fibre CSA in the elderly was accompanied by 25% lower total number of muscle fibres (Lexell et al., 1988). The main determinant of fibre number reduction with ageing is due to denervation. Until approximately 60 years of age, motor units are preserved, after which declines with age are observed, associated with a neuropathy of the stimulating motor neuron (Ling et al., 2009). Motor neurons combine with a series of muscle fibres to form a motor unit, allowing innervation from brain to muscle for contraction. Increasing age leads to the death of some of these motor neurons resulting in a denervation of all the muscle fibers, leading to muscle fibre atrophy and death. The consequence of this denervation is ultimately a reduction in muscle mass (Figueiredo et al., 2006).

Although denervation is unique to ageing, there are several anabolic processes that decrease, and catabolic processes that accelerate with age. These include oxidative damage, inflammatory and anabolic hormones, and the interaction of external factors such as physical activity and diet. These are discussed below.

1.3.2 Mitochondria and Oxidative phosphorylation

There is compromised oxidative phosphorylation in ageing muscle (Trifunovic and Larsson, 2008; Joseph et al., 2012), consequences due to the reduction in

mitochondrial density and function (Short et al., 2005; Sun et al., 2016). The decline in mitochondrial DNA number is associated with high levels of free radical production and accumulation of mutations in mitochondrial DNA with increasing age that eventually results in a decreased amount of mitochondrial protein (Larsen et al., 2005; Short et al., 2005). The decreased mitochondrial protein, accumulated mutation and higher ROS finally results in a reduction in the capacity to resynthesize ATP through oxidative mechanisms (Amara et al., 2008; Chistiakov et al., 2014) which is a precursor of muscle catabolism. A study in mice lacking SOD1 (Cu, Zn superoxide dismutase) has shown the age related neuromuscular degeneration (Larkin et al., 2011; Sakellariou et al., 2014). The reduced mitochondrial content and PGC-1 alpha expression along with increment in mitochondrial apoptotic susceptibility has been observed with the increment in free radical production; which might be associated with sarcopenia (Chabi et al., 2008). Lower mitochondrial capacity and efficiency has been found to be correlated with slower walking speed, a clinical indicator for sarcopenia (Coen et al., 2012).

1.3.3 Inflammatory molecules and growth hormones

There is alteration in the level of inflammatory molecules with ageing. The level of several cytokines and regulatory molecules such as TNF-alpha, IL-1, IL 6, myostatin, and human leukocyte antigen changes with increasing age. One of the molecules that highly contributes to sarcopenia is IL-6; the level of which has been reported to increase with ageing. The increased IL-6 level with ageing has been further linked to a decrement in bone mineral density and muscle mass (Papadopoulos et al., 1997; Ershler and Keller, 2000; Visser et al., 2002), thus, it is very likely that it might be associated with sarcopenia. Higher level of IL-6 were positively correlated with age, fat mass and waist circumference, while inversely associated with handgrip strength (Dutra et al., 2017). While it should be noted that IL-6 also has an anti-inflammatory role, reported to help in both local and systemic acute inflammatory response (Xing et al., 1998). IL-6 mRNA level increases in contracting muscles and further increases with exercise (Ostrowski et al., 1998; Keller et al., 2001; Steensberg et al., 2001). Similarly, TNF-alpha accelerates the catabolic pathways in skeletal muscle (Wang et al., 2014) and has been reported to cause apoptosis of type I and type II muscle

fibres (Pistilli et al., 2006). There is an inverse relation between TNF alpha and thigh muscle cross sectional area and handgrip strength in the elderly (Schaap et al., 2009). Other inflammatory molecules altered with ageing and thereafter deteriorates the skeletal muscle function include growth hormone, interleukin-10 and interleukin-15.

Anabolic hormones decrease and catabolic hormones increase with ageing. For example, there is a lower level of testosterone particularly in elderly males that is thought to contribute to muscle loss with age (Harman et al., 2001; Wu et al., 2008). The role of testosterone in sarcopenia is also evidenced by the fact that exogenously administered testosterone has resulted in increases in muscle mass and muscle strength(Harman et al., 2001; Chiang et al., 2018). There is also a reduction in anabolic hormones such as growth hormone in elderly people. These declines in growth hormone and muscle mass have been shown to be improved with exogenous administration of GH in the elderly (Blackman et al., 2002).

A reduction in level of IGF has been reported with ageing (Landin-Wllhelmsen et al., 1994) which has been further linked with poor knee strength, slow walking speed and self- reported mobility problems (Cappola et al., 2001). Similarly, it has also been found that there is an increment in the level of IGF-1 with high-intensity training in the elderly (de Souza Vale et al., 2009). The low level of IGF-1 associated with increasing age and the increase in IGF-1 following strength training in the elderly, suggests that IGF-1 might contribute to sarcopenia.

1.3.4 Nutrition and Physical activity

Loss of appetite is common with ageing (Morley and Silver, 1988; Malafarina et al., 2013). Appetite is normally assessed from the results of food intake, nutritional assessment, body mass or BMI measurements (Mattes, 2005). Loss of appetite has been linked to decreased body mass and nutritional deficiencies (Wilson et al., 2005; Brownie, 2006 2010). Nutritional deficiencies have been related with impaired

quality of life, immune function along with falls, frailty, muscle weakness and mortality (Wilson et al., 2005; Agarwal et al., 2013; Bischoff-Ferrari et al., 2016). Consistent with these observations of nutrient intake accounting for aspects of sarcopenia, is the observation that nutrient supplementation (particularly increased protein intake) can increase lean mass in sarcopenic people (Solerte et al., 2008; Tieland et al., 2012; Deutz et al., 2014).

Physical inactivity is also one of the major causes of sarcopenia. Several longitudinal and cross sectional studies have reported a low level of physical inactivity with increasing age and its association with sarcopenia (Baumgartner et al., 1999; Szulc et al., 2004; Lee et al., 2007). There is a negative correlation between muscle mass and leisure-time physical activity (Lee et al., 2007), with higher levels of leisure time physical activity correlated with higher muscle mass and less total and truncal fat (Raguso et al., 2006). Similarly, there is a decrease in sarcopenia risk with daily walking and moderate intensity exercise (Park et al., 2010; Marzetti et al., 2017). The loss of lean mass with ageing reduces with increased energy expenditure through physical activity (Genton et al., 2011). The importance of both diet and physical activity is emphasized through the interaction of both external influences, such that nutrient supplementation (e.g. proteins and essential amino acids) wit exercise in elderly populations have resulted in progressive gains in muscle mass and muscle strength (Gryson et al., 2014; Beaudart et al., 2017).

The above factors represent most of the potential factors that contribute to sarcopenia. Although some external factors (i.e. diet and physical activity) can be modified, the prevalence of sarcopenia, dependent on the rate of physiological deterioration, is undoubtable under the influence of genetic variation. As has been observed in those at the elite end of physical fitness, genetics plays a considerable role in the accretion of muscle mass, and is likely therefore to contribute to the prevalence of sarcopenia.

1.3.5 Genetic factors

There are more than 200 genetic variants that are reported for the association with health and fitness related phenotypes (Bray et al., 2009). Despite more SNPs being studied, 30 gene variants have commonly been associated with skeletal muscle mass and strength related phenotypes and muscle performance (Hughes et al., 2011; Garatachea and Lucía, 2013; Zarebska et al., 2013). These include SNPs that code for a) structural proteins within the muscle or tendon (e.g. COL1A1, PTK2, ACTN3) b) favourable enzyme isoforms (e.g. ACE) c) hormonal or messenger proteins linked with catabolic or anabolic process (e.g. VDR, ESR1) d) negative regulators of muscle growth (MSTN, ACVR1B) e) myotrophic factors (CNTF, TRHR) f) body composition regulators (FTO). In terms of genetic factors, specifically, that can contribute to sarcopenia, few studies have investigated muscle mass and muscle strength phenotypes and their SNP associations in the elderly. To date, there has been four studies that have investigated the association of SNPs with sarcopenia; limited to *VDR*, *IL6* and *ACTN3* polymorphisms. Roth et al (2002) first investigated the association of *VDR* in elderly females and found that genotype with FF group has 2.17-fold higher risk for being sarcopenic compared to f-allele carriers. This finding was also replicated in a male population by the same group (Walsh et al., 2016) with similar results; a 1.3 fold risk for sarcopenia with FF homozygotes. *ACTN3* R577X has been previously investigated for several skeletal muscle phenotypes (Delmonico et al., 2008; Roth et al., 2008). Recently, *ACTN3* R577X has been found to be associated with a 2 fold risk of sarcopenia with those elderly individuals expressing the XX genotype compared to RR homozygotes (Cho et al., 2017). Another pleiotropic gene variant, *IL6*, was also investigated for an association with sarcopenia. Although despite being conducted in nursing home residents, no associations were reported (Tasar, 2018). Unlike the previous studies that used low ASM as the cut-off for sarcopenia; this study in Turkey used both low muscle mass and muscle function for defining sarcopenia.

1.3.5.1 Identifying the candidate genes of interest for this thesis

As there are limited observations of the genetics of sarcopenia in elderly population, in order to identify potential genes of interest for inclusion within this thesis, a broader range of literature was reviewed, including phenotypes associated with

adaptation to training, associations with strength and muscle mass related phenotypes or an over representation in elite power athletes. Below the process and results of the review are described.

Previously, heritability of muscle mass and muscle strength were studied to understand the role of genetic components on those phenotypes. Although the heritability approach has been successful in determining the phenotypes that are strongly under genetic control, it does not provide sufficient information on the specific gene variant/s that are associated with muscle phenotypes. More recent studies are therefore, mostly focused on identifying the specific gene variants that are associated with skeletal muscle phenotypes. In doing so, several cross-sectional and longitudinal studies have been done (Charbonneau et al., 2008; Yoshihara et al., 2009; Garatachea and Lucía, 2013). Investigating and identifying the specific gene variants that could contribute to the difference in skeletal muscle phenotypes is the difficult part of this approach. A complete theoretical knowledge on how the gene variant can affect the functional aspect of protein might provide an easy way to identify the candidate gene, however, it is a tough and time consuming task to identify as much as thousands of gene variants with this approach. Although the alternative robust approach, GWAS, utilizes a large sample and is popular these days to identify thousands of genes associated with singular phenotypes, the chance of false positive result is also quite high. An alternative approach to GWAS that has recently been adopted in investigating the suggestive association of polymorphisms with skeletal muscle phenotypes is the utilization of Genetic Predisposition Score approach (He et al., 2018). This allows the cumulative genetic influences of multiple SNPs to be considered without the expense of the GWAS. As it is not possible to complete the GWAS analysis in the present thesis due to limited resources and difficulty in testing the large sample, the study utilizes the case-association approach. With consideration of expenses and equipment available for the study, 24 candidate SNPs were chosen for the thesis.

Initially, while doing the literature review during the study design, 36 candidate genes were selected. Due to the time constraint, budget and the equipment design

(only 24 SNPs genotyping possible in maximum), the number of candidate SNPs was reduced to 24 in the present thesis (Table 1.5), based on several criteria such as:

1) the number of studies supporting the associations between some SNPs and the skeletal muscle phenotypes.

2) whether contradictory findings for some SNPs with the similar skeletal muscle phenotypes existed.

3) likelihood of some genetic variants being functional with the findings from existing gene transcriptional analysis.

4) the necessity for replication in independent elderly population with some of the skeletal muscle phenotypes.

5) investigating the novel possible associations of some SNPs with skeletal muscle related phenotypes .

Table 1.5 Previous studies investigating association between SNPs and skeletal muscle phenotypes

SNPs	Gene/polymorphism function/ location	Muscle phenotypes studied/physical performance/ population	Main results	References
ACTN3 rs1815739	component of sarcomeric Z-discs located on chromosome 11 and is a nonsense mutation R577X in ACTN3 gene resulting in lack of protein expression due to production of stop codon at residue 577 (North et al., 1999)	Lower mid-thigh cross sectional area (aged 58-70 years, Japan)	XX demonstrated significantly lower than RX/RR	(Zempo et al., 2010)
		KE shortening and lengthening peak torque	XX group had significantly reduced than RR/RX groups	(Walsh et al., 2008)
		Decline in walking time after 5 years	XX male had significantly less walking distance time	(Delmonico et al., 2008)
		40m sprint time	R allele as favourable allele and contributes for faster time in additive manner	(Moran et al., 2007)

		Explosive power of leg in elite volleyball players Sarcopenia	No association XX as risk group for sarcopenia	(Ruiz et al., 2011) (Cho et al., 2017)
ACE rs4341 (I/D)	D allele increases angiotensin II activity located on human chromosome 17 and contains a 287bp insertion/deletion polymorphism in intron 16 (Glenn et al., 2009).	lean mass, and body weight; appendicular fat free mass in older women isometric and isokinetic strength in healthy young men; handgrip strength in athletes Handgrip strength and vertical jump ability in adolescents	Higher body mass measures were associated with D allele DD genotype was associated with higher strength II genotype is associated with higher strength and jump performance	(Charbonneau et al., 2008) (Williams et al.; Costa et al., 2009b) (Moran et al., 2006)
CNTF rs1800169	located on chromosome 11 and is a G-A substitution (1357 G → A) in the second exon of *CNTFR* gene (Walsh et al., 2009). AA genotype produces non-functional protein and CNTF level declines with ageing	Concentric peak torque knee extensors and elbow flexors concentric knee flexors strength in middle aged women Handgrip strength	Heterozygotes GA were stronger than GG individuals A-allele carriers were weaker than GG AA homozygotes had 3.8 kg weaker handgrip strength than G-allele carriers.	(Roth et al., 2001) (De Mars et al., 2007b) (Arking et al., 2006)
CNTFR rs2070802	is present in intron 5 , 37bp upstream of exon 6 for CNTFR (De Mars et al., 2007a). CNTF interacts with CNTFR for signalling	60-78 years male	T-allele carriers possessed higher KE and KF in male	(De Mars et al., 2007b)
ESR1 rs1999805 rs4870044	encodes for the oestrogen that function for bone mass and bone growth	Lean mass	Loss of oestrogen after menopause was associated with low lean mass	(Poehlman et al., 1995)
FTO rs9939609	associated with obesity related phenotypes is located on Chromosome 16 within first intron of *FTO* gene (Ben Halima et al., 2018).	BMI Calf circumference	AA was associated with higher BMI, muscle mass, and obesity related phenotypes	(Sonestedt et al., 2011), (Jacobsson et al., 2012; Al-Serri et al., 2018)

HIF1A rs11549465	is located on chromosome 14 and in exon 12 of the *HIF1A* gene (Hlatky et al., 2007) upregulated during hypoxia condition and controls expression of genes involved in apoptosis, cell proliferation and differentiation C-T transition with T associated with higher transactivation activity	maximal oxygen consumption with exercise training in elderly Comparison of frequency distribution of genotypes between the groups	TT was associated with higher V02 max TT was over represented in weightlifters and wrestlers No association with HIF1A variants	(Prior et al., 2003) (Ahmetov et al., 2008) (Eynon et al., 2010)
ID3 rs11574	Is located on chromosome 1 resulting in alanine to threonine substitution in C terminus of Id3 gene (Doran et al., 2010). has a helix –loop-region with specific C-terminus with which it binds to E12 and E47 and averts their dimerization with tissue specific class II bHLH proteins	fat mass, BMI, waist circumference (WC), and waist-hip ratio (WHR) in humans	A-allele was associated with changes in cross sectional BMI and fat mass	(Svendstrup et al., 2018)
IGF1 rs35767	is located in promoter of the IGF1 gene in chromosome 12 where 192bp of CA repeats is found (Rietveld et al., 2004). promotes myoblast proliferation, differentiation during normal muscle growth and muscle injury by coordinating with other growth factors	Body composition	CC genotype was associated with higher trunk and total fat and lower lean and muscle mass	(Kostek et al., 2010)
IL6 rs1800795	is located in promoter region of IL6 gene which is found in chromosome 7 and G allele linked with higher transcription (Murphy et al., 1997).	Weightlifters and jumpers Knee muscle strength and frailty Exceptional longevity	G allele overrepresentation No association No association	(Ruiz et al., 2010) (Walston et al., 2005; Pereira et al., 2011) (Fuku et al., 2015)
MTHFR rs1801131 rs1537516 17421511	*MTHFR* rs1801131 (A1298C) results decrease in MTHFR enzyme activity (Cui et al., 2012).	Sprint and strength athletes VO_2max post training	C allele overrepresentation C-allele carriers had significantly improvement in VO_2 Max	(Zarebska et al., 2014) (Cięszczyk et al., 2016)
PTK2 rs7843014, rs7460	components of cell costameres, provide an integral link between ECM, cytoskeleton and muscle fibres in skeletal muscle	Exceptional longevity	rs7843014 CC and rs7460 TT association with longevity	(Garatachea et al., 2014)

		Specific force in healthy men	AA homozygotes had higher VL specific force	(Erskine et al., 2012; Stebbings et al., 2017)
TRHR rs7832552	is located in the region 8q.23.1 on the surface of thyrotrophic pituitary cells binding site for TRH and thereafter controls the level of T4 that plays important role in skeletal muscle development	lean body mass exceptional longevity	T allele was associated with higher lean body mass No associations	(Liu et al., 2009; Lunardi et al., 2013) (Fuku et al., 2015)
TTN rs10497520	missense mutation, C-T transition, contribute to variation in TTN isoforms (Rankinen et al., 2003; Timmons et al., 2010). protein helps in myocyte development, function, assembly and organization of thick and thin filaments during myofibrillogenesis	marathon running performance and muscle fascicle length isometric knee strength in CAD patients elderly population 20-83 years for isometric knee extension	T-allele carriers had better marathon personal best times. No association with strength C-allele is predisposing allele for knee strength in elderly population	(Stebbings et al., 2018) (Thomaes et al., 2013) (He et al., 2018)
VDR rs2228570	known as Fok1 polymorphisms, involves C-T transition in exon 2 of VDR gene in chromosome 12 (Hou et al., 2015). helps in calcium accumulation in sarcoplasmic reticulum and impact in muscle protein synthesis	quadriceps strength, hamstring strength, peak torque, FFM FFM/Sarcopenia	F (C) allele has been linked to reduced fat-free-mass and men F allele with reduced concentric and isometric knee strength than f-allele carriers FF homozygotes in higher risk than f-allele carriers and f allele associated with higher FFM	(Roth et al., 2004) (Windelinckx et al., 2007) (Roth et al., 2004; Walsh et al., 2016)
MSTN rs1805086	is located in chromosome 2, located in exon 2 of the three-exon gene, involves an A-to-G change in the codon that encodes the 153th amino acid of myostatin (Ferrell et al., 1999). modulates the myoblast proliferation and acts as the inhibitor of the muscle tissue growth	muscle strength	R153 allele of MSTN with decreased strength a negative influence of 1RM leg press and muscle mass of women at old age R allele linked to maximal isometric	(Seibert et al., 2001; Corsi et al., 2002) (González-Freire et al., 2010) (Kostek et al., 2009;

			contraction of elbow muscle flexors and ability to produce peak power during muscle contractions	Santiago et al., 2011)
		Exceptional longevity in Japanese population	No association	(Fuku et al., 2015)
		Strength training induced hypertrophy in Chinese population	KR has significantly higher increment in bicep and quadriceps thickness than KK genotypes	(Li et al., 2014) (Santiago et al., 2011)
		Vertical jump		
COL1A1 rs1800012	known by Sp1 polymorphism, located in the intronic part of *COL1A1* gene, associated with transcription start site, and -1997G/T polymorphism is located in promoter region of this gene (Jin et al., 2009) encodes type I collagen protein polymorphism results the increment in alpha-1 chain	hand grip strength and biceps strength in elderly 70+ population	s allele is associated with the lower strength	(Van Pottelbergh et al., 2001)
ACVR1B rs2854464	rs2854464 is located in a putative miR-24-binding site in the 3' untranslated region (UTR) of the *ACVR1B* mRNA (Windelinckx et al., 2011). encodes the Activin A receptor type 1b protein, regulates the expression level of several genes implicated in controlling muscle growth		rs2854464 A allele associated with higher knee strength	(Windelinckx et al., 2011)
			over representation of A allele in sprint and power Caucasians athletes	(Voisin et al., 2016)
ACVR1B rs10783485			A-allele with SMM	(He et al., 2018)
			C-allele as strength increasing allele for isometric knee flexion	(Windelinckx et al., 2011)
NOS3 rs1799983	is located in Chromosome 7 and associated with G>T transition at codon 298 at 7th exon and results substitution of (Glu298Asp) (Heidari et al., 2017).	distribution in athletic population and long distance swimmers	T allele was overrepresented in the power athletes	(Gómez-Gallego et al., 2009; Sessa et al., 2011; Eider et al., 2014;

	encodes the enzyme eNOS which catalyzes the synthesis of NO T allele is associated with increased NO activity	stroke volume	T-allele was associated with higher stroke volume and lower HR during submaximal dynamic exercise in postmenopausal women	Zmijewski et al., 2018) (Hand et al., 2006)

1.4 Aims and objectives

In short, the over-all aim of the current thesis was to investigate the association of selected SNPs with sarcopenia and to investigate the association of SNPs with skeletal muscle phenotypes. More precisely, the objectives were:

1) To identify a meaningful definition of sarcopenia that can distinguish the sarcopenia group from non-sarcopenia groups based on skeletal muscle phenotypes

2) To investigate the association of genetic polymorphisms with sarcopenia

3) To investigate the associations of specific gene variants with skeletal muscle size/strength phenotypes

4) To investigate the predictive power of Genetic Predisposition Score (GPS) for explaining the variance of skeletal muscle phenotypes

1.5 Overview of thesis

The present thesis is sub-divided into 7 chapters. Chapter 1 (presented above) is the literature review and background of the study. Chapter 2 describes the methodology applicable for chapters 3, 4, 5 and 6. In brief, Chapter 2 describes the muscle mass and muscle strength measurement techniques, and the calculation and assessment of each skeletal muscle phenotype used throughout the thesis. It also describes the methodology used to assess physical performance, through the One Leg Standing Balance Test. Finally, this chapter explains the procedures of DNA sample collection and, DNA extraction followed by genotyping procedures. Chapter 3 investigates a meaningful definition of sarcopenia among 3 previous definitions and a novel approach based on skeletal muscle mass and handgrip strength. Chapter 4 identifies a meaningful definition of sarcopenia based on the discriminating power of each definition for skeletal muscle phenotypes used in the present thesis. Thereafter, the aim of Chapter 4 is to examine the association of specific gene variants with sarcopenia used as a distinct phenotype. It was hypothesized that individuals in the sarcopenia group would carry risk alleles for either lower muscle

mass or muscle strength or both. Chapter 5 investigates the association of SNPs with skeletal muscle phenotypes. More precisely, in Chapter 5 the individual influence of 24 genetic polymorphisms will be assessed for muscle size (VL-thickness, VL_{ACSA}, biceps brachii thickness), muscle strength (Handgrip Strength, MVC_{KE}, and MVC_{EF}) phenotypes, and the One Leg Standing Balance Test. It was hypothesized that the association will be found between selected gene variants and the skeletal muscle phenotypes.

Subsequently in chapter 6, the polygenic approach is utilized to explain the variance of skeletal muscle phenotypes. The GPS data-driven approach is used to study the effect of multiple gene variants in predicting skeletal muscle phenotypes. Since muscle mass and muscle strength are influenced by multiple genes, it is expected that a greater phenotype variance could be explained by the polygenic approach rather than investigating associations with single candidate polymorphisms.

Finally, the discussion chapter, Chapter 7, reviewed the results obtained within each chapter of this thesis into a coherent narrative that considers the findings within the context of current literature. It explains how the sarcopenia group is different from non- sarcopenia with neuromuscular parameters under the study. Identification and explanation for the risk allele/genotype for sarcopenia is also discussed. Similarly, it also explains how the selected candidate SNPs on the present study effects skeletal muscle size and strength of the elderly Caucasian female. In linking the results of the prior chapters, this chapter also considers potential future directions for research in this area.

2 . General Methodology

2.1 Participant recruitment

Participants comprised 60-91 years old Caucasian females (n= 307, 70.7±5.7 years, 66.3±11.2 kg, 1.60±0.06cm) (Mean ± SD) that were recruited between May 2016 to September 2017 from South Cheshire and the surrounding areas through advertisements and word of mouth. All participants were independently living, ambulatory and had no history of severe muscle and bone issues, such as osteoporosis, rheumatoid arthritis and cancer, nervous system disorders, such as Alzheimer's, convulsions, epilepsy, or cardiovascular-related diseases. Study protocols were in accordance with the guidelines of the Declaration of Helsinki (World Medical Association, 2013) and approved by the Ethics Committee of Manchester Metropolitan University. Informed written consent was obtained from all the participants prior to involvement in the present thesis study.

2.2 Protocol overview

Participants attended for testing on a single visit for 3 hours per participant during which time they completed anthropometric tests (height, mass, body composition and muscle size measures), functional tests of muscle strength and balance, followed by collection of either a venous blood or saliva sample. DNA was extracted from the collected samples and genotyped for 24 polymorphisms. Physical activity of participants was assessed using Physical Activity Scale for Elderly (PASE) questionnaire. The order of testing was as follows: questionnaires, anthropometrics, muscle phenotype assessment (function, balance and size), body composition and finally phlebotomy.

2.2.1 Anthropometrics

Standing height was measured with participants' unshod using a stadiometer to the nearest 0.1 cm. Body Mass was measured to the nearest 0.1 kg on a digital scale unshod in minimal clothing.

2.2.2 Body composition

Fat and fat-free mass were quantified using Bioelectrical Impedance Analysis (BIA) (Model 1500; Bodystat, Isle of Man, UK). Participants were instructed to remove any metallic attachments such as watches and bracelets before the test. They were then instructed to lie on a physiotherapist bed in a supine position with both upper and lower limbs slightly abducted from the body for about 4-5 minutes. Two adhesive electrodes were placed on the dorsum of the hand and foot on the right side of the body. A negligible electrical current (frequency: 50kHz; amplitude: 0.4mA), which is too low for the participant to feel, was then passed between these electrodes. Whole-body impedance, resistance to an applied current, Fat-Free Mass (FMM), Fat mass, water percentage and Body Mass Index (BMI, body mass/height^2) were recorded using BIA. Skeletal muscle mass was estimated using a previously established formula (Janssen et al., 2000a) as:

Equation 1: Skeletal Muscle Mass (SMM) = [(Ht^2/Rx0.401) + (sex x 3.825) + (age x -0.071)] + 5.102

Where Ht is height in cm, R is resistance from BIA and age in years. For sex, a male is scored as 1 and female as 0.

Skeletal Muscle Index (SMI) was calculated as skeletal muscle mass (SMM) divided by the height as;

Equation 2: SMI= SMM/Ht^2 where SMM is in kg and height is in m.

2.2.3 One Leg Standing Balance Test

One Leg Standing balance test (OLST) was performed to access the balancing ability and balance impairment of participants. OLST is a simple, predictive and inexpensive marker for screening the low functional level and frailty associated with ageing (Vellas et al., 1997a). Participants were instructed to stand barefoot, then to flex either their left or right knee to 90 degree allowing the foot to clear the floor, and balance on one leg as long as possible, up to a maximum duration of 30 s (Bohannon

et al., 1984), with time recorded via stop-watch. If participants did not reach 30 seconds, they were asked to repeat the test twice more and the maximum of the three attempts was recorded as their OLST time. Participants alternated attempts between left and right leg, leaving 1 min between trials, to reduce the impact of accumulated fatigue. The OLST was performed first with eyes open (EO) and eyes closed (EC) condition (Bohannon et al., 1984).

2.2.4 Handgrip Strength

Handgrip strength (HGS) was measured using a digital load cell handgrip dynamometer (JAMAR plus, JLW Instruments, Chicago, USA) with a previously validated protocol (Roberts et al., 2011). Participants were instructed to stand in an upright position with the dynamometer held with the arm straight, and flexion at 90 degrees to the shoulder (Figure2.1). Verbal encouragement was provided to encourage each participant to squeeze the handgrip dynamometer with maximum force, which was maintained for 5 seconds. The highest grip strength of three maximal efforts was recorded for the study. The left and right were alternated, with 1 minute break between trials.

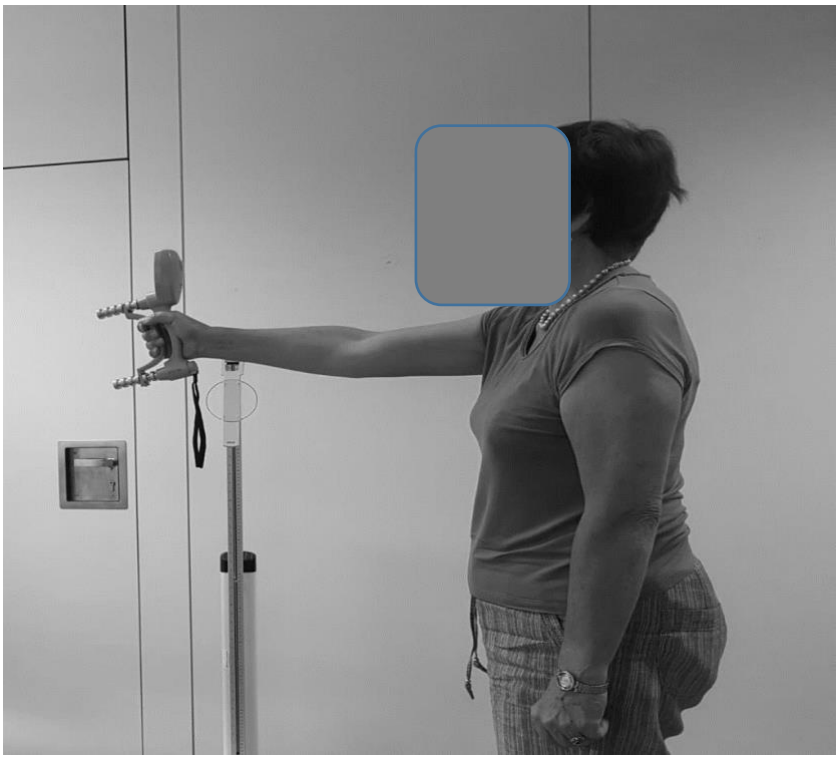

Figure 2.1 Handgrip Strength measured using handgrip dynamometer. Strength was obtained from a participant of the work described in the current thesis

2.2.5 Vastus Lateralis Muscle Size

B-mode ultrasonography (My LabTwice, Esaote Biomedical, Italy) was used to determine Vastus Lateralis (VL) muscle size. With the participants standing, the origin and insertion of the VL muscle was identified as the proximal and distal myotendinous junction of the VL, respectively, using ultrasound (7.5 MHZ, linear array probe, 38mm). The origin and insertion of the VL were assessed in a standing position to the accumulation of subcutaneous fat in some participants making identification of the VL origin impossible in the supine position. The VL length was measured with a measuring tape as the distance from origin (head of femur) to insertion (VL myotendinous junction). The lateral and medial borders of the VL were identified using ultrasound to identify the mid-sagittal line of the VL. The participants were then seated subsequent ultrasound procedures. A sagittal plane scan was performed at 50% of VL length, on the mid sagittal line of the VL.

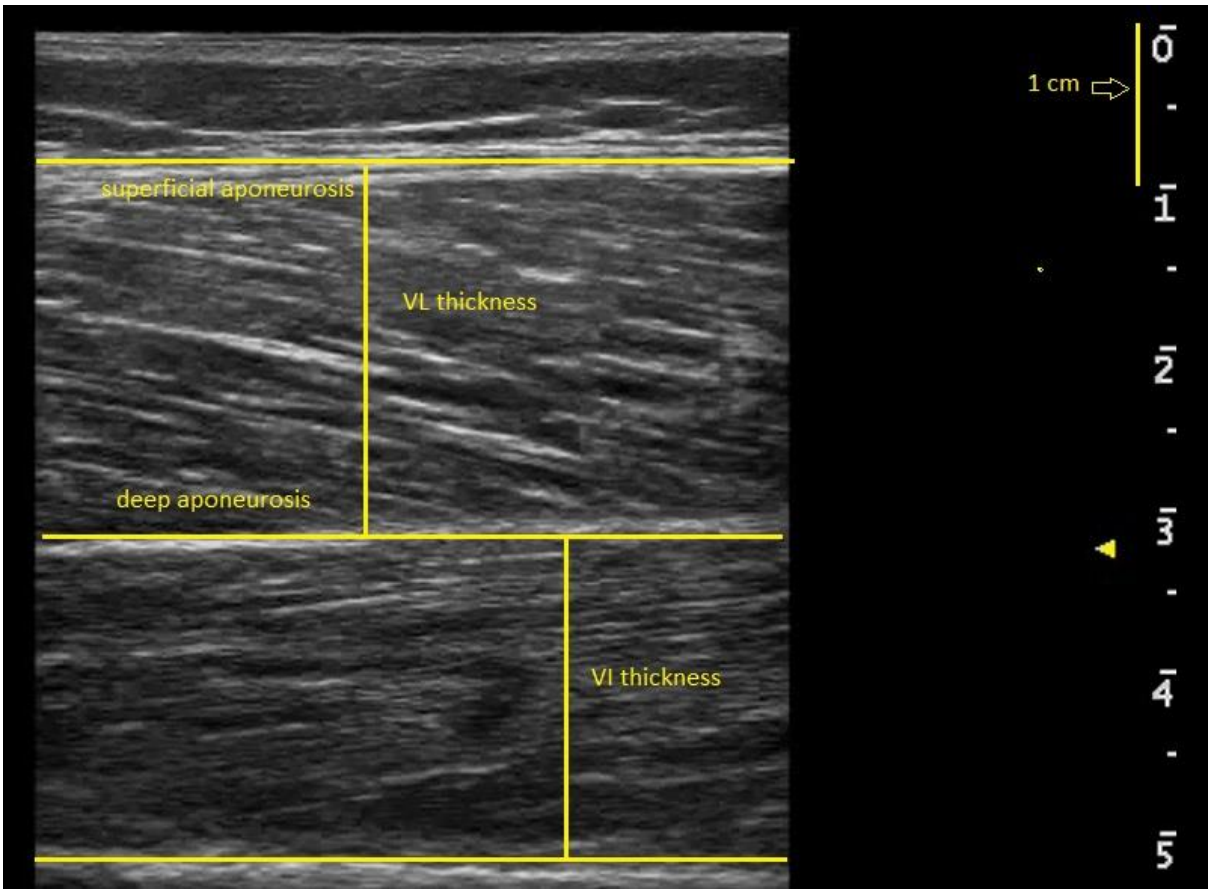

Figure 2.2Measurement of Vastus lateralis thickness using sagittal plane B-mode ultrasound scans. Scan was obtained from a participant of the work described in the current thesis.

For Anatomical Cross-Sectional Area of Vastus Lateralis Muscle (VL_{ACSA}), a transverse plane ultrasound scan was performed at 50% of VL length, as this corresponds to the VL length at which maximum ACSA is found (Morse et al., 2007). Using echo absorptive markers every 3 cm from the medial to the lateral border of VL muscle, the ultrasound probe was steadily moved over the echo-absorptive markers from medial to the lateral edge of VL. Minimal pressure was applied during scanning to avoid compression of the muscle. The ultrasound was recorded as a digital video file from which individual images were acquired using capture software (Adobe Premier, Adobe). Captured images were acquired at contiguous intervals between each shadow cast by the echo-absorptive markers. The entire VL_{ACSA} was reconstructed in a single canvas from each captured image. For the measurement, digitizing software (Image J, NIH) was used as the visible aponeurosis around the VL.

The reliability and validity of this method were reported high (>0.99) previously when compared with MRI (Reeves et al., 2004).

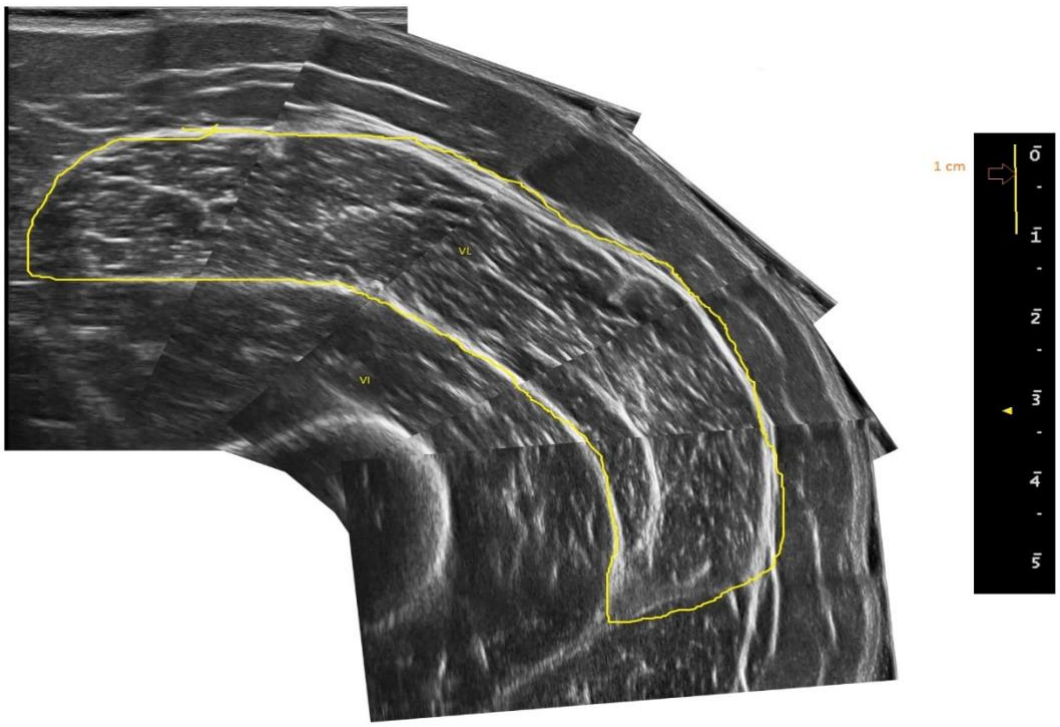

Figure 2.3VL_{ACSA} measured using single transverse plane B-mode ultrasound scans and contour matched. VL= Vastus Lateralis, VI= Vastus Intermedius. Scan was obtained from a participant of the work described in the current thesis.

2.2.6 Biceps brachii thickness

B-mode ultrasonography (My LabTwice, Esaote Biomedical, Italy) with a 38mm probe (7.5 MHz, linear array probe) was used to measure biceps brachii thickness following a previously established method (Miyatani et al., 2004). Participants were seated with the dominant arm hanging, relaxed at their side; the proximal (acromion process) and distal ends (olecranon) of the humerus were identified using ultrasound scanning and palpation. Thereafter, a sagittal plane scan was performed at 60% length from the proximal end of the humerus, identifying the upper and lower aponeurosis of the biceps brachii muscle (Ogasawara et al., 2012). Minimal pressure was applied to the ultrasound probe while scanning to avoid compression of the muscle. Ultrasound was recorded in real time, from which an image was captured from the recorded video. Biceps thickness was recorded as the distance

between the superficial and deep aponeurosis, taken at the proximal, middle and distal end of the captured image using digitizing software (Image J, NIH). The average of the three measurements was recorded as the bicep thickness.

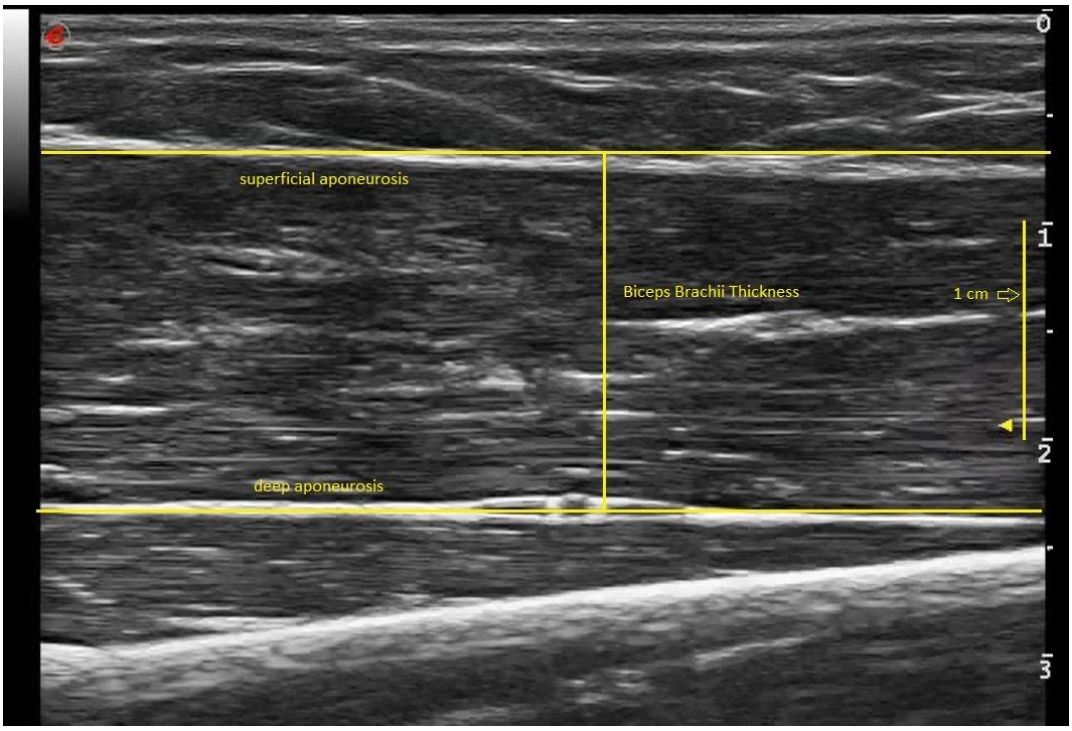

Figure 2.4 Sagittal plane ultrasound at 60% length of the humerus showing biceps brachii from a participant of the work described in the current thesis.

2.2.7 Isometric Knee Extension Maximum Voluntary Contraction

Isometric Knee Extension Maximum Voluntary Contraction Force (MVC_{KE}) was recorded using a load cell (Zemic, Eten-Leur, Netherlands) with all participants in a seated position in a custom-built dynamometer with knee angle maintained at 120 degrees (straight is considered as 180 degrees). The load cell was calibrated using known loads of 500g-5kg, in 500g increments, prior to every strength testing session. The dominant leg was securely fastened using low compliance, nylon straps to a force transducer, at some known distance (5cm in most cases) above the lateral malleolus (identified by palpation). Participants were instructed to perform MVC_{KE} with real-time visual feedback and verbal encouragement from the principal investigator. Three trials were performed, with breaks of 1 minute between trials to reduce any influence of fatigue (Armatas et al., 2010). The force produced was

digitized using an analogue-to-digital converter displayed and recorded on a PC (MyLabView, National Instruments, Berkshire, UK). MVC_{KE} was calculated as:

MVC_{KE} = Force (Output) X (Tibia length- strap distance from ankle)

2.2.8 Isometric Maximum Voluntary Contraction -Elbow flexion

Isometric elbow flexion Maximum Voluntary Contraction (MVC_{EF}) was recorded using the same approach and equipment as for the MVC_{KE}. The participants were seated in the custom-built dynamometer and dominant hand was securely fastened to a force transducer with a shoulder flexed. Participants were instructed to perform MVC_{EF} with elbow angle maintained at 60 degrees (straight is considered as 0 degree) whilst receiving real-time visual feedback and verbal encouragement from the principal investigator. Three trials were performed, with breaks of 1 minute between trials to reduce the influence of fatigue (Armatas et al., 2010). The force produced was digitized using an analogue-to-digital converter, displayed and recorded on a PC (MyLabView, National Instruments, Berkshire, UK) and recorded in Newton (N).

2.2.9 Physical Activity Scale for Elderly questionnaire

Physical Activity Scale for Elderly (PASE) questionnaire was used to assess the physical activity of participants. PASE is a valid tool for the measurement of physical activity, health and physical function and energy expenditure in older individuals (Washburn and Ficker, 1999). The low to moderate correlations between the PASE score with accelerometers in the different populations have been reported (Dinger et al., 2004; Hagiwara et al., 2008; Svege et al., 2012). Participants completed the questionnaire in the lab on the testing day. The questionnaire is a 7-day recall questionnaire identifying time spent undertaking activities such as sitting, moderate intensity activities, recreational activities, strenuous activities and endurance and muscle strength related exercises. The questionnaire includes questions related to time spent in household work, gardening, caring for dependent person, and work (paid or voluntary). The total PASE score was computed by multiplying the amount of time spent in each activity (hours/ week) or participation (yes/no) in an activity

by the empirically derived item weights and summing over all activities (Washburn and Ficker, 1999).

2.2.10 Genetic Analysis

2.2.10.1 DNA sample collection

Two techniques for DNA collection were adopted during the whole testing. All participants were encouraged to provide a forearm venous sample (*n* = 189 participants), however, in the case were participants were afraid of the needle or there was difficulty in getting the blood sample, a saliva sample was collected (*n* = 116 participants). Samples from two participants were not accessible and were not collected.

Five mL of blood was collected from a superficial forearm vein by a trained phlebotomist (the principal investigator in this case) in 5mL EDTA tubes (BD Vacutainer Systems, Plymouth, UK). Samples were stored at -20 ° C before further processing. During the DNA extraction process, the blood was first aliquoted in 2mL micro-centrifuge tubes (Eppendorf AG, Hamburg, Germany). Saliva samples were collected using DNA Saliva Kits (Oragene.DNA, OG-500, Canada) using manufacturer's instructions with participants abstained from food for a period of 3 hours before collection. They were provided water to prevent dryness of mouth and enhance saliva production prior collection. After saliva collection, the samples were stored at room temperature in a laboratory until DNA extraction. All the samples collected in EDTA tubes/DNA Saliva Kit were coded and labelled anonymously in accordance with the Human Tissue Act 2004.

2.2.10.2 DNA extraction

Genomic DNA, from both the Blood sample and Saliva sample, was extracted using the Qiagen QIAcube spin protocol (Qiagen, Crawley, UK). The extraction process was performed in accordance with the guidelines provided by the manufacturer. Qiagen DNA Blood Mini kit buffers (Qiagen, Crawley, UK) were used for the extraction. While extracting DNA from blood/saliva samples, cell lysis was done with protease

and an AL buffer was used during incubation at 56° C for 10 minutes. Centrifugation was then performed followed by the addition of ethanol. The resultant lysate was centrifuged at 8000 rpm for 60 s to allow binding of silica gel membrane. Additional buffer centrifugation cycles were performed to remove the proteins, nucleases and other impurities. Elution of the remaining solution was carried out with 200 µL of AE buffer into a 1.5 mL micro-centrifuge tube. These procedures were standardised in the automated Qiagen QIAcube that could extract DNA from a maximum of 12 samples at a time. DNA quantity was measured from NanoDrop prior genotyping from the 48 random samples and was found to be between 18-65.8 ng/ µL with average 40.2±12.91 ng/ µL. Typical yields from whole blood and saliva with this protocol are described as good quality with A_{260}/A_{280} ratios of 1.2-1.7 (Glasel, 1995).

2.2.10.3 Genotyping

The extracted DNA samples were genotyped for 24 polymorphisms (Table 2.1). Two techniques were adopted for genotyping. EP1 Fluidigm system was used for the initial genotyping but in instances when errors occurred (~1%), such as duplicate samples were not in 100% agreement, a second run was performed using StepOnePlus real-time PCR.

The work flow process for EP1 included the transferring of samples and assays into the IFC, loading the IFC on IFC Controller RX to automatically set up reaction chambers, placing the IFC onto the FC1 cycler and start the PCR protocol, reading the IFC on the EP1 reader and analysing the software to view and interpret the result.

In brief, four runs were performed and genotyping was determined using Fluidigm 192.24 Dynamic Array IFC (Integrated Fluidic Circuit, Fluidigm Corp., CA, USA) in accordance with the manufacturer's instructions. Each assay (4 µL) compromised 2.0 µL of assay loading reagent [2x] (Fluidigm), 1.0 µL SNP genotyping Assay Mix [40X] (Applied Bio-systems), 0.2 µL ROX [50X] (Invitrogen, Carlsbad, CA), and 0.8 µL DNA-free water. Each sample (4 µL) contained 1.6 µL genomic DNA, 2.0 µL GTX press Master Mix [2X] (Applied Biosystems), 0.2 µL Fast GT Sample Loading Reagent [20X] (Fluidigm), and 0.2 µL DNA-free water. Non-template controls (NTCs) were included

in each run. Each of the assays (3.75 µL) and samples (4 µL) were pipetted into separate inlets on the frame of the chip as manufacturer's instructions. The assays and samples were loaded into the chip and mixed using the Integrated IFC Controller RX software. When the mixing was completed, the chip was taken out and then loaded into a thermal cycler (FC1 Fluidigm) and the GT 192X24 Fast v1.pcl protocol was run. Thereafter, fluorescence levels of the VIC and FAM dyes were measured for each sample-SNP combination using the EP1 reader (Fluidigm) and genotypes were called using the Fluidigm SNP genotyping analysis software (Fluidigm) and manually inspected for unusual patterns.

In circumstances where the genotyping was not successful in the above process, StepOnePlus Real-Time PCR system (Applied Biosystems) was used for amplification and genotyping for those samples. For genotyping, reaction volume of 10 µL was used which contained 0.2 µL of participant DNA (blood/saliva), 5 µL of TaqMan genotyping master mix (Applied Biosystems, Paisley, UK), 4.3 µL of nuclease free H2O (Qiagen) and 0.5 µL of TaqMan SNP genotyping assay (Applied Biosystems). The protocol included: an initial 10 minutes at 95 °C followed by 40 cycles of denaturation for 15 seconds at 92 °C, primer annealing and extension for 1 minute at 60°C and the plate read. Genotype was determined by using StepOnePlus analysis software version 2.3 (Applied Biosystems). Genotypes were identified based on reporter dyes VIC and FAM intensity and visualized using cluster plots. All samples were analysed in duplicate with 100% agreement to minimise the genotyping errors (Tintle et al., 2009).

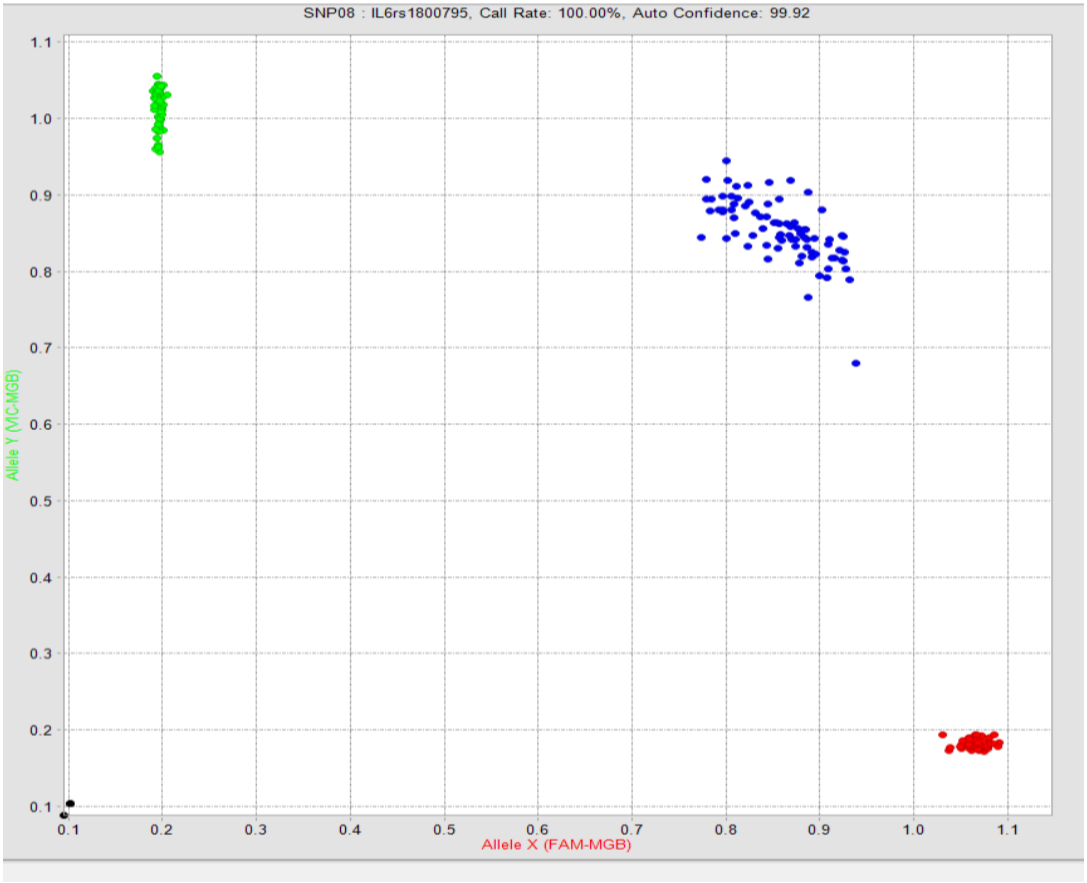

Figure 2.5 Example allelic discrimination plots for IL6 rs1800795 obtained using the EP1 system. Green denotes CC, blue denotes GC and red as GG for IL6 rs1800795 in this example.

2.2.11 Statistical Analysis

A one-way analysis of variance (ANOVA) was conducted to determine any significant differences in physical characteristics (stature, BMI, mass and age) between genotype groups (Table). When genotype groups were combined into a dominant and recessive model, an independent sample t-test was used to identify the differences in physical characteristics. Chi-square test was performed to check the compliance for the Hardy-Weinberg equilibrium (Table 2.1).

Table 2.1Genotype frequency and participant's physical characteristics with genotypes

SNPs	Frequency	χ^2	p-value	Stature	BMI	Mass	Age	p-value
IL6 rs1800795								
CC (n=61)	20%			1.59±0.06	25.6±4.0	65.8±11.2	71.3±6.0	≥0.089
CG (n=145)	47.5%	0.356	0.837	1.60±0.05	25.8±4.4	65.9±11.6	70.0±5.2	
GG (n=99)	32.5%			1.60±0.07	26.1±4.0	67.2±11.0	71.5±6.1	
FT0 rs9939609								
AA (n=48)	15.7%			1.59±0.05	26.63±5.70	67.3±14.1	70.4±4.6	≥0.343
AT (n=151)	49.5%	0.228	0.892	1.60±0.06	25.70±3.77	66.2±10.5	70.9±5.7	
TT (n=106)	34.8%			1.59±0.07	25.92±3.85	66.0±10.9	70.7±6.1	
MTHFR								
rs17421511				1.62±0.10	26.5±4.5	70.6±16.0	72.6±4.8	≥0.129
AA (n=7)	*2.3%*	*0.001*	*0.99*	1.60±0.06	26.4±4.7	67.8±12.8	70.9±6.0	
AG (n=78)	*25.6%*			1.59±0.06	25.7±3.9	65.7±10.5	70.6±5.6	
GG (n=220)	*72.1%*							
ACVR1B								
rs10783485				1.60±0.05	26.3±4.4	66.9±12.0	70.2±5.6	≥0.138
GG (n=128)	42.1%	2.036	0.361	1.60±0.07	25.6±4.0	65.8±10.5	71.4±5.9	
GT (n=147)	48.4%			1.60±0.07	25.6±3.9	66.0±11.2	69.8±5.1	
TT (n=29)	9.5%							
NOS3 rs1799983								
GG (n=118)	38.7%			1.60±0.06	26.2±4.4	66.9±11.5	70.6±5.9	≥0.583
GT (n=144)	47.2%	0.008	0.996	1.60±0.06	25.7±4.0	70.7±5.9	70.7±5.8	
TT (n=43)	14.1%			1.58±0.09	25.8±4.1	71.3±4.7	71.3±4.7	
ACVR1B								
rs2854464				1.60±0.05	26.2±4.3	66.7±11.8	70.6±5.7	≥0.406
AA (n=153)	50.2%	0.150	0.928	1.60±0.07	25.7±4.1	66.0±11.2	71.2±6.0	
AG (n=128)	42.0%			1.61±0.06	25.4±2.9	65.6±8.0	69.6±3.8	
GG (n=24)	7.8%							
PTK2 rs7460								
AA (n=72)	23.6%			1.59±0.05	25.9±3.8	65.7±9.7	70.2±6.4	≥0.257

AT (n=153) TT (n=80)	50.2% 26.2%	0.005	0.998	1.60±0.07 1.61±0.06	26.0±4.5 25.7±3.8	66.6±12.2 66.2±10.8	71.3±5.7 70.2±5.0	
ESR1 rs1999805 AA (n=100) AG (n=148) GG (n=57)	 32.8% 48.5% 18.7%	 0.029	 0.985	 1.60±0.06 1.59±0.07 1.61±0.05	 26.0±4.0 26.0±4.2 25.6±4.3	 66.4±10.9 66.3±11.0 66.3±12.7	 70.8±5.8 70.9±5.6 70.4±5.7	 ≥0.469
PTK2 rs7843014 AA ((n=105) AC (n=142) CC (n=57)	 34.5% 46.7% 18.8%	 0.534	 0.766	 1.61±0.06 1.60±0.07 1.59±0.05	 26.0±4.3 25.7±4.2 26.1±3.9	 67.0±11.8 66.0±11.4 65.7±10.2	 70.7±5.2 70.9±5.7 70.5±6.5	 ≥0.086
VDR rs2228570 AA (n=103) AG (n= 154) GG (n=48)	 33.8% 50.5% 15.7%	 0.585	 0.747	 1.60±0.06 1.60±0.07 1.60±0.05	 26.2±4.0 25.9±4.4 25.3±3.6	 66.7±11.2 66.4±11.7 65.3±10.2	 71.3±6.0 70.4±5.4 70.5±5.8	 ≥0.461
ID3 rs11574 CC (n=178) CT (n=111) TT (n=16)	 58.4% 36.4% 5.2%	 0.059	 0.971	 1.60±0.06 1.59±0.07 1.60±0.06	 25.8±4.2 26.1±4.2 25.7±3.0	 66.1±11.8 66.7±10.9 66.0±8.3	 70.4±5.6 70.7±5.3 75.5±7.6	 ≥0.002
CNTF rs1800169 AA (n=3) AG (n=77) GG (n=225)	 1.0% 25.2% 73.8%	 1.662	 0.436	 1.56±0.05 1.60±0.05 1.60±0.06	 26.7±3.4 25.9±4.4 25.9±4.1	 65.0±9.5 66.3±11.5 66.3±11.3	 70.5±1.9 70.8±5.9 70.8±5.7	 ≥0.444
ACE rs4341 CC (n=63) CG (n=142) GG (n=100)	 20.7% 46.5% 32.8%	 0.921	 0.631	 1.60±0.05 1.60±0.07 1.60±0.06	 25.8±4.4 25.9±4.2 26.0±3.9	 65.1±12.1 66.6±11.4 66.7±10.6	 70.8±5.0 71.1±6.3 70.2±5.1	 ≥0.157
CNTFR rs2070802 AA (n=216) AT (n=79) TT (n=10)	 70.8% 25.9% 3.3%	 0.686	 0.710	 1.60±0.06 1.60±0.06 1.60±0.05	 25.8±4.2 67.4±11.7 69.9±9.7	 65.8±11.2 67.4±11.7 69.9±9.7	 70.8±5.5 70.6±5.7 72.4±8.7	 ≥0.332

MTHFR rs1801131								
GG (n=27)	8.9%			1.61±0.06	25.6±3.6	66.8±11.4	70.5±5.7	
GT (n=133)	43.8%	0.224	0.894	1.60±0.05	26.2±4.5	67.1±12.1	71.0±5.8	≥0.333
TT (n=144)	47.3%			1.60±0.06	25.7±3.9	65.5±10.6	70.6±5.6	
ESR1 rs4870044								
CC (n=158)	52.0%			1.60±0.06	25.9±3.7	66.0±10.6	71.0±5.4	
CT (n=122)	40.1%	0.004	0.998	1.60±0.06	26.3±4.8	67.1±12.6	70.7±6.1	≥0.343
TT (n=24)	7.9%			1.61±0.05	25.0±3.2	64.1±9.04	69.7±5.5	
COL1A1 rs1800012								
AA (n=10)	3.3%			1.598±0.0569	23.1±2.6	59.2±7.6	71.2±5.5	
AC (n=89)	29.3%	0.008	0.996	1.602±0.054	26.5±4.6	68.1±12.6	71.2±5.2	≥0.038
CC (n=205)	67.4%			1.599±0.056	25.8±4.0	65.9±10.7	70.5±5.8	
ACTN3 rs1815739								
CC (n=103)	33.9%			1.592±0.0567	26.2±3.7	66.6±10.4	72.0±6.3	
CT (n=133)	43.8%	3.899	0.142	1.606±0.055	26.1±4.2	67.5±11.6	70.8±5.6	≥0.071
TT (n=68)	22.4%			1.596±0.055	25.5±4.3	64.8±11.3	69.9±5.2	
HIF1A rs11549465								
CC (n=241)	79.0%			1.600±0.056	25.8±4.3	66.0±11.5	70.6±5.8	
CT (n=62)	20.3%	0.869	0.648	1.599±0.057	26.1±3.3	66.9±9.4	71.7±5.0	≥0.047
TT (n=2)	0.7%			1.597±0.004	33.0±10.3	84.1±25.9	63.8±3.4	
MSTN rs1805086								
CC (n=0)	0.0%							
CT (n=10)	3.3%	0.085	0.959	1.618±0.0509	26.2±3.6	68.7±11.4	71.3±4.9	≥0.299
TT (n=295)	96.7%			1.599±0.056	25.9±4.2	66.2±11.3	70.7±5.7	
MTHFR rs1537516								
AA (n=4)	1.3%	0.154	0.926	1.568±0.031	26.2±4.9	64.9±13.9	68.1±6.1	
AG (n=56)	18.4%			1.607±0.058	25.3±3.9	65.4±10.2	70.6±6.3	≥0.071
GG (n=245)	80.3%			1.598±0.055	26.0±4.2	66.5±11.5	70.8±5.5	
TTN rs10497520								
CC (n=235)	77.1%			1.599±0.055	26.0±4.2	66.6±11.6	70.7±5.8	
CT (n=66)	21.6%	0.069	0.966	1.602±0.0586	25.7±4.0	65.9±10.2	70.9±5.5	≥0.167
TT (n=4)	1.3%			1.595±0.0465	22.2±3.5	56.4±7.3	67.8±2.5	

TRHR rs7832552 CC (n=137) CT (n=130) TT (n=37)	 45.0% 42.8% 12.2%	 0.510	 0.775	 1.600±0.0537 1.601±0.059 1.592±0.0517	 26.6±4.0 25.9±3.9 27.0±5.1	 65.4±10.6 66.6±11.3 68.7±13.5	 69.8±4.9 71.7±6.0 71.1±6.8	 ≥0.024
IGF1 rs35767 AA (n=4) AG (n=76) GG (n=225)	 1.3% 24.9% 73.8%	 0.740	 0.691	 1.62±0.044 1.60±0.056 1.60±0.056	 28.6±8.3 25.2±3.4 26.1±4.3	 74.9±19.7 64.8±9.4 66.7±11.7	 71.2±4.9 70.3±6.0 70.9±5.6	 ≥0.129

3．Evaluation of previously established definitions of sarcopenia for assessing neuromuscular phenotypes in elderly women

3.1 Introduction

Sarcopenia is an important predictor of adverse outcomes such as limited mobility, increased risk of falls, decreased quality of life (QoL), hospitalization and mortality, and contributes tens of millions to health care costs in the UK (McNamee et al., 2001; Cruz-Jentoft et al., 2010; Fahy, 2012). The prevalence of sarcopenia within the elderly population is attributable to an ageing- associated decline in both muscle mass and strength (Thom et al., 2005; Chien et al., 2008; Toran et al., 2012).

The decline of muscle mass becomes obvious after the 5th decade of life with 1-2% declines reported annually (Marcell, 2003; Buford et al., 2010) and can amount to a 30-40% lower skeletal muscle mass in 80-year-old people than in young adults (Frontera et al., 2000). For a long time, sarcopenia ('poorness of flesh') was defined as the presence of low muscle mass (Rosenberg, 1997). It has however, been found that muscle strength or power correlates more with performance of daily life activities in old age than muscle mass (Skelton et al., 1994; Bean et al., 2003; Maden-Wilkinson et al., 2015). Indeed, muscle strength may well decrease more so than the related decrease in muscle mass (Goodpaster et al., 2006; McPhee et al., 2018), as such, some definitions include handgrip strength (Cruz-Jentoft et al., 2010).

To date however, there is no consensus in the operational definition of sarcopenia. Due to this inconsistency in the use of definitions, prevalence of sarcopenia varies from 3.3% to 20% in the same population when studied with different definitions (Dupuy et al., 2015). This heterogeneity in prevalence can be attributed to the different cut-offs, diagnostic method, and the characteristics of the elderly population and reference population (Pagotto and Silveira, 2014).

Any definition of sarcopenia should be able to discriminate meaningful neuromuscular phenotypes between sarcopenic and non-sarcopenic elderly groups, particularly those phenotypes that have been associated with impairments in activities of daily living or prevalence of falls. Within the present thesis, a definition of sarcopenia is required that will allow for distinction of genotypes between sarcopenic and non-sarcopenic elderly women. Sarcopenia has previously been

defined based on being able to discriminate group differences in isometric maximum voluntary contraction strength knee extension (MVC_{KE}) (Dupuy et al., 2015) and to predict falls incidence in the elderly (Scott et al., 2014b; Bischoff-Ferrari et al., 2015). Although knee extension strength is a determinant of Activities of Daily Livings (ADLs), the presentation of sarcopenia also needs to consider the size of lower limb muscle (as a predictor of whole limb power output) (Macaluso and De Vito, 2004), balance performance (as a predictor of falls and injuries) (Bogle Thorbahn and Newton, 1996) and potentially also upper limb muscle size and strength (Yeung et al., 2017).

Appendicular muscle cross sectional area (CSA) is often reported as lower in the elderly (Overend et al., 1992), however, appendicular lean body mass or skeletal muscle mass are often used to define sarcopenia (Cruz-Jentoft et al., 2010). These measures of lean mass, presented relative to height as appendicular lean mass index (ALMI) and skeletal muscle index (SMI), allow for a description of the changes with age to the muscular system more globally than single CSA measures. Although, Dual-energy X-ray absorptiometry (DEXA) is commonly used for measuring ALMI, the use of Bioelectrical Impedance Analysis (BIA) has been suggested as a valid and low-cost alternative for measuring Skeletal Muscle Index (SMI) (Wang et al., 2016). The present chapter, aimed to evaluate three previous, and a fourth novel approach of defining sarcopenia, to study differences in prevalence and neuromuscular function. With relevance to this thesis, the overarching aim was to identify a definition of sarcopenia that can be subsequently be used for identifying phenotype and genotype associations in the present Caucasian elderly females. Of the three previously established definitions all were based on skeletal muscle mass (SMM) index, the first, as SMM/height^2 (SMI_A) the second as SMM/body mass (%SMM); and the third was based on SMI and Handgrip strength (HGS) cut-offs as suggested by European Working Group on Sarcopenia in Older People (EWGSOP). The fourth approach was based on a composite Z-score derived from SMI and HGS, and defining sarcopenia as individuals in the least second quintile of the composite Z-score.

3.2 Materials and methods

3.2.1 Study design

All participants attended for testing on a single session at the MMU Cheshire Campus, Crewe. The testing session was conducted in the following order: anthropometry, handgrip strength, isometric maximum voluntary contraction knee extension (MVC_{KE}) and elbow flexion (MVC_{EF}), ultrasound of the biceps brachii and vastus lateralis muscles and a standing balance test.

3.2.2 Methods

Detailed descriptions of participant recruitment, assessment of skeletal muscle size measures and muscle strength and balance performance; and DNA sample collection and genotyping are included in Chapter 2, thus a brief description of these methods are detailed below.

3.2.2.1 Participants

Three hundred and seven active elderly Caucasian females (age 70.7 (5.7) years, mass 66.3 (11.3) kg, height 1.60 (0.06) m; (mean (SD)) volunteered to participate in this study from surrounding areas of MMU. Participants met the inclusion criteria (described in Chapter 2) and provided written informed consent prior to testing.

3.2.2.2 Skeletal Muscle Mass Index

Skeletal muscle mass was calculated using measures of whole-body impedance and resistance to an applied current quantified using Bioelectrical Impedance Analysis (BIA) (Model 1500; Bodystat, Isle of Man, UK). Skeletal muscle mass was estimated using a previously established formula (Janssen et al., 2000a) as:

Equation 1: Skeletal Muscle Mass (SMM) = $[(Ht^2/Rx0.401) + (sex \times 3.825) + (age \times -0.071)] + 5.102$

Where Ht is height in cm, R is resistance from BIA and age in years. For sex, a male is scored as 1 and female as 0.

Skeletal Muscle Index (SMI) was calculated as skeletal muscle mass (SMM) divided by the height as;

Equation 2: SMI= SMM/Ht^2 where SMM is in kg and height is in m.

3.2.2.3 Muscle Strength phenotypes

Skeletal muscle strengths were measured for handgrip, elbow flexor and knee extensor muscles. HGS was measured by Handgrip strength dynamometer and MVC_{KE} and MVC_{EF} with a customized built dynamometer (detailed in Chapter 2).

3.2.2.4 Muscle size phenotypes

The cross-sectional area of the vastus lateralis (VL_{ACSA}) and thickness of the biceps muscle were measured with B-mode ultrasound as described in the Chapter 2 at 50% femur length, and 60% humerus length, respectively.

3.2.2.5 One leg Standing Balance Test

One Leg Standing balance test (OLST) was performed as described in Chapter 2. Briefly, participants were instructed to stand barefoot and to flex either their left or right knee to 90^0 to ensure the foot was not in contact with the floor, and balance on one leg as long as possible. The time was recorded with a stop-watch with 30 seconds as the maximum duration of the test (Bohannon et al., 1984). If they did not reach the 30 seconds, they were asked to repeat the test 3 times and the maximum of the three was recorded for the study.

3.2.2.6 Assessment of sarcopenia

Sarcopenia was assessed in the participants with 4 different definitions. For all definitions, SMI was calculated using BIA as described in Chapter 2. The first definition, SMI_A previously used by (Chien et al., 2008) was calculated as SMM/$height^2$. Participants were defined as sarcopenic if SMI_A was <6.42 kg/m^2. The

second definition, %SMM was calculated as SMM/body mass*100. Participants were defined as sarcopenic if SMM<22.1% (Janssen et al., 2000b). Definition 3 used the measures of low SMI and low HGS as suggested by the EWGSOP; for which individuals with SMI <6.76 kg/m^2 and HGS<20kg were considered as sarcopenic (Cruz-Jentoft et al., 2010). Definition 4, Z-score, uses the composite Z-score calculated by summing the Z-score of SMI and HGS consistent with the EWGSOP definition of sarcopenia. Unlike the EWGSOP definition, which defines specific cut-off thresholds for sarcopenia, sarcopenia was defined as individuals in the lowest second quintile of the composite Z-score and non-sarcopenia (normal) as the individuals at the highest second quintile.

3.2.3 Statistical Analysis

Statistical analyses were carried out using SPSS Version 23.0 for Windows (IBM Corp., Armonk, NY, USA). To determine parametricity, Kolmogorov-Smirnov test was used for sarcopenia and non-sarcopenia groups and Levene's test for homogeneity of variance. If parametric assumptions were met with sarcopenia definitions, an independent sample t-test was used for the comparison. In instance, when parametric assumptions were violated, a Mann-Whitney test was utilized and Monte-Carlo p-value was reported. It should be noted that some participants did not complete all tests due to faults during data capture or inaccessibility for the specific tests. Data are presented as Mean±SD. Alpha level $p< 0.05$ was considered as statistically significant.

3.3 Results

3.3.1 Prevalence of sarcopenia

The prevalence of sarcopenia according to each definition was: SMI_A 60.6%, %SMM 14.7%, EWGSOP 1.3% and Z-score 40% (Table 3.1).

3.3.2 Comparison of Neuromuscular outcomes with sarcopenia definitions

Based on sarcopenia definitions, Z-score was able to differentiate 5 neuromuscular outcome measures, with decreasing order: 4 for $SMI_{A,}$ and 3 for % SMM and EWGSOP. Sarcopenia defined by Z- score was able to differentiate all the neuromuscular outcome measures between the two groups. When classification was based on composite Z-score, significant differences between sarcopenia and non-sarcopenia groups for all the neuro-muscular outcome measures; $VL_{ACSA,}$ biceps thickness, MVC_{KE}, MVC_{EF} and OLST were observed (Table 3.1). With this definition, VL_{ACSA} is significantly lower by 17.5 % ($p < 0.001$); MVC_{KE} by 22.8% ($p < 0.001$); biceps thickness by 9.4% ($p < 0.001$) and OLST by 19.9 % ($p < 0.001$) compared to non-sarcopenia.

Table 3.1Population and sarcopenia group characteristics with different sarcopenia definition

		SMI_A		**%SMM**		**EWGSOP**		**Z-score**	
Sarcopenia Prevalence (%)	n=307	n=18660.6%	n=121	n=45 14.7%	n=262	n=4 1.3%	n=303	n=123 40%	n=123
	General characteristics	S	NS	S	NS	S	NS	S	NS
Age (years)	70.7±5.7	71.0±5.3	70.3±6.3	71.6±5.6	70.6±5.7	76.8±7.0*	70.6±5.7	72.5±5.9**	68.8±4.8
Body Mass (Kg)	66.3±11.3	63.4±9.3**	70.9±12.5	77.4±13.1**	64.4±9.8	68.5±10.9	66.3±11.3	62.6±10.0**	69.9±12.0
BMI (Kg/m^2)	25.9±4.2	24.6±3.2**	27.9±4.7	30.2±5.5**	25.2±3.4	28.2±2.9	25.9±4.2	24.9±3.7**	27.1±4.7
HGS (Kg)	29.9±5.0	29.1±4.4**	31.1±5.6	28.4±4.9*	30.2±5.0	17.8±2.2**	30.0±4.9	26.1±3.7**	33.8±4.1
SMI (kg/m^2)	6.56±0.81	6.05±0.51**	7.33±0.53	6.04±0.92**	6.64±0.75	6.29±0.18	6.56±0.81	5.95±0.59**	7.17±0.67
VL_{ACSA} (cm^2)	16.3±3.4	15.5±3.1**	17.6±3.3	16.84±3.71	16.3±3.3	15.9±0.7	16.3±3.3	14.7±3.0**	17.9±3.2
Bicep thickness (cm)	1.76±0.34	1.72±0.34*	1.82±0.31	1.819±0.362	1.75±0.33	1.51±0.09	1.76±0.33	1.67±0.35**	1.85±0.34
MVC_{EF} (N)	117±29	111±25**	127±32	107±21*	119±29	68±11**	118±28	105±24**	131±31
MVC_{KE} (N)	1649±550	1590±526*	1738±576	1471±558*	1679±545	846±321*	1657±547	1433±463**	1856±558
OLST(s)	23.9±9.7	23.7±9.8	24.2±9.5	18.5±11.5**	24.8±9.0	9.2±13.8*	24.1±9.5	21.2±11.1**	26.5±7.4

* and ** denotes the group is significantly different from non-sarcopenia group at $p<0.05$ and $p<0.001$ respectively. VL_{ACSA}-Vastus Lateralis Anatomical Cross Sectional Area, MVC_{KE}-Isometric Maximum Voluntary Contraction for Knee Extension, MVC_{EF}- Isometric Maximum Voluntary Contraction for Elbow Flexion, OLST-One Leg Standing Test, S- Sarcopenia group, NS- Non sarcopenia group

3.4 Discussion

The current chapter identified that different definitions of sarcopenia result in a widely different prevalence of sarcopenia, ranging from 1.3% to 60.6% in the present population of women aged >60 years. Of the four-sarcopenia definitions studied, the novel Z-score approach could distinguish the most meaningful neuromuscular outcome measures between the sarcopenic and non-sarcopenic group.

In the present study, the prevalence of sarcopenia ranged from 1.3% to 60.6% depending on sarcopenia criteria used. This heterogeneity prevalence is consistent with previous study, (Bijlsma et al., 2013), and confirms that the criteria, definition and threshold will determine prevalence with greater variety shown when comparing different study populations. Below, each definition is discussed, based on the prevalence, and ability to show distinct differences in neuromuscular phenotypes between the sarcopenic and non-sarcopenic populations.

The SMI definition of sarcopenia has previously resulted in prevalence ranging from 2.8-42% (Janssen, 2006; Chien et al., 2008; Tichet et al., 2008). At the extremes, this lower prevalence in sarcopenia is due to threshold levels being set too conservatively (SMI 6.2kg/m^2 previously) (Tichet et al., 2008), compared to 6.42kg/m^2 (SMI_A) in present study definition based on previous (Chien et al., 2008). Although the present study adopted a less stringent level of SMI, this approach was successful in being able to distinguish sarcopenic and non-sarcopenic groups in 4/5 neuromuscular outcome measures.

The second definition of sarcopenia using % SMM resulted in a prevalence of 14.7% in present elderly female population. This prevalence falls within a range similar to reported previously using the same approach. For example, the large population NHANESS III study, which estimated that 10% of US women above 60 years are sarcopenic (Janssen et al., 2002) and 23.6% prevalence in elderly French women (Tichet et al., 2008). In the present study, % SMM definition was able to distinguish

group differences in 3/5 neuromuscular outcome measures between sarcopenic and non-sarcopenic groups.

The prevalence of sarcopenia (1.3%) in elderly female in the present study using the EWGSOP definition was consistent with studies reported in different populations. For instance, the prevalence of sarcopenia was found to be 2.5% in Taiwanese women (Wu et al., 2014) 4.5% in German females (Kemmler et al., 2015), 5% in Australian males and females (Scott et al., 2014a) and 7.4% in Japanese female (Yoshida et al., 2014) using the EWGSOP definition. While others have reported, higher prevalence with EWGSOP compared to the present study. It should be noted that higher prevalence has been reported ranging between 22-48% in some of the studies conducted across Europe, Asia and South America (Arango-Lopera et al., 2012; Velázquez Alva et al., 2013; Volpato et al., 2013; Yamada et al., 2013; ter Borg et al., 2016). These higher prevalances tend to be in populations incorporating the oldest old, their own young reference group (rather than cut offs) and those living non-independently. The EWGSOP definition used in the present chapter was able to distinguish 3/5 neuromuscular phenotypes, and although partially successful as a discriminating factor, was not tenable as a method within the present population as subsequent genotype analysis would be impossible with such a small population of sarcopenic participants (n = 4).

In the present chapter, the HGS and SMI Z-score approach was developed based on the fact that a) differences in SMI alone do not reflect the accelerated loss of muscle strength (over size) in the elderly, and 2) it would allow for the clear distinction of sarcopenic from non-sarcopenic, into quintiles, allowing for a “stressed phenotype” approach to subsequent genotype analysis. A similar quintile approach was adopted to define cut-offs for lower muscle mass/body fat (Davison et al., 2002; Batsis et al., 2013), but to the author’s knowledge this is the first description of sarcopenia using a composite Z-score consistent with the EWGSOP. In the present chapter, Z-score approach is considered meaningful based on the ability to discriminate the neuromuscular outcome measures between the sarcopenia and non-sarcopenia groups. Furthermore, any subsequent genotype analysis would be possible given the numbers of participants categorised as sarcopenic and non-sarcopenic.

Although the EWGSOP is an accepted threshold for identifying sarcopenia population based on cut-offs for HGS and SMI, it is however, too conservative approach for the present elderly females, as both thresholds have to be met to define an individual as sarcopenic. As the aim of the thesis is to be able to discriminate sarcopenic from non-sarcopenic based on neuromuscular impairments, and genotype, the Z-score approach using SMI and HGS, was able to: a) identify a wider population as sarcopenic, and b) show group differences in all neuromuscular measures between those identified as sarcopenic and non-sarcopenic.

Having considered the definitions above, the subsequent section considers the neuromuscular differences between sarcopenic and non-sarcopenic individuals as identified using the quintiles Z score approach. Elderly women described as sarcopenic using the Z-score approach, were weaker (MVC_{KE} and MVC_{EF}), had lower muscle mass (VL_{ACSA} and biceps brachii thickness) and worse single leg balance (OLST). The specific physiological mechanisms as to why the two groups are different in the present study are consistent with what we understand about the mechanisms of ageing i.e. changes in hormonal status (Curtis et al., 2015), physical activity (Troiano et al., 2008) neurological factors, or genetic factors (Garatachea and Lucía, 2013). Based on their specific decrements in strength, balance and muscle mass, the present elderly sarcopenic women (despite not being considered saropenic based on the EWGSOP at present) are more likely to be at risk of higher falls (Landi et al., 2012b), have higher incidence of frailty (Clegg et al., 2013), be less likely to be able reach functional thresholds for activities of daily living (Choi et al., 2013; Shiozu et al., 2015), and are at a high risk of mortality (Norman et al., 2011). These sarcopenic women, as classified here, although healthy at the moment could be meaningfully categorised as sarcopenic for subsequent phenotype and genetic association studies in this thesis, based on the neuromuscular differences identified between the groups.

3.5 Conclusion

The present chapter concluded that sarcopenia prevalence varied with the use of different definitions and definition based on composite Z-score can differentiate

more features of sarcopenia individuals. It is the only definition that differentiates all the neuromuscular phenotypes investigated in the present study that are important for the maintenance of independence in the old age between the two groups.

4 . Genetic associations with sarcopenia in elderly Caucasian females

4.1 Introduction

As observed in Chapter 3, there is a variance in the prevalence of sarcopenia within elderly women, such that depending on the definition of sarcopenia used, between 1-61% of elderly women would be considered sarcopenic. The fact that some elderly women do not show symptoms of sarcopenia, whilst others of the same age do, suggests that some individuals are susceptible to sarcopenia at an earlier age than others. The severity of sarcopenia is likely due to a combination of factors including physical activity, diet and sedentary behaviour (Carmelli and Reed, 2000; Gerdhem et al., 2005; Bruce, 2017; de Camargo Smolarek et al., 2018). The heritability of muscle mass and muscle strength as a contributor to the maintenance of muscle mass during ageing cannot be neglected (Zhai et al., 2004). The heritability of muscle mass and muscle strength have been reported, as high as 66% and 82%, respectively, in large population studies of healthy adults (Arden and Spector, 1997; Thomis et al., 1998; Abney et al., 2001). Genome Wide Association Studies (GWAS) and case-control association studies have identified associations between several gene variants and muscle mass and muscle strength phenotypes ranging from young to elderly populations (Tan et al., 2012; Garatachea and Lucia, 2013). Taken together, the high heritability of muscle mass and muscle strength, and the findings from GWAS and association studies, it is possible that individuals carrying a greater proportion of favourable gene variants possess a greater ability to preserve muscle function despite their old age; hence can maintain independence until later life and are less susceptible to sarcopenia in their early stage of ageing.

To date, there has been a considerable number of studies describing the association of single gene variants with muscle mass and muscle strength (Table 1.5). There are however, only a limited number that have investigated the genetic associations with sarcopenia, in total these include: *VDR* FokI (Roth et al., 2004; Walsh et al., 2016), *IL6* (Tasar, 2018) and *ACTN3* R577X (Cho et al., 2017) polymorphisms. Considering there are now more than 200 gene variants suggested to be associated with physical performance and health-related skeletal muscle phenotypes (Bray et al., 2009), it is reasonable to assume that SNPs previously associated with skeletal muscle phenotypes, could also be associated with sarcopenia.

The present chapter therefore, aimed to investigate the association of 24 SNPs selected initially (process described in Chapter 1) and sarcopenia, using the definition obtained in chapter 3, in elderly females.

4.2 Methods

4.2.1 Participants

60-91 years old Caucasian females (n= 307, 70.7±5.7 years, 66.3±11.3 kg, 1.60±0.06 m, (Mean ± SD)) were recruited for the study. All the participants provided written informed consent and met the inclusion criteria as mentioned in the Chapter 2 prior to taking part in this study.

All the procedures utilised in this Chapter have been described in detail in Chapter 2, thus only a brief description is provided here.

4.2.2 Skeletal Muscle Mass Index

Skeletal muscle mass was calculated using measures of whole-body impedance and resistance to an applied current quantified using Bioelectrical Impedance Analysis (BIA) (Model 1500; Bodystat, Isle of Man, UK). Skeletal muscle mass was estimated using a previously established formula (Janssen et al., 2000a) as:

Equation 1: Skeletal Muscle Mass (SMM) = $[(Ht^2/Rx0.401) + (sex \times 3.825) + (age \times -0.071)]+ 5.102$

Where Ht is height in cm, R is resistance from BIA and age in years. For sex, a male is scored as 1 and female as 0.

Skeletal Muscle Index (SMI) was calculated as skeletal muscle mass (SMM) divided by the height as;

Equation 2: $SMI= SMM/Ht^2$ where SMM is in kg and height is in m.

4.2.3 Handgrip Strength

Handgrip strength (HGS) was measured using a digital load cell handgrip dynamometer. The highest grip strength of three maximal efforts was recorded for the study. The left and right hand were alternated, with 1 minute break between trials.

4.2.4 DNA sample collection and genotyping

Blood (189 samples (~ 62%)) and saliva (116 samples (~38%)) were obtained using standard protocols. Blood was drawn from a superficial forearm vein and then stored at -20 °C until further processing. For the saliva sample, saliva was collected in Oragene.DNA OG-500 collection tubes (DNA Genotek Inc., Ontario, Canada) following the company's protocol and stored at room temperature until DNA extraction. DNA was extracted by the QIAcube method; subsequent to which genotyping of 24 SNPs described in the Chapter 1, Table 1.5 was performed.

4.2.5 Assessment of Sarcopenia

Sarcopenia was defined with the Z-score definition from Chapter 3, which is based on the composite Z-score. In short, a composite Z-score was calculated by the summation of Z-score of SMI and Z-score of HGS and the individuals in the lowest second quintile of the composite Z-score were defined as the sarcopenia group in the present study.

4.2.6 Statistical analysis

The data were tested for parametricity before completing any statistical analyses. Kolmogorov-Smirnov was used to assess the normal distribution of the population and Levene's test for the homogeneity of variance of HGS and SMI. The frequency distribution of each SNP was assessed for compliance with Hardy-Weinberg equilibrium (HWE) using chi-square tests. Binary logistic regression was performed to investigate the association of sarcopenia and the SNPs studied, with age used as covariate. In instances where the number of homozygous participants was low, this homozygous group was combined with the heterozygous group and a two-group analysis was performed. When there was a tendency of association ($0.05<p<0.15$)

(Fischer et al., 2004; Danilovic et al., 2007), as was the case with *ACE* rs4341, the homozygous groups were combined with the heterozygous group in a recessive and dominant model and then the analyses were re-run. Odds Ratios for the risk allele for sarcopenia were estimated for each SNP. Benjamini-Hochberg correction was performed to reduce the chance of type I error for multiple testing (Benjamini and Hochberg, 1995). All the tests were performed in SPSS Version 23.0. $p<0.05$ was considered significant.

4.3 Results

All genotype data were compliant with Hardy Weinberg equilibrium as shown in Table 2.1 ($p>0.05$). The genotype frequency distribution for each SNP between sarcopenia and non-sarcopenia group is presented in Table 4.1. The two SNPs, *ACE* rs4341 and *HIF1A* rs11549465 were found to be associated with sarcopenia (*ACE* rs4341 before multiple correction). As there were few participants (n=2) with the TT genotype in *HIF1A* rs11549465, they were combined with the heterozygous group and analysis was performed. Following binary logistic regression, using age as a covariate, *HIF1A* rs11549465 CC individuals had 2.5 times higher risk of being sacropenic than T-allele carriers (OR= 2.45, CI: 95% (1.26-4.78), p=0.008). Additionally, *ACE* rs4341 CC homozygotes were had almost a 2 times greater risk of being sarcopenic than G-allele carriers (OR= 1.95, CI: 95% (1.002-3.80), p= 0.049) (1.26-4.78), p=0.008). Besides the above two SNPs, no other SNPs showed significant association with sarcopenia (Table 4.1).

Table 4.1 Genotype frequency distribution between sarcopenia and non-sarcopenia groups

SNPs	Sarcopenia (N=123) n (%)	Non-sarcopenia (N=123) n (%)	Odds ratio	CI 95%	p-value
IL6 rs1800795					
CC	27 (21.9%)	25 (20.3%)	1.10	0.63-1.94	0.731
CG	53 (43.1%)	60 (48.8%)	CC+CG Vs		
GG	43 (35.0%)	38 (30.9%)	GG		
FT0 rs9939609					
AA	27 (22.0%)	20 (16.2%)	0.66	0.34-1.28	0.218
AT	56 (45.5%)	59 (48.0%)	AA Vs		
TT	40 (32.5%)	44 (35.8%)	AT+TT		

MTHFR rs17421511 AA AG GG	1 (0.8%) 28 (22.8%) 94 (76.4%)	4 (3.3%) 34 (27.6%) 85 (69.1%)	1.53 AA+AG Vs GG	0.84-2.79	0.168
ACVR1B rs10783485 GG GT TT	54 (43.9%) 59 (48.0%) 10 (8.1%)	52 (42.3%) 58 (47.2%) 13 (10.6%)	0.82 GG Vs GT+TT	0.48-1.40	0.460
NOS3 rs1799983 GG GT TT	51 (41.5%) 56 (45.5%) 16 (13.0%)	44 (35.8%) 62 (50.4%) 17 (13.8%)	0.69 GG Vs GT+TT	0.40-1.12	0.181
ACVR1B rs2854464 AA AG GG	64 (52.0%) 52 (42.3%) 7 (5.7%)	62 (50.4%) 50 (40.7%) 11 (8.9%)	0.90 AA VS AG+GG	0.53-1.52	0.683
PTK2 rs7460 AA AT TT	33 (26.8%) 61 (49.6%) 29 (23.6%)	26 (21.1%) 61 (49.6%) 36 (29.3%)	0.78 AA+AT Vs TT	0.43-1.41	0.401
ESR1 rs1999805 AA AG GG	38 (30.9%) 64 (52.0%) 21 (17.1%)	44 (35.8%) 55 (44.7%) 24 (19.5%)	1.279 AA Vs AG+GG	0.73-2.24	0.391
PTK2 rs7843014 AA AC CC	41 (33.3%) 53 (43.1%) 28 (22.8%)	43 (35.0%) 62 (50.4%) 18 (14.6%)	1.81 AA+AC Vs CC	0.90-3.63	0.097
VDR rs2228570 AA AG GG	45 (36.6%) 59 (48.0%) 19 (15.4%)	38 (30.9%) 68 (55.3%) 17 (13.8%)	0.85 AA Vs AG+GG	0.49-1.49	0.567
ID3 rs11574 CC CT TT	68 (55.3%) 45 (36.6%) 10 (8.1%)	75 (61.0%) 45 (36.6%) 3 (2.4%)	2.38 CC+CT Vs TT	0.58-9.85	0.230
CNTF rs1800169 AA AG GG	1 (0.8%) 30 (24.4%) 92 (74.8%)	2 (1.6%) 29 (23.6%) 92 (74.8%)	0.92 AA+AG Vs GG	0.50-1.69	0.793
ACE rs4341 CC CG GG	31 (25.2%) 59 (48.0%) 33 (26.8%)	19 (15.5%) 56 (45.5%) 48 (39.0%)	1.95 CC Vs GG+GC	1.00-3.80	0.049

CNTFR rs2070802 AA AT TT	92 (74.8%) 28 (22.8%) 3 (2.4%)	81 (65.8%) 37 (30.1%) 5 (4.1%)	0.61 AA Vs AT+TT	0.34-1.09	0.097
MTHFR rs1801131 GG GT TT	11 (8.9%) 53 (43.1%) 58 (47.1%)	12 (9.8%) 49 (39.8%) 62 (50.4%)	1.15 GG Vs GT+TT	0.46-2.87	0.230
ESR1 rs4870044 CC CT TT	60 (48.8%) 56 (45.5%) 7 (5.7%)	64 (52.0%) 49 (39.8%) 9 (7.3%)	1.17 CC Vs CT+TT	0.69-1.99	0.566
COL1A 1rs1800012 AA AC CC	2 (1.6%) 30 (24.4%) 90 (73.1%)	6 (4.9%) 39 (31.7%) 78 (63.4%)	1.76 AA+AC Vs CC	0.99-2.12	0.056
ACTN3 rs1815739 CC CT TT	43 (35.0%) 54 (43.9%) 25 (20.3%)	41 (33.3%) 53 (43.1%) 29 (23.6%)	1.56 TT VS CT+CC	0.81-3.01	0.184
HIF1A rs11549465 CC CT TT	104 (85.5%) 19 (15.5) 0 (0.0%)	89 (72.4%) 32 (26.0%) 2 (1.6%)	2.45 CC Vs CT+TT	1.26-4.78	0.008
MSTN rs1805086 CC CT TT	0 (0.0%) 1 (0.8%) 122 (99.2%)	0 (0.0%) 4 (3.3%) 119 (96.7%)	4.13 CC Vs CT	0.451-37.80	0.209
MTHFR rs1537516 AA AG GG	0 (0.0%) 26 (21.1%) 97 (78.9%)	4 (3.3%) 17 (13.8%) 102 (82.9%)	0.80 AA+AG Vs GG	0.41-1.58	0.526
TTN rs10497520 CC CT TT	95 (77.3%) 26 (21.1%) 2 (1.6%)	93 (75.6%) 28 (22.8%) 2 (1.6%)	0.91 CC+CT Vs TT	0.49-1.69	0.755
TRHR rs7832552 CC CT TT	53 (43.1%) 56 (45.5%) 13 (10.6%)	60 (48.8%) 45 (36.6%) 18 (14.6%)	0.65 CC+CT Vs TT	0.28-1.47	0.30
IGF1 rs35767 AA AG GG	1 (0.8%) 28 (22.8%) 94 (76.4%)	2 (1.6%) 32 (26.0%) 89 (72.4%)	1.20 AA+AG Vs GG	0.65-2.20	0.567

n represents number of participants in the specific genotype groups and p value denotes the association between the SNPs and sarcopenia and grey genotype groups denotes reference group in regression.

4.4 Discussion

The present chapter aimed to investigate the genetic association with sarcopenia in an elderly Caucasian female population and identified *HIF1A* rs11549465 and *ACE* rs4341 polymorphisms as being associated with sarcopenia. The present data showed that *HIF1A* rs11549465 CC homozygotes had a 2.5-fold higher risk of sarcopenia compared to T-allele carriers. For *ACE* rs4341, CC homozygotes had a 2-folds higher risk of being in the sarcopenia group compared to G–allele carriers. Genotype frequencies for polymorphisms are presented in the current chapter (Table 4.1).

In the present elderly population, *HIF1A* rs11549465 CC genotype group was observed as the genotype risk group for sarcopenia. The reason for the association of the nuclear transcription factor HIF1A protein could be explained by its possible role in skeletal muscle physiology. HIF1 protein is stable in hypoxia, mediates several biological processes such as apoptosis, cell proliferation and differentiation, and controls the genes involved in those processes (Epstein et al., 2001; Hashimoto and Shibasaki, 2015). A higher transactivation capacity of *HIF1A* with the TT variant has also previously been reported (Tanimoto et al., 2003), so it is possible that individuals with TT genotypes could have balanced mechanisms of muscle cell apoptosis, and enhance the muscle cell proliferation and differentiation, which ultimately could result in higher muscle mass and muscle strength in the non-sarcopenia group. This is supported by previous studies that have associated TT homozygotes with favourable muscle phenotypes and muscle function in sporting performance. For instance, with regards to muscle strength phenotypes, TT homozygotes were observed to be significantly overrepresented in weightlifters and wrestlers (Gabbasov et al., 2013) and power-oriented athletes (Cięszczyk et al., 2011; Drozdovska et al., 2013b). This suggests that individuals requiring more muscle mass and muscle strength in such sports would have some beneficial effects

of the TT variant of *HIF1A* rs11549465. Sarcopenia described in this thesis, as elderly women with lower composite HGS and SMI Z-score, also have smaller muscle mass and weaker muscle strength (as observed in chapter 3); hence, the identification of *HIF1A* rs11549465 CC genotype as a risk factor for sarcopenia in the present chapter.

In the present chapter, the *ACE* rs4341 CC genotype group has been found to be at higher risk of being sarcopenic. The biological role of the ACE enzyme and the change in the ACE activity with *ACE* rs4341 polymorphism may explain the observed association. This polymorphism has been reported to be in linkage disequilibrium with *ACE* I/D polymorphism, with the C allele behaving as I allele. Biologically, the *ACE* D allele increases the conversion of Angiotensin I to Angiotensin II and is widely expressed in skeletal muscle (Reneland and Lithell, 1994). Angiotensin II helps in modulating skeletal muscle hypertrophy in response to mechanical loading (Gordon et al., 2001) likely by AT1 receptors. *ACE* DD has been previously associated with higher muscle mass/size and overrepresentation in sports demanding higher skeletal muscle strength. For instance, DD genotype was associated with larger quadriceps muscle volume in both men and women pre-training (Charbonneau et al., 2008), higher isometric and isokinetic strength in knee extensors pre-training (Williams et al., 2005), and significant gains in knee extensor strength in post-training (Folland et al., 2000; Giaccaglia et al., 2008; Pereira et al., 2013). Similarly, studies have also evidenced the higher representation of D allele in power-oriented athletes (Woods et al., 2001; Tsianos et al., 2004; Costa et al., 2009b). Therefore, it is possible that D allele is positively associated with muscle mass and muscle strength, and hence could act as the protective allele whereas; I may act as the risk allele for sarcopenia.

It should be noted that the present chapter utilises a Z-score based sarcopenia definition for studying the possible associations with the SNPs. Using the other sarcopenia definitions and comparing the results with same set of SNPs might yield similar or different results. Future studies should therefore, focus on using the several established sarcopenia definitions; compare, and associate the similar set of SNPs with all the definitions. The use of the Z-score (and quintiles approach) allowed

sufficient participants to be defined as sarcopenic, for subsequent inclusion within the genetic analysis. The previous definitions of sarcopenia similarly took this approach as discussed in the previous chapter.

Within this chapter, it was found that only 2 of the investigated 24 SNPs were associated with sarcopenia, despite hypothesising that more SNPs would be associated with sarcopenia before the investigation. Since the present study used a Z-score approach and classified participants between sarcopenia and non-sarcopenia based on cumulative Z-score of HGS and SMI, it is possible that the recruitment of active participants might not be able to discriminate accurately between the sarcopenic and non-sarcopenic groups according to genotype, which may explain the lack of associations with many of the SNPs. Another possible reason could be due to insufficient power. Additionally, the skeletal muscle phenotypes, muscle mass and muscle strength, are polygenic in nature (Hughes et al., 2011), hence the influence of a single SNP for sarcopenia might be less in the present study. A common shortcoming of sarcopenia research is the use of "healthy" older participants, so that although only a small number of SNPs were associated with sarcopenia, the results are presented in the knowledge of the limitations within the heterogeneity of the participants recruited here and in other sarcopenia studies. Further research should consider either stressing the sarcopenic phenotype by presenting data from non-independently living elderly participants, or associating SNPs with more distinct neuromuscular phenotypes that show greater impairments through ageing, such as quadriceps muscle size and strength.

4.5 Conclusion

The present chapter identified the novel associations of *HIF1A* rs11549465 CC and *ACE* rs4341 II genotypes as the risk genotype group for sarcopenia in an elderly female population. Identification of gene variants associated with sarcopenia might help in screening the population prone to sarcopenia in old age.

5 . Influence of genetic polymorphism in skeletal muscle phenotypes in elderly Caucasian females

5.1 Introduction

Sarcopenia is a complex process and is associated with the decline in skeletal muscle function (Cruz-Jentoft et al., 2010). Twin studies have suggested the high contribution of genetic factors on inter-individual variation in muscle mass and muscle strength (Thomis et al., 1998; Abney et al., 2001 ; Silventoinen et al., 2008), therefore there is the possibility of associations of Single Nucleotide Polymorphisms (SNPs) with muscle mass and strength in the elderly. Chapter 4 identified the association of two SNPs, *ACE* rs4341 and *HIF1A* rs11549465, with sarcopenia in the present elderly females; however, the preceding chapter (Chapter 3) has identified that sarcopenia can affect as few as 1.3%–60.6% of the elderly population depending on the definition. Although sarcopenia is a meaningful clinical definition, the difference in skeletal muscle phenotypes (muscle mass/muscle strength) between the two groups are relevant to quality of life (QoL) and Activities of Daily Livings (ADLs) (Rizzoli et al., 2013; Beaudart et al., 2015a; Yoshimura et al., 2017). Regardless of whether sarcopenic levels are reached, lower muscle mass has been linked with functional impairment and physical disability in older people (Janssen et al., 2002). Similarly, lower knee strength is linked with higher falls and injuries (Takazawa et al., 2003; Chung-Hoon et al., 2016) and lower handgrip strength is associated with impaired mobility, functional decline and higher levels of mortality (Bohannon, 2015; Stessman et al., 2017; McGrath et al., 2018).

Inter-individual variability exists between muscle size and muscle strength; up to 18% (Wakahara et al., 2010) and 20% (Stebbings et al., 2014) population variability was reported for appendicular lean muscle size and vastus lateralis muscle volume respectively, and up to 16% coefficient of variation (CV) for specific force (Erskine et al., 2009; Stebbings et al., 2014) in younger adults. Within the elderly, this variance implies that those at the weaker or lower end of the distribution are likely to experience a loss of independence at an earlier age. It is likely that the presentation of muscle strength and muscle size phenotypes in the elderly are associated with numerous SNPs.

There are presently numerous studies associating single SNPs with skeletal muscle phenotypes in a variety of populations, ranging from young adult athletes to elderly (Table 1.5, Chapter 1). However, there are numerous instances where these SNPs show contrasting results depending on the population investigated. In older adult populations for instance, *ACE I/D* is associated with skeletal muscle mass phenotypes (Charbonneau et al., 2008) in one study, while not associated in another (Pereira et al., 2013). Within female elderly populations, there are presently no investigations into the role of multiple SNPs on the plethora of skeletal muscle mass/size and strength phenotypes.

In terms of identifying meaningful phenotypes to investigate SNP associations, the previous chapter has observed the differences between sarcopenic and non-sarcopenic populations for Isometric Knee Extension Maximum Voluntary Contraction (MVC_{KE}), Isometric Elbow Flexion Maximum Voluntary Contraction (MVC_{EF}), Handgrip Strength (HGS), biceps brachii and Vastus lateralis (VL) muscle size measures (Chapter 3). Specific to the present chapter, VL muscle atrophy is representative of muscle loss associated with ageing (Lexell et al., 1988) and loss of knee extensor strength correlates with functional impairments in the elderly (Martien et al., 2015). In addition to lower limb musculature, the upper limb muscle size and muscle strength are also prone to decline with ageing (Janssen et al., 2000b; Keller and Engelhardt, 2013). Identification of new gene variants or replicating the previous findings in the present elderly population could be useful in targeting the sarcopenia group with appropriate interventions.

Therefore, the present chapter aimed to investigate the association of SNPs on skeletal muscle phenotypes, specifically muscle size (biceps brachii thickness, VL thickness, VL_{ACSA}) and muscle strength (HGS, MVC_{EF} and MVC_{KE}), in a Caucasian elderly female population.

5.2 Methods

Detailed descriptions of participant recruitment, assessment of skeletal muscle size and strength measures and DNA sample collection and genotyping is included in Chapter 2, thus only brief descriptions are provided below.

5.2.1 Participants

Three hundred and seven active elderly Caucasian females (age 70.7 (5.7) years, mass 66.3 (11.3) kg, height 1.60 (0.06) m; mean(SD)) volunteered to participate in this study from surrounding areas of MMU. Participants met the inclusion criteria (described in Chapter 2) and provided written informed consent prior to testing.

5.2.2 Skeletal muscle properties

5.2.2.1 Muscle strength phenotypes

Skeletal muscle strength was measured for handgrip, elbow flexor and knee extensor muscles. Handgrip strength was measured by Handgrip dynamometer and Isometric Maximum Voluntary Contraction of Elbow Flexion and Knee extension with a customized built dynamometer (detailed in Chapter 2).

5.2.2.2 Muscle size phenotypes

Skeletal muscle size phenotypes included VL at 50% length (VL thickness, VL_{ACSA}) and biceps brachii thickness at 60% length of the humerus bone. Measurements of VL_{ACSA} and biceps brachii thickness were conducted using B-mode ultrasound as described in Chapter 2.

5.2.2.3 Sample collection, DNA extraction and genotyping

Blood (189 samples (~ 62%)) and saliva (116 samples (~38%)) were obtained using standard protocols. Blood was drawn from a superficial-forearm vein and then stored at -20 °C until further processing. Saliva was collected in Oragene OGR-500 collection tubes (DNA Genotek Inc., Ontario, Canada) following the company's

protocol and stored at room temperature until DNA extraction. DNA was extracted by the QIAcube method, subsequent to which genotyping was performed as described in Chapter 2.

5.2.3 Statistical analysis

The frequency of all the selected polymorphisms was checked for compliance with Hardy-Weinberg equilibrium using chi-square tests. ANCOVA was used to test any genotype effects on skeletal muscle phenotypes (muscle size, muscle strength) with age used as covariate. When too few participants were in one genotype group, the group was combined with the heterozygous group. All significant associations identified in the main ANCOVA analyses were subject to post-hoc pairwise comparisons using the Benjamini-Hochberg correction. When there was a tendency for association ($0.05<p<0.15$) (Fischer et al., 2004) the two groups with similar means were combined and then ANCOVA was re-run for the analysis. All statistical analyses were performed using SPSS version 23.0 and statistical significance was accepted when $p\leq0.05$. Data are presented as mean (SD).

5.3 Results

All the SNPs were in Hardy-Weinberg equilibrium ($p\geq0.15$) and the genotype frequencies of all polymorphisms are presented in Table 2.1. In the following section, only the SNPs associated with skeletal muscle phenotypes are presented.

Muscle phenotype differences were observed in elderly women who expressed favourable genotype groups for the following SNPs: HGS (*PTK2* rs7843014, *COL1A1* rs1800012 and *PTK2* rs7460; Figure 1), MVC_{EF} (*ACVR1B* rs2854464, *HIF1A* rs11549465, *PTK2* rs7460 and *MTHFR* rs1801131; Figure 2), MVC_{KE} (*CNTF* rs1800169 and *NOS3* rs1799983; Figure 3), bicep thickness (*ACE* rs4341 and *ACVR1B* rs10783485; Figure 4), VL thickness (*TRHR* rs7832552 and *HIF1A* rs11549465; Figure 5) and VL_{ACSA} (*TRHR* rs7832552, *ACVR1B* rs10783485*, HIF1A* rs11549465 and *FTO* rs9939609 Figure 6). For the SNPs associated with skeletal muscle phenotypes, elderly women in the favourable genotype groups were 5-12% stronger and had 3-7% larger muscle (Table 5.1) than their counterparts with less favourable genotypes.

Of the 24 SNPs analysed, 12 showed differences in muscle phenotypes between those elderly with and without favourable genotypes (Table 5.1).

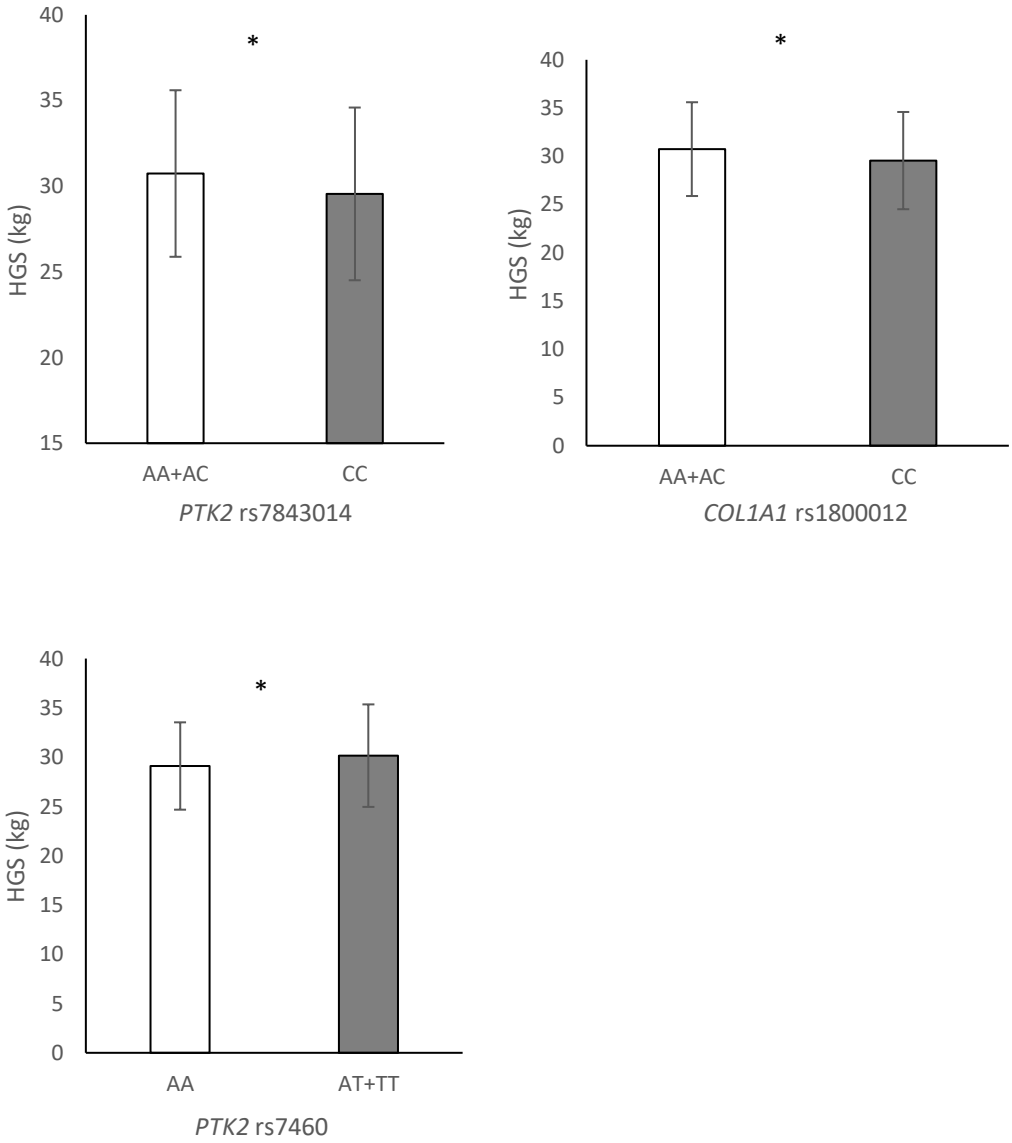

Figure 5.1 *Association of SNPs and Handgrip strength (HGS). Comparison of HGS between genotype groups for PTK2 rs7843014 (AA+AC=247 Vs CC=57), COL1A1 rs1800012 (AA+AC= 99 Vs CC=205) and PTK2 rs7460 (AA=72 Vs AT+TT=233) polymorphisms. * denotes significant difference*

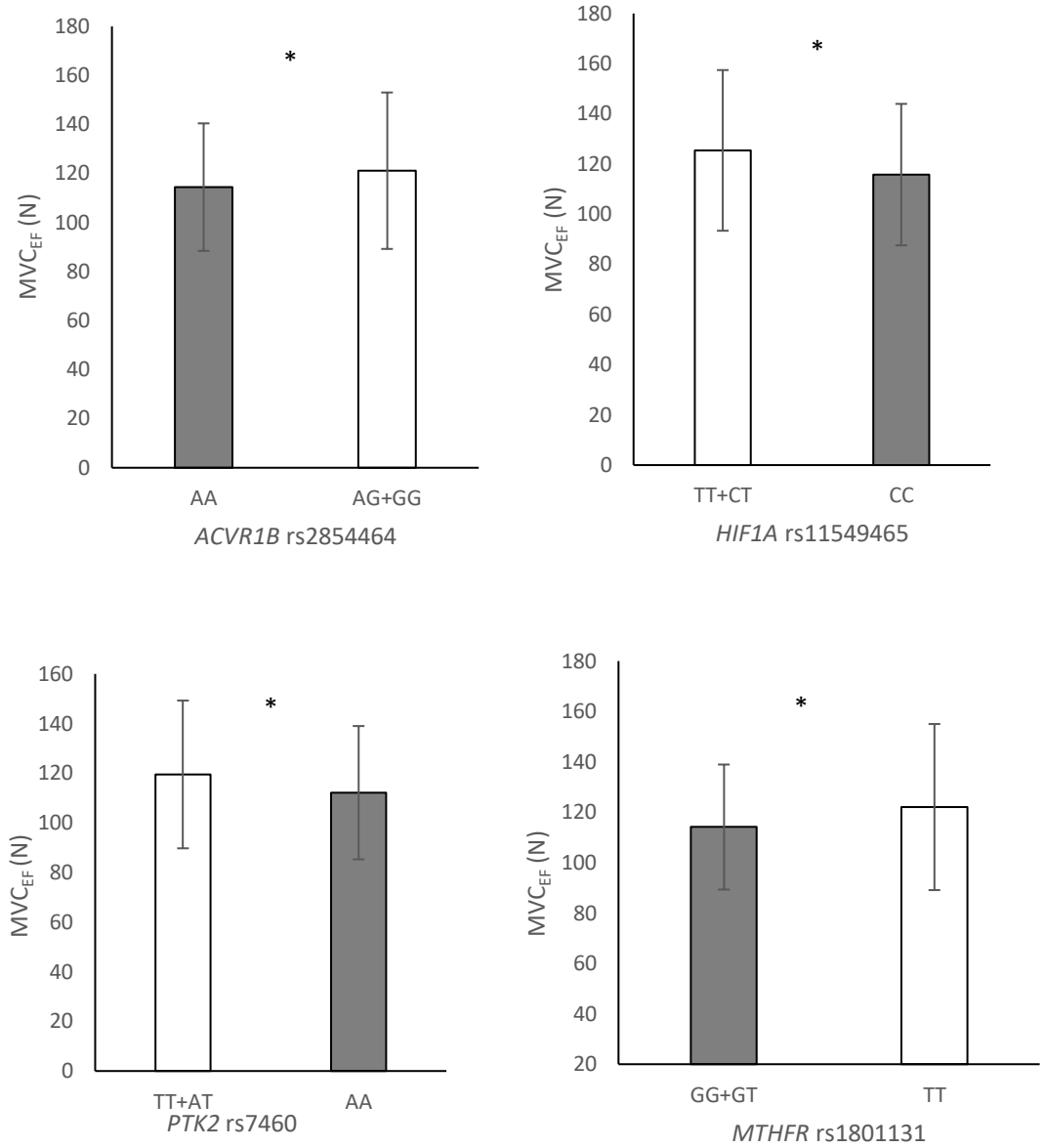

*Figure 5.2Association of SNPs and Isometric elbow flexion maximum voluntary contraction (MVC_{EF}). Comparison of MVC_{EF} between genotype groups for ACVR1B rs2854464 (AA=153 Vs AG+GG=151), HIF1A rs11549465 (TT+CT=63 Vs CC=241), PTK2 rs7460 (TT+AT=233 Vs AA=71) and MTHFR rs1801131 (GG+GT=159 Vs TT=144). * denotes significant difference.*

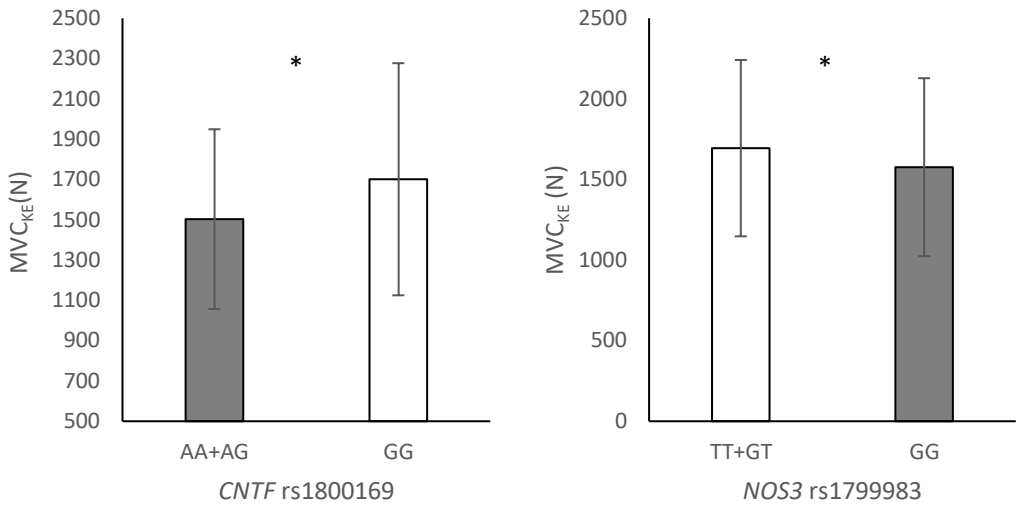

*Figure 5.3Association of SNPs and Isometric Knee Extension Maximum Voluntary contraction (MVCKE). Comparison of MVC_{KE} between genotype groups for CNTF rs1800169 (AA+AG=80 Vs GG=222) and NOS3 rs1799983 (TT+GT= 185 Vs GG= 117) polymorphisms. * denotes significant difference.*

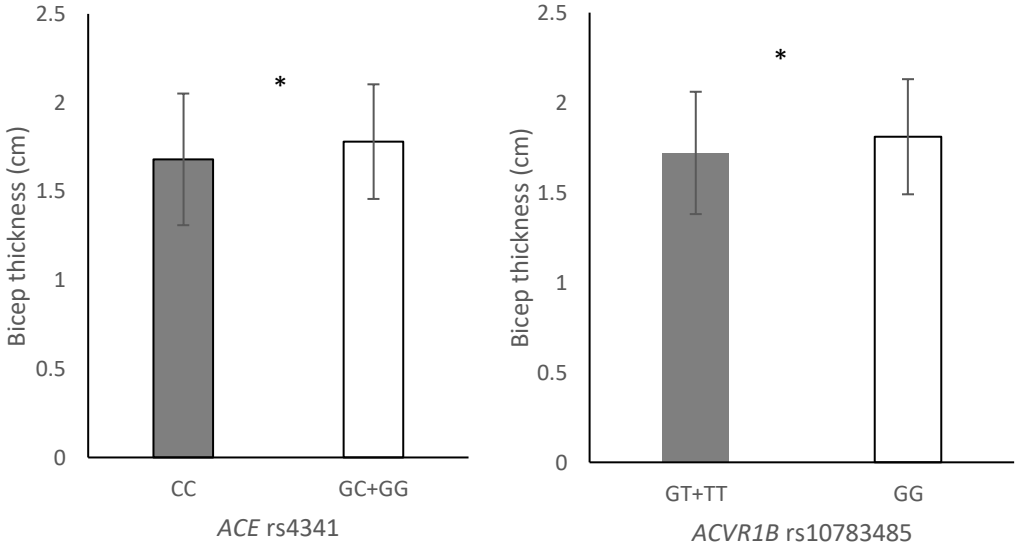

*Figure 5.4: Association of SNPs and bicep brachii thickness. Comparison of biceps brachii thickness between genotype groups for ACE rs4341 (CC=61 Vs GG+GC= 231) and ACVR1B rs10783485 (GT+TT=167 Vs GG=124) polymorphisms. * denotes significant difference.*

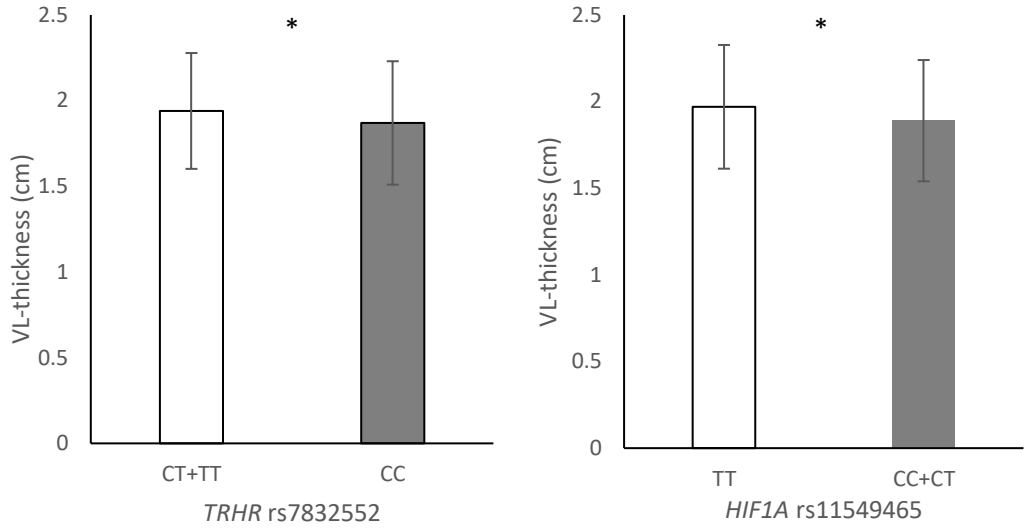

*Figure 5.5: Association of SNPs and Vastus lateralis Thickness. Comparison of VL thickness between genotype groups for TRHR rs7832552 (CT+TT= 159 Vs CC=130) and HIF1A1 rs11549465 (TT+CT=288 Vs CC=2) polymorphisms. * denotes significant difference.*

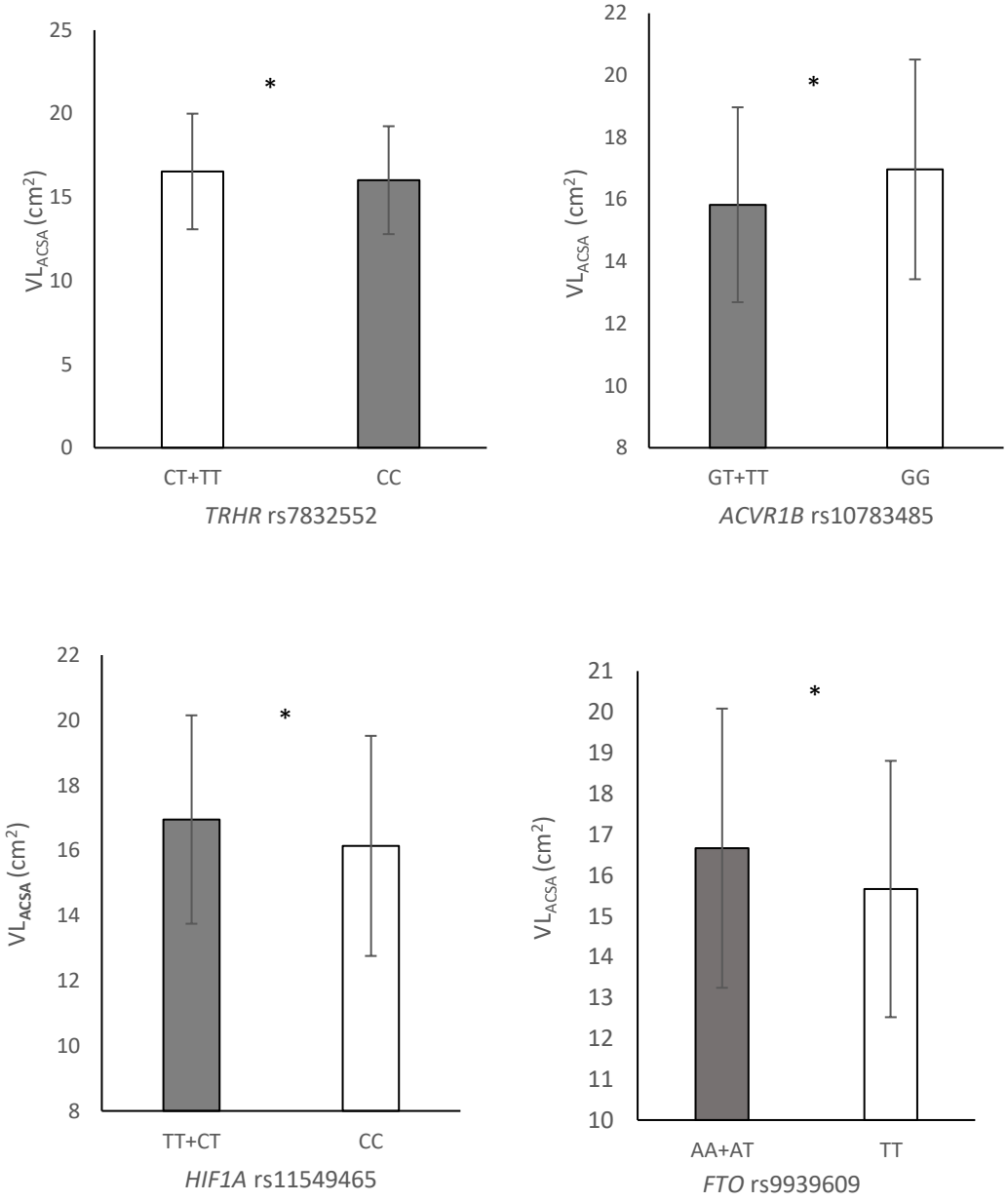

*Figure 5.6Association of SNPs and Vastus Lateralis Anatomical Cross sectional area (VL_{ACSA}). Comparison of VL_{ACSA} between genotype groups for TRHR rs7832552 (CT+TT=159 Vs CC=130), ACVR1B rs10783485 (GT+TT=168 Vs GG=121), HIF1A rs11549465 (CC=228 Vs CT+TT=62) and FTO rs9939609 (AA+AT= 188 Vs TT=102) polymorphisms. * denotes significant difference.*

Table 5.1 Associations between genotype and skeletal muscle phenotypes in elderly Caucasian female.

Polymorphisms	Genotypes	Phenotypes	% difference	p
***TRHR* rs7832552**	CT+TT Vs CC	VL thickness	3.6	0.036
		VL_{ACSA}	3.0	0.032
***HIF1A* rs11549465**	CT+TT Vs CC	VL_{ACSA}	5.0	0.037
	CC+CT Vs TT	VL thickness	4.0	0.044
	CT+TT Vs CC	MVC_{EF}	7.7	0.009
***PTK2* rs7460**	AT+TT Vs AA	MVC_{EF}	6.0	0.031
		HGS	3.5	0.042
***PTK2* rs7843014**	AC+AA Vs CC	HGS	5.0	0.018
***ACVR1B* rs10783485**	GT+TT Vs GG	VL_{ACSA}	7.0	0.010
		Biceps brachii thickness	5.0	0.030
***ACVR1B* rs2854464**	AG+GG Vs AA	MVC_{EF}	5.5	0.033
***FTO* rs9939609**	AT+TT Vs AA	VL_{ACSA}	6.0	0.013
***NOS3* rs1799983**	TT+GT Vs GG	MVC_{KE}	7.0	0.042
***CNTF* rs1800169**	AA+AG Vs GG	MVC_{KE}	12.0	0.004
***ACE* rs4341**	GC+GG Vs CC	Biceps brachii thickness	5.6	0.039
***COL1A1* rs1800012**	AA+AC Vs CC	HGS	4.0	0.013
***MTHFR1* rs1801131**	GT+GG Vs TT	MVC_{EF}	6.5	0.019

Grey shading denotes the favourable groups for skeletal muscle phenotypes.
Abbreviations: HGS - Handgrip strength, VL_{ACSA} - Vastus lateralis anatomical cross sectional area, MVC_{KE} - Isometric knee extension maximum voluntary contraction, MVC_{EF} – Isometric Elbow flexion maximum voluntary contraction.

5.4 Discussion

The current chapter aimed to investigate associations between 24 SNPs and skeletal muscle phenotypes in elderly females related to muscle size (biceps brachii thickness, VL-thickness and VL_{ACSA}) and muscle strength (HGS, MVC_{EF} and MVC_{KE}). There were significant associations of *ACVR1B* rs2854464, *MTHFR1* rs1801131,

HIF1A rs11549465 and *PTK2* rs7460 with MVC_{EF}, and of *PTK2* rs7843014, *COL1A* rs1800012 and *PTK2* rs7460 with HGS. Similarly, significant associations of both *CNTF* rs1800169 and *NOS3* rs1799983 with MVC_{KE} were observed. For muscle size measures, there were significant associations of *ACE* rs4341 and *ACVR1B* rs10783485 with biceps brachii thickness, *ACVR1B* rs10783485, *TRHR* rs7832552, *HIF1A* rs11549465, and *FTO* rs9939609 with VL_{ACSA}, and *TRHR* rs7832552 and *HIF1A* rs11549465 with VL-thickness. The present study therefore reports novel associations with skeletal muscle strength in an elderly Caucasian population, in addition to independently replicating some previous reports.

In the present elderly female population, the genetic variants associated with skeletal muscle phenotypes can be described by the biological roles of the genes. When the potential mechanisms for the influence of the SNPs is considered, there are six thematic areas that are consistent across both muscle size and muscle strength: 1) structural protein, 2) epigenetic regulator, 3) transcriptional regulator, 4) antagonist of muscle growth, and 5) body composition regulator, and 6) myotrophic factor.

5.4.1 Structural protein

The present study has identified the association of structural protein gene variants *PTK2* rs7460, *PTK2* rs7843014 and *COL1A1* rs1800012 with the skeletal muscle phenotypes under investigation; *PTK2* rs7460 with HGS and MVC_{EF}, *PTK2* rs7843014 and *COL1A1* rs1800012 with HGS. These genes encode for a component of muscle structural proteins and the extracellular matrix and thus might provide strength and integrity for the muscle fibre. The genotypes identified as favourable for skeletal muscle phenotypes in the present elderly population have been previously associated with exceptional longevity for *PTK2* rs7460 TT and *PTK2* rs7843014 CC (Garatachea et al., 2014) and bone mineral density (BMD) in juvenile idiopathic arthritis (Kostik et al., 2013) for *COL1A1* rs1800012. The presence of favourable genotypes could probably be attributed to a favourable presentation of a structural protein. For example, *PTK2* rs7843014 CC (associated with higher HGS in the present study) has been associated with low focal adhesion kinase (FAK), the lower level of

which has been speculated to lead to normal cell division, and linked with longevity (Garatachea et al., 2014), consistent with the evidence that muscle mass and muscle strength are key determinants of mortality (Rantanen et al., 2000; Ruiz et al., 2008). Higher FAK expression has been linked with metastasis and cancer (Lark et al., 2005) suggesting an improper integrity.

The *COL1A1* rs1800012 A-allele carriers group had higher handgrip strength in the present study. There is evidence that the (A) allele of the Sp1-COL1A1 binding site polymorphism is linked with a higher rate of DNA-protein binding, leading to transcription increase, elevated expression of COL1A1 protein in osteoblasts culture (Mann et al., 2001) and results in a higher proportion of collagen alpha 1. Therefore, *COL1A1* A-allele carriers have a higher percentage of collagen alpha 1, and recent meta-analysis has shown professional soccer players appear less prone to soft tissue injury (Wang et al., 2017a). It is possible, therefore, that there is some effect of the *COL1A1* rs1800012 A-allele that can preserve the integrity of the muscle and explain the higher strength observed in elderly women.

5.4.2 Epigenetic regulator

MTHFR governs the housekeeping methylation reactions and nucleic acid formation (Bailey and Gregory Iii, 1999) and is the key player in epigenetic mechanisms (Garcia-Gimenez et al., 2012). The 677C>T mutation of the *MTHFR* gene (*MTHFR* rs1801131) has been associated with increased level of plasma homocysteine concentrations (Frosst et al., 1995; Brattström et al., 1998). *MTHFR* rs1801131 CC homozygotes are likely to have higher homocysteine plasma concentrations (Castro et al., 2004) which have been linked with reduced physical activity (Dankner et al., 2007) and lower quadriceps strength (Kuo et al., 2007). This is consistent, with the finding that the CC genotype is associated with lower HGS in the present elderly women.

5.4.3 Transcriptional regulator

The present study has identified associations of transcription factor and transcription regulator gene variants *HIF1A* rs11549465, *NOS3* rs1799983 and *ACE*

rs4341 with the skeletal muscle phenotypes under investigation in the elderly female population; *HIF1A1* rs11549465 with VL-thickness, VL_{ACSA}, and MVC_{EF}, *NOS3* rs1799983 with MVC_{KE} and *ACE* rs4341 with biceps brachii thickness.

It has been speculated that observed gene variants might change the transcription of genes affecting skeletal muscle and thus can explain the higher muscle size and muscle strength in the elderly women studied. For instance, transcription factor HIF1A is the sub-unit of heterodimeric transcriptional factor HIF1 that induces the transcription of genes involved in cellular proliferation and survival (Lee et al., 2004; Hashimoto and Shibasaki, 2015) with *HIF1A* rs11549465 T allele associated with enhanced trans-activation capacity (Tanimoto et al., 2003). Furthermore, previous studies have also observed the T-allele to be found more commonly in weightlifters and wrestlers (Gabbasov et al., 2013) and power oriented athletes (Cięszczyk et al., 2011; Drozdovska et al., 2013b), and also associated with maximal oxygen consumption post exercise training in elderly Caucasians (Prior et al., 2003). It is therefore possible that there is enhanced transactivation capacity with the T allele in the present elderly population and a corresponding higher VL_{ACSA}, VL-thickness and HGS.

Similarly, *NOS3* encodes endothelial NOS (eNOS) that catalyzes the synthesis of NO that affects the process of skeletal muscle fibre conversion (Martins et al., 2012), mitochondrial energy production (Brown, 2007) and normal muscle hypertrophy (Smith et al., 2002). Higher NO activity has been associated with *NOS3* rs1799983 T-allele (Tesauro et al., 2000; Persu et al., 2002) which has been identified as the favourable allele in athlete populations (Eider et al., 2014). It is therefore likely that *NOS3* rs1799983 T- allele carriers have higher NO activity and have higher knee strength in the present elderly population.

ACE rs4341 D-allele carriers has been associated with thicker biceps brachii thickness in the present elderly female population. *ACE* rs4341 D allele is associated with higher ACE activity resulting effective conversion of Angiotensin-I to Angiotensin II. Angiotensin II has been linked with modulating skeletal muscle hypertrophy in response to mechanical loading (Gordon et al., 2001). Previous

studies have also linked the D allele as favourable for skeletal muscle phenotypes such as having a higher proportion of Type II fibres (Zhang et al., 2003), larger quadriceps muscle volume (Charbonneau et al., 2008), better athletic performance (Tsianos et al., 2004; Costa et al., 2009b) and higher muscle strength (Hopkinson et al., 2004; Williams et al., 2005). Therefore, it is possible that in present elderly women the association of the D allele with thicker biceps brachii thickness could be due to higher gene activity of ACE.

5.4.4 Antagonist of muscle growth

ACVR1B genotypes, *ACVR1B* rs10783485 GG and *ACVR1B* rs2854464 G-allele carriers are observed as the favourable genotypes for skeletal muscle phenotypes in the present elderly population and could be explained by the biochemical role of ACVR1B. The *ACVR1B* gene encodes the activin A receptor type 1b protein, which is a member of the TGF beta family and known to be involved in molecular pathways regulating myostatin and activin signalling, signalling pathways identified as a negative regulator of muscle growth (McPherron et al., 1997; Thomas et al., 2000). The findings of the current study are similar to the previous studies reporting *ACVR1B* rs10783485 G allele in 20-90 years Caucasian male (Windelinckx et al., 2011) and *ACVR1B* rs2854464 G-allele (He et al., 2018) favourable for knee strength in elderly population. It is therefore possible that the genotype groups that showed significantly larger muscle size and higher muscle strength in the present elderly female population (*ACVR1B* rs10783485 GG for biceps brachii thickness and VL_{ACSA}; *ACVR1B* rs2854464 G-allele carriers for MVC_{EF}) might show less inhibition of those muscle-signalling pathways.

5.4.5 5.4.5. Body composition regulator

Body composition indices such as BMI, fat-free mass and other obesity related phenotypes are strongly regulated by the FTO gene (Frayling et al., 2007; Sonestedt et al., 2011; Livshits et al., 2012). The importance of FTO during skeletal muscle development and differentiation was observed in FTO-deficient mice with impaired skeletal muscle development. FTO increases during myogenic differentiation, and silencing of FTO leads to myogenic suppression (Wang et al., 2017c). A recent study

has found an association between FTO and appendicular lean mass, with a decrement in appendicular muscle mass when fat mass was controlled (Cordero et al., 2018). The present chapter is consistent with the previous studies showing associations of the *FTO* rs9939609 A-allele with body composition parameters such as fat mass and lean body mass (Sonestedt et al., 2011; Livshits et al., 2012) and BMI (Jacobsson et al., 2012; Al-Serri et al., 2018). Accordingly, the A-allele is associated with greater muscle size (VL_{ACSA} in this case) in the present elderly women population.

5.4.6 Myotrophic factor

CNTF rs18000169 and *TRHR* rs7832552 are gene variants identified as favourable for the skeletal muscle phenotypes. CNTF is a signalling molecule with neurotrophic and myotrophic role (Forger et al., 1993; Ip et al., 1993). The association of *CNTF* rs18000169 with muscle strength, specifically with MVC_{KE}, in the present elderly population can be explained by the biological role of CNTF. Myogenesis process upregulates and atrophy mediators downregulates with CNTF treatment (Tsompanidis et al., 2016). CNTF level decreases with ageing and exogenous administration of CNTF in older rats has shown to improve muscle strength (Guillet et al., 1999). A functional gene variant *CNTF* rs1800169 with AA genotype produces the non-functional protein (Takahashi et al., 1994). The present study is consistent with most of the studies (Roth et al., 2001; Arking et al., 2006; Walsh et al., 2009) who reported GG genotype as the favourable genotypes for skeletal muscle phenotypes. It is therefore likely that the present elderly women with GG genotype might have more functional protein that could contribute to effective myogenesis and hence they are stronger than A-allele carriers.

TRHR leads to the release of thyroxin, and helps in the skeletal muscle development. TRHR also plays an important role in decreasing age-related alteration in tissue function (Larsson et al., 1994). The change in the thyroid hormone level results in notable symptoms of muscle weakness (Salvatore et al., 2014). A Genome Wide Association Study (GWAS) has found the association of TT genotype with higher lean body mass in US Caucasians (Liu et al., 2009). Up-regulated luciferase activity was

associated with the T allele compared to the C allele in C2C12 skeletal muscle cell lines of mice (Fuku et al., 2015). Therefore, it is likely that TT genotype may be associated with higher expression of thyroid hormone and thus associated with favourable skeletal muscle phenotypes in the present elderly population.

Several muscle size and strength measures are reported in this study; however, no single gene variant was associated with all those measures. The probable reason of no association with every muscle measure could be the modest influence of those specific gene variants on the specific muscle measures, while the influence could not be observed in other muscle measures. It is to be noted that the commonly studied *ACTN3* R577X gene variant did not show any association with any of the studied phenotypes/measures. There are many studies reporting no association of *ACTN3* with skeletal muscle phenotypes (Ruiz et al., 2011). *ACTN3* seems related to sprint speed in younger adults, and therefore probably rate of force development or the ability to produce force at high contraction velocities (Erskine et al., 2014; Broos et al., 2016), and not muscle strength per se. Perhaps assessing muscle function during faster, dynamic contractions in the elderly would show an association with *ACTN3*. One possible reason for no association could be the recruitment of independently living, and recreationally active participants, with which the discriminating power of genotypes was not able to distinguish the muscle phenotypes.

5.5 Implications

Identifying the gene variants that might affect both upper limb and lower limb strength, is necessary in the perspective of targeting the development of treatments against muscle wasting disorders such as cachexia and sarcopenia. Upper limb strength and lower limb strength are very important in the maintenance of activities of daily living (Rantanen et al., 2002). Therefore, the gene variants identified in the present study that show associations with skeletal muscle phenotypes could be beneficial in screening a population for those most prone to develop sarcopenia. The proper therapies and interventions can be developed with the understanding of gene variants that have influence on skeletal muscle phenotypes in an elderly population.

5.6 Conclusion

The present study has identified novel gene variants that are associated with muscle phenotypes in elderly women. However, the lack of associations of some of the possible SNPs believed to be associated with skeletal muscle phenotypes also adds to the growing body of literature. Furthermore, the findings of this chapter have applications in a variety of areas, specifically ageing, for whom completion of activities of daily living in their old age may be improved as a consequence of good understanding their individual-specific muscle mechanics.

6 . Genetic predisposition scores partially predict skeletal muscle phenotypes in elderly Caucasian females

6.1 Introduction

Heritability of muscle mass (66%) and muscle strength (82%) (Arden and Spector; Thomis et al., 1998; Abney et al., 2001) are high, suggesting a strong control of genetics in skeletal muscle phenotypes. Cross-sectional studies or case control studies have identified more than 200 genes associated with physical performance and health-related fitness phenotypes (Bray et al., 2009). Although several Single Nucleotide Polymorphisms (SNPs) have been studied with skeletal muscle related phenotypes, only a few of them have been replicated successfully (Loos et al., 2015); while some of them could not be replicated or have been tested on single occasions. This suggests that observed skeletal muscle phenotypes are not related to single gene variants (Tan et al., 2012) but are likely to be polygenic. It can therefore, be assumed that many individual gene variants might contribute to skeletal muscle phenotypes and should be considered together to obtain a more complete understanding of the genetic influence on muscle phenotypes. Although most of the associations between individual SNPs and skeletal muscle phenotypes in the presently studied elderly population did not reach significance (Chapter 5), considering all gene variants together might help to understand the combined effect of other gene variants.

There are many approaches to study the polygenic influence in skeletal muscle phenotypes (Charlier et al., 2016). (Hughes et al., 2011; Charlier et al., 2016) used the polygenic concept for the first time to study the multiple genes contribution to elite endurance athletes. The genetic algorithm was used whereby each polymorphism receives a score (known as Genetic Score, GS) based on their influence in the skeletal muscle phenotypes; with this approach, each favourable allele gets a score of 1 in an additive manner. For instance, if R is the favourable allele in the *ACTN3* rs1815739 polymorphism, then RR=2, RX=1, XX=0. Subsequently, the Total Genotype Score (TGS_{total}) can be calculated summing the GS for the selected polymorphisms, which is then transformed to lie within the range 0-100 (Santiago et al., 2010). Some studies have identified that athletes competing at the elite level in sports and activities demanding higher muscle mass/strength, possess significantly higher TGS than non-athlete populations (Ruiz et al., 2009; Santiago et

al., 2010; Massidda et al., 2014). Although there have been many modifications in the construction of the polygenic model for studying skeletal muscle strength/mass and performance, the overall basis of this approach is giving a genotype score based on the positive/negative effect of alleles on phenotypes (Charlier et al., 2016). Including all of the measured SNPs within the TGS can induce noise, therefore an alternative approach is to only include those SNPs that have shown an association with the phenotypes, an approach named as data-driven Genotype Predisposition Score (GPS_{dd}) (Thomaes et al., 2011; Thomaes et al., 2013; He et al., 2018).

The decline in muscle size and strength with age, can lead to significant loss of independence and physical function (Cruz-Jentoft et al., 2010). Several studies have shown the association of muscle mass and strength with SNPs in an ageing population (Tan et al., 2012; Garatachea and Lucía, 2013). An understanding of the polygenic profile of neuromuscular phenotypes may be beneficial for the understanding of disability and sarcopenia in old age. To the best of the author's knowledge, no study has examined the predictive ability of a Genetic Predisposition Score (GPS) based on the gene variants related to muscle phenotypes in elderly females, to explain individual differences in muscle mass and muscle strength. In this chapter, the author uses the GPS_{dd} approach to investigate the influence of multiple gene variants in the skeletal muscle phenotypes in Caucasian elderly females aged over 60 years. This approach has been utilized in several studies to study the genetic influence in peak VO_2 (Thomaes et al., 2011); muscle size, muscle strength and trainability (Thomaes et al., 2013); VO_2 max training response (Bouchard et al., 2010), and knee extension strength (Charlier et al., 2016; He et al., 2018).

Therefore, the aim of the present chapter are divided into three parts 1) to assess the predictive power of data-driven GPS (GPS_{dd}) on skeletal muscular phenotypes, 2) to assess if the traditional TGS approach is associated with the skeletal muscle phenotypes, and 3) to study if there is difference in TGS between the sarcopenia and non-sarcopenia groups in elderly Caucasian female.

6.2 Methods

6.2.1 Participants

Three hundred, 60-91 years old Caucasian females (70.7±5.7 years, 66.3±11.3 kg, 1.60±0.06 m) (Mean±SD) who were ambulatory and had no history of severe muscle and bone issues such as osteoporosis, rheumatoid arthritis and cancer, and nervous system disorder like Alzheimer's, convulsions, epilepsy, and cardiovascular related diseases volunteered for this study. Study protocols were in accordance with the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Manchester Metropolitan University. Informed written consent was obtained from all the participants prior to involvement in this study.

6.2.2 Procedures

All participants attended for testing on a single session at the MMU Cheshire Campus, Crewe. The testing session was conducted in the following order: anthropometry, handgrip strength, isometric knee extension maximum voluntary contraction (MVC_{KE}), ultrasound of bicep and Vastus Lateralis muscle and DNA sample collection (blood/saliva). DNA extraction and genotyping were performed later.

6.2.2.1 Handgrip strength

Handgrip strength (HGS) was measured using a digital load cell handgrip dynamometer (JAMAR plus, JLW Instruments, Chicago, USA) with a validated protocol (Roberts et al., 2011). Briefly, participants were verbally encouraged to squeeze the handle of dynamometer with maximum strength three times in a standing position with the dynamometer maintained at a right angle to the shoulder. Each trial was performed with at least 1-minute rest between efforts and the highest of the three trials was recorded for the study.

6.2.2.2 Isometric Knee Extension Maximum Voluntary Contraction

Isometric Knee Extension Maximum Voluntary Contraction (MVC_{KE}) was measured as described in Chapter 2 (Methodology). Briefly, for assessment of MVC_{KE},

participants were seated in a customized built dynamometer (MMU, UK) with knee at 120 degree (straight is considered as 180 degree). The dominant leg was securely fastened to the force transducer above the lateral malleolus (identified by palpation) at the known distance. Participants were instructed to perform MVC_{KE} with real-time visual feedback and verbal encouragement from the principal investigator. This was repeated 3 times with a short rest interval and the highest value of three total attempts was recorded for the MVC_{KE}.

6.2.2.3 Biceps brachii thickness

Biceps brachii thickness was measured at 60% length of the humerus bone as described in Chapter 2.

6.2.2.4 Vastus lateralis muscle area

Skeletal muscle phenotype, VL_{ACSA} was measured at 50% VL length as described in Chapter 2. In short, the ultrasound was recorded as a digital video file, from which individual images were acquired, using capture software (Adobe Premier, Adobe), between each shadow cast by the echo-absorptive markers. The entire VL_{ACSA} was reconstructed in a single canvas from each captured image and measured using digitizing software (Image J, NIH).

6.2.2.5 Sample collection, DNA extraction and genotyping

Blood and saliva samples were collected as described in the Chapter 2. Briefly, blood was drawn from a superficial-forearm vein by the principal investigator and then stored at -20°C until further processing. For the saliva sample, saliva was collected in Oragene.DNA OG-500 collection tubes (DNA Genotek Inc., Ontario, Canada) following the company's protocol and stored at room temperature until DNA extraction. DNA was extracted by the QIAcube method; subsequent to which genotyping was performed as described in Chapter 2.

6.2.3 Statistical Analysis

Chi square was performed to examine any deviations of the SNPs from the Hardy-Weinberg equilibrium. GS was assigned as described previously (Williams and Folland, 2008). A backward regression was performed to identify the SNPs significantly associated with skeletal muscle phenotypes with age as a covariate. Phenotype related predisposing alleles are regarded as positively associated with the skeletal muscle phenotypes. Thereafter, GPS was calculated from those selected subsets of SNPs as mentioned previously (Thomaes et al., 2011; He et al., 2018). Finally, linear regression was carried out with GPS as an independent variable and age as confounding factor where it was observed significant; and skeletal muscle phenotypes as dependent variable.

In the case of traditional TGS model, TGS was calculated from the SNPs whose allele direction are well described in previous studies (for *ACTN3* rs1815739, *ACE* rs4341, *CNTF* rs1800169, *TRHR* rs7832552, *HIF1A* rs11549465, *NOS3* rs1799983, *MSTN* rs1805086, *VDR* rs2228570 and *FTO* rs9939609) as favourable and unfavourable alleles for the skeletal muscle phenotypes.

For both the GPS and TGS approach, independent samples t-test was carried out between the sarcopenia and non-sarcopenia group. Statistical significance was set at $p<0.05$ for the analyses. All the statistics were performed in IBM SPSS Version 23.

6.3 Results

All the SNPs under the study were compliant with Hardy Weinberg Equilibrium as tested by the Chi square test; shown in the table 2.1 (in Chapter 2 Methodology). A general characteristic of the participants is given in table 6.1 below.

GPS approach

The GPS models with age explained 17.2%, 8.7%, 2.2% and 14.5% variance of HGS, MVC_{KE}, biceps brachii thickness and VL_{ACSA} size. GPS_{dd} alone explained 5.0%, 2.2%, 2.2% and 8.2% variance of HGS, MVC_{KE}, biceps brachii thickness and VL_{ACSA} size (Table 2). Out of 24 SNPs in the current study, 10 SNPs were found to be associated

with at least one of the skeletal muscle phenotypes under investigation. *PTK2* rs7460 was found to be associated with all the skeletal muscle phenotypes, and in descending number of phenotype associations: *PTK2* rs7843014 and *HIF1A* rs11549465 with 3, *TRHR* rs7832552 with 2 and *ACE* rs4341, *CNTF* rs1800169, *CNTFR* rs2070802, *FTO* rs9939609, *MTHFR* rs1801131, and *MTHFR* rs17421511 with 1 phenotype (table 6.3). The other SNPs did not show any significant associations with the phenotypes considered in this chapter.

TGS approach and sarcopenia

While studying the association of skeletal muscle phenotypes with TGS, the present chapter found an association between the TGS (calculated from the established 9 SNPs from previous studies) and $VL_{ACSA;}$ F (1,283)=6.383, p=0.012); while it did not find any association between TGS and other skeletal muscle phenotypes; for HGS (F (1,298)=0.198, p=0.657)), MVC_{KE} (F (1,295)=1.66, p=0.199)) and bicep brachii thickness (F (1,284)=2.634, p=0.106)).

Similarly, the present chapter did not find any difference in TGS between sarcopenia and non-sarcopenia group; t(240)=0.317, p=0.752.

Table 6.1 General characteristics of participants

Variables	Mean ± SD (n=300)
Body Mass (kg)	66.32±11.27
Age (years)	70.74±5.66
Handgrip Strength(kg)	29.97±4.97
Biceps brachii thickness (cm)	1.76±0.34
Vastus lateralis anatomical cross sectional area (cm^2)	16.32±3.38
Isometric Knee Extension Maximum Voluntary Contraction (N)	1651±546

Table 6.2Relation between GPS models and skeletal muscle phenotypes

		GPS		AGE				R^2	
	estimate	B value	Partial correlation	p-value	estimate	B value	Partial correlation	p-value	
VL_{ACSA}	0.769	0.278	0.286	<0.001	-0.174	-0.290	-0.298	<0.001	0.145
Biceps brachii thickness	0.063	0.149	0.149	0.012	-	-	-	-	0.022
HGS	1.031	0.209	0.224	<0.001	-0.322	-0.367	-0.374	<0.001	0.172
MVC_{KE}	95.83	0.142	0.147	0.011	-25.11	-0.260	-0.263	<0.001	0.087

Abbreviations: VL_{ACSA}- Vastus Lateralis Muscle Anatomical Cross Sectional Area; HGS- Handgrip Strength; MVC_{KE}- Isometric Knee Extension Maximum Voluntary Contraction

Table 6.3 SNPs and skeletal muscle phenotypes

SNPs	VL_{ACSA}	Bicep thickness	HGS	MVC_{KE}
TRHR rs7832552	*	-	-	-
HIF1A rs11549465	*	-	*	-
ACE 4341	-	*	-	-
PTK2 rs7460	*	*	*	*
PTK2 rs7843014	*	*	-	-
MTHFR1 rs17421511	-	-	*	-
MTHFR1 rs1801131	-	-	*	-
CNTF rs1800169	-	-	-	*
CNTFR rs2070802	*	-	-	-
FTO rs9939609	*	-	-	-

* and - denote significant and no association of SNPs with muscle phenotypes, respectively. Abbreviations: VL_{ACSA}-Vastus Lateralis-Anatomical Cross Sectional Area; $MVC_{KE,}$ Isometric Maximum voluntary contraction- Knee Extension, HGS-Handgrip Strength.

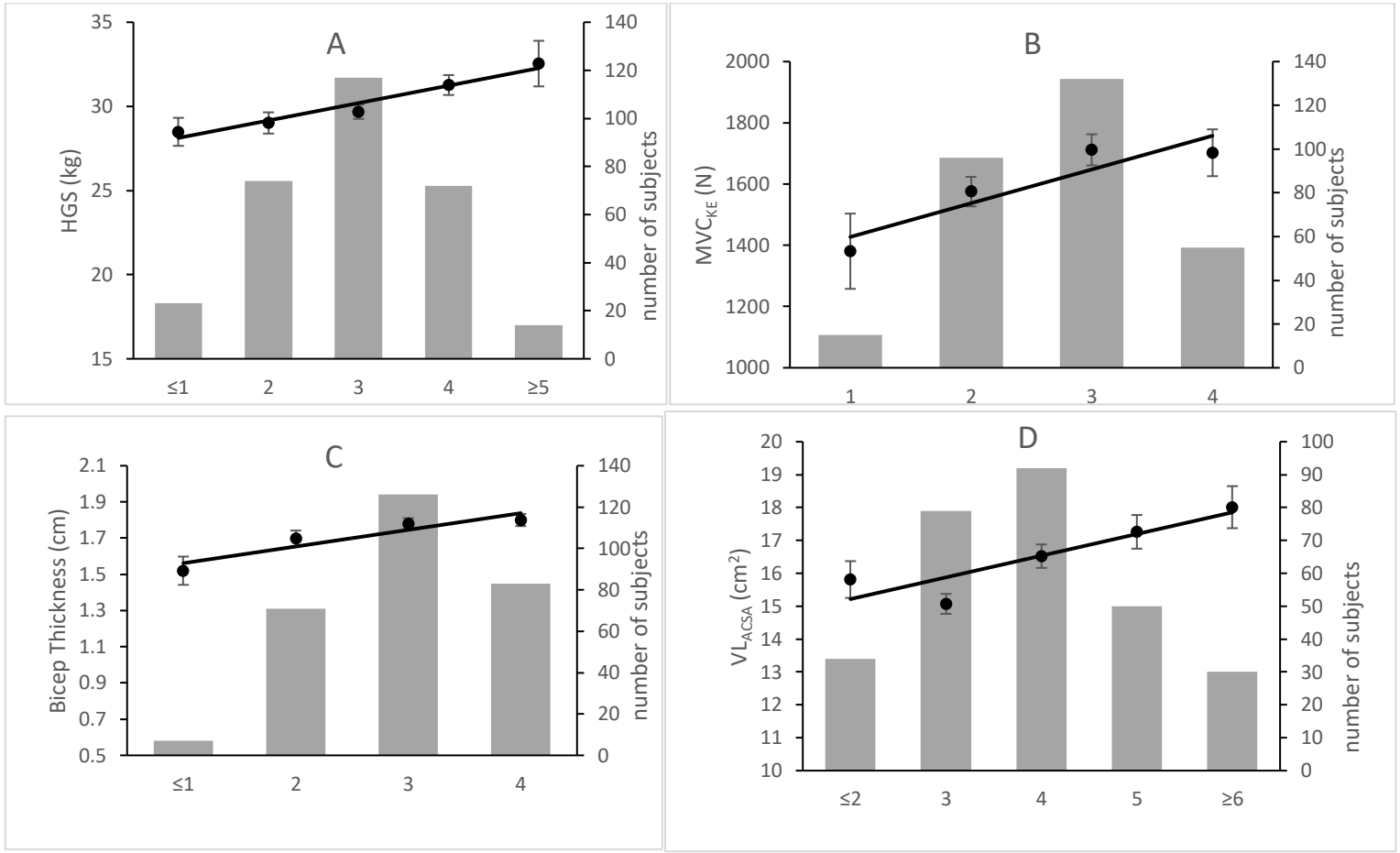

Figure 6.1 Distribution of genetic predisposition score (GPS) with Skeletal Muscle Phenotype

Participant frequency distribution (bars) and GPS (line) for A) HGS (Handgrip strength) B) MVC_{KE} (Isometic Knee extension -Maximum Voluntary Contraction) C) Biceps brachii thickness D) VL_{ACSA} (Vastus Lateralis-Anatomical Cross Sectional Area). Dot and error bar represents absolute mean and standard error of the mean respectively

6.4 Discussion

The present chapter has used the construction of GPSs to explain the effect of gene variants in skeletal muscle phenotypes; muscle mass and muscle strength, in elderly Caucasian females. Unlike previous research that has studied whole body muscle mass (SMM/FFM) and knee strength with a single gene variant or with a polygenic approach, this study focused on the specific muscle size and function within the lower limb (Vastus Lateralis), and upper limb (biceps and forearm) for the first time. It was hypothesised that considering the polygenic nature of skeletal muscle phenotypes, multiple SNPs (24 SNPs in this case) might better explain the genetic influences than when investigated individually. Although the GPSs in this study were calculated based on the limited subset of significant SNPs (GPS_{dd} approach), they explained 2.2%, 8.2%, 5.0% and 2.2% of the variance in bicep thickness, VL_{ACSA}, HGS and MVC_{KE} respectively, in the present elderly Caucasian females. Some of the selected SNPs in this chapter are previously reported to be associated with skeletal muscle phenotypes while some of them are studied for the first time; hence, this chapter is partially replicating the previous findings for some SNPs and suggesting some novel gene variants with skeletal muscle phenotypes in elderly females. It can be implicated from the present data that elderly females possessing more of the predisposing alleles for skeletal muscle mass/strength could maintain their independence and less likely to reach sarcopenic thresholds until much later in life.

It should be considered that the established associations between the GPSs and phenotypes are derived from the same population of elderly women. The rationale for using the same population to create the TGS was established by some of the previous studies (Charlier et al., 2016; He et al., 2018)and allows for the accumulated influence of marginal effect SNPs within a relatively small population. This approach has however been criticised due to the fact that within group GPS correlations may inflate the level of association for a combined GPS. One approach to validate the associations within the present population was to produce a Total Genotype Score (TGS) using a historical evidence based approach as described in several studies (Williams and Folland, 2008; Drozdovska et al., 2013a). Despite being an established approach to conduct GPS and allowing individual data driven SNP allocation to the TGS, the GPSdd used in the present chapter was also compared to the traditional approach (TGS). The present chapter found association between the TGS and

VL_{ACSA} among four phenotypes investigated. It was found using the historical approach that firstly, only nine SNPs could be used (as opposed to 24 in the GPSdd approach) as there was not sufficient evidence for allocating the genotype score in the present elderly females, but secondly that association were observed for VL_{ACSA} consistent with the GPSdd approach. It is acknowledged therefore that cross validation is required in an independent population as discussed below in limitations.

6.4.1 Genetic predisposition scores

This study has assessed the GPS for the first time explaining skeletal muscle phenotypes in a large number of elderly females. A subset of SNPs was selected based on backward regression analysis, a procedure which has been applied by other groups (Bouchard et al., 2010; Buxens et al., 2011; Thomaes et al., 2013; Charlier et al., 2016; He et al., 2018). This study is consistent with other studies that have shown that a higher GPS is positively associated with higher knee strength (Charlier et al., 2016; He et al., 2018) and gains in peak VO_2 max post training (Thomaes et al., 2011). One of the studies so far that has studied regional muscle size measures, rectus femoris diameter measured by ultrasonography, has shown that higher GPS was associated with bigger rectus femoris diameter (Thomaes et al., 2013). The difference in the SNP pool, subject characteristics and the study design might contribute to the difference in predictive power of GPS among several studies. The present study is the first to assess the GPS for vastus lateralis and biceps muscle size; and found that GPS alone predicted 8.2% and 2.2 % variance respectively in the elderly population. As VL is representative of the general quadriceps atrophy with age (Maden-Wilkinson et al., 2013), the GPS predicted variance of 8.2% in this elderly population suggests genetics can partially explain the variance of muscle size; hence, could predict the adverse outcome of sarcopenia in old age. The data on the elderly females in this study shows that if there is an increment in 1 favourable allele, the area of the VL increases by nearly 0.77cm^2. Similarly, GPS predicted that 5.0% of the variance of HGS with 4 subsets of significant SNPs showing that with an increment of 1 predisposing allele, there is an increment in 1kg of HGS. Similarly, GPS explained 2.2% variance in biceps brachii

thickness in elderly population and shows that an increment in 1 favourable allele increases the biceps brachii thickness by approximately 0.06 cm.

While considering the traditional TGS approach based on 9 SNPs, this chapter identified that TGS is associated with VL_{ACSA}, while did not associate with other skeletal muscle phenotypes. This finding is consistent with a previous study that has reported that TGS is associated with athlete status (Drozdovska et al., 2013a). The lack of association with other phenotypes could be due to less influence of genetics on those phenotypes or inclusion of limited number (n=9) of SNPs in the present chapter.

The present study did not find any difference in TGS between the sarcopenic and non-sarcopenic groups. The possible explanation for no difference could be due to the inclusion of less number of SNPs in the present elderly females. Previous studies have reported that the muscle mass/strength phenotypes are multifactorial in nature (Prior et al., 2007). Therefore, the consideration of other environmental factors along with inclusion of many SNPs that can affect skeletal muscle phenotypes might provide better understanding of genetics between the two groups.

6.4.2 Gene variants contributing to Genetic Predisposition Profiles

Several GWAS and case-controlled association studies have reported the association of specific gene variants with skeletal muscle phenotypes; muscle mass and muscle strength. Among the gene variants, some of them are replicated successfully in most of the population, while some of them could be replicated with limited success (Tan et al., 2012). GWAS are often utilized to select the regions in DNA that might be associated with skeletal muscle phenotypes (Liu et al., 2009). Alternatively, the GPS model can be useful to identify any suggestive associations. With the GPS, this study has suggested new associations of some SNPs for the skeletal muscle phenotypes under investigation in the present elderly population. Similarly, our study also confirms some previous associations.

To the author's knowledge, this study is the first to report associations between the muscle size and absolute muscle strength phenotypes in any age group with *PTK2* polymorphisms. The present study has found *PTK2* rs7460 T is a favourable allele for all phenotypes under this study, while *PTK2* rs7843014 C is a predisposing allele for muscle mass phenotypes. The *PTK2* gene encodes for focal adhesion kinase (FAK) protein, and the mentioned polymorphisms have previously been investigated with muscle-specific force in young adults (Erskine et al., 2012; Stebbings et al., 2017) and also with exceptional longevity (Garatachea et al., 2014). Lower expression of FAK observed with *PTK2* rs7460 TT and *PTK2* rs7843014 CC genotypes has been linked with exceptional longevity in a Spanish elderly population (Garatachea et al., 2014); alternatively, higher levels of FAK has been reported in muscle wasting condition such as cancer cachexia (Gabarra-Niecko et al., 2003). Therefore, it is possible that the *PTK2* rs7460 T and *PTK2* rs7843014 C alleles are muscle mass and muscle strength predisposing alleles in this present elderly population (Table 6.3).

Another novel finding from the present study is the suggestive association of skeletal muscle size with *FTO* rs9939609; with the A-allele favouring a larger vastus lateralis muscle. Similar associations of the A-allele have been observed with obesity-related phenotypes in previous GWAS and SNP association studies (Frayling et al., 2007; Livingstone et al., 2016; Al-Serri et al., 2018) and with fat mass and lean body mass (Sonestedt et al., 2011; Livshits et al., 2012). The present study also identified *CNTF* rs1800169 G as a pre-disposing allele for $MVC_{KE,}$ which is consistent to the finding of a previous study (Roth et al., 2001). A functional gene variant *CNTF* rs1800169 with AA genotype has been observed to produce a non-functional protein variant (Takahashi et al., 1994); hence the result of a functional protein in the group possessing GG genotype might explain the higher strength in the present elderly. Similarly, the present study also observed *CNTFR* rs2070802; with the T allele as the predisposing allele for muscle size, specifically VL_{ACSA}. The finding of the present study concurs with others reporting the positive association of TT/T genotype/allele with muscle strength phenotypes (De Mars et al., 2007b). Muscle

mass is the main determinant of muscle strength (Maughan et al., 1983b); hence the association of the *CNTFR* rs2070802 T allele with VL_{ACSA} is convincible in this population. *HIF1A* rs11549465 T allele has been found as a predisposing allele for both muscle size and muscle strength phenotypes in this study. The T-allele is associated with enhanced transactivation capacity (Tanimoto et al., 2003) and found to be overrepresented in weightlifters, wrestlers (Gabbasov et al., 2013), and power-oriented athletes. This suggests individuals demanding more strength for such sports possess significantly higher number of *HIF1A* rs11549465 T–allele than the normal population. This is consistent with the observation from the present study that *HIF1A* rs11549465 T-allele is associated with higher muscle size and strength phenotypes in the present elderly population.

TRHR rs7832552 was associated with muscle size phenotypes (VL-thickness and VL_{ACSA}), with T as predisposing allele. This finding is in line with studies supporting the T allele as the favourable for muscle mass; for example, a GWAS has identified individuals homozygous for T allele to have an average of 2.5 kg higher lean body mass compared to the heterozygotes and C-allele homozygotes, and this was replicated in three independent samples (Liu et al., 2009). In the present study, *ACE* rs4341 polymorphism was associated with muscle size, specifically the G allele was associated with thicker biceps brachii thickness. This polymorphism has been reported to be in linkage disequilibrium with the *ACE* I/D polymorphism (Glenn et al., 2009). The *ACE* D allele is positively related with greater lean mass, body mass and quadriceps muscle volume (Charbonneau et al., 2008) or appendicular fat free mass in older women (Lima et al., 2011). As biceps brachii thickness is representative of muscle size parameters, this study also supports that *ACE* D (G) allele is associated with the thicker muscle size in the present elderly population. Similarly, *MTHFR* rs1801131 polymorphism has been associated with Handgrip strength with T as predisposing allele in elderly population. It has previously been observed that the presence of the C allele appears to be advantageous in sprint-strength and strength athletes, suggesting that exercise induced hypomethylation helps in higher expression of the genes promoting muscle growth (Eynon et al.,

2011). However, it needs to be validated how the *MTHFR* rs1801131 C variant affects the hypomethylation process. On the other hand, *MTHFR* rs1801131 CC genotype has been linked with a higher homocysteine plasma concentration (Castro et al., 2004). Previous studies have evidenced that higher homocysteine levels are associated with reduced physical activity (Dankner et al., 2007) and quadriceps strength (Kuo et al., 2007). The plausible reason to observe T-allele as the predisposing allele for strength in the present study could be due to the detrimental effect of the C-allele that could be mediated by homocysteine levels.

6.5 Limitations

This study has some limitations. There are controversies in some of favourable directions of the SNPs for association with muscle strength, hence the coding might be wrong or the author might have coded from a false positive result. This might have a negative effect on the validity of the GPS model, or could contribute to the lower explained variance in the present data compared to others. Similarly, this study always scored heterozygotes with intermediate values in consistency with a previous study (He et al., 2018). However, it is likely that some of the heterozygotes might have its affect as comparable to homozygotes (complete dominance) or even better than homozygotes (over dominance).Some of this limitation was overcome by including a historical TGS based approach, however this was only partially successful due to there being only sufficient evidence to include 9 SNPs as opposed to the 24 from the GPSdd approach. It should also be noted that in this study each predisposing allele carries an equal weight in the summed GPS_{dd} model. This ignores the fact that different gene variants might affect the muscular phenotypes unequally. Therefore, weighing the contribution of each SNPs for the observed phenotypes might be more effective than assigning the equal weight for all SNPs (Massidda et al., 2014; Charlier et al., 2016). The observed muscular phenotypes are also affected by environmental factors such as lifestyle, particularly present or previous levels of physical activity which could potentially induce epigenetic modifications. In those instances, GPS might be unfavourable to predict the exact

variance observed in the participants. Epigenetic processes such as methylation and phosphorylation might affect gene expression differently; hence, accounting for those modifications could also improve the understanding of the polygenic effect on muscle related phenotypes. It can also been seen that some of the SNPs such as *ACTN3* rs1815739 R-allele which have been replicated so many times and known as the predisposing allele for skeletal muscle function did not show any association in this study. This might be due to the interactions with other SNPs considered in this study. Similarly, the *ACTN3* genotype is reported to be associated with high velocity contraction of muscle in most of the studies (Erskine et al., 2014; Broos et al., 2016) which is different from isometric knee extensor strength included in the present study. Furthermore, many SNPs have been identified to affect skeletal muscle phenotypes; hence, accounting all the SNPs might produce different results. Similarly, sub-group validation would be impossible within sarcopenic group in the present study, due to small numbers after dividing the same population into test group and validation group. With all of those (commonly encountered) limitations of the GPS approach being considered it is likely that future studies investigating similar phenotypes and participants, would expect higher or lower levels of explained variance compared to those reported here. Replication studies are therefore encouraged to validate the present findings on the sarcopenic muscle.

6.6 Conclusion

In the current chapter, a GPS_{dd} alone was able to explain up to 8.2% and 5.0% variance in skeletal muscle size (VL_{ACSA}) and muscle strength (HGS) phenotypes respectively in elderly females. This study identified genetic variants *PTK2* rs7843014, *PTK2* rs7460, *ACE* rs4341, *FTO* rs9939609, *CNTF* rs1800169, *CNTFR* rs2070802, *MTHFR* rs1801131, *MTHFR* rs17421511, *HIF1A* rs11549465 and *TRHR* rs7832552 to be associated with some of the skeletal muscle phenotypes under investigation. Similarly, VL_{ACSA} was the phenotype that was associated with both traditional TGS and GPSdd approaches. The present chapter also concluded that there is no difference in TGS between the sarcopenia and non-sarcopenia groups.

Identifying the gene variants that might affect the skeletal muscle phenotypes could be beneficial in targeting the development of treatments against muscle wasting disorders such as cachexia and sarcopenia.

7 . Discussion

7.1 Main findings and implications for future research

The main findings of the present thesis established how sarcopenia prevalence varied with different definitions, as observed in previous studies (Bijlsma et al., 2013). Within Chapter 3, a meaningful definition was established based on how the neuromuscular characteristics are differentiated between sarcopenic and non-sarcopenic groups, whilst maintaining a reasonable population sample for subsequent genetic analysis. A novel approach was identified using a Z-score consistent with guidelines established by the EWGSOP that was able to differentiate the sarcopenia and non-sarcopenia groups using neuromuscular phenotypes. Using this robust definition of sarcopenia, Chapter 4 revealed novel associations of sarcopenia with the genetic variants, *ACE* rs4341 and *HIF1A* rs11549465. In Chapter 5, association of gene variants with several skeletal muscle phenotypes, that were differentiated by sarcopenia definitions in chapter 3, were investigated. Twelve SNPs were found to be associated with skeletal muscle phenotypes of strength (MVC_{KE}, MVC_{EF} and HGS) and muscle size (VL_{ACSA}, VL thickness and biceps brachii thickness). Based on these observations, a polygenic approach was undertaken in Chapter 6, and determined that GPS_{dd} approach was able to explain up to 8.2% variance of muscle size measures (VL_{ACSA}) and up to 5.0% of muscle strength (HGS).

7.1.1 Sarcopenia prevalence and neuromuscular outcome measures

Prior to investigating the genetic influence in sarcopenia, it was important to identify a definition of sarcopenia that can differentiate the most meaningful neuromuscular outcome measures between sarcopenia and non-sarcopenia groups. However, the lack of consensus in the operational definition leads to varying prevalence in the same population (Dupuy et al., 2015). Hence, Chapter 3 investigated the definition of sarcopenia based on discriminating power between two groups with 4 definitions: Definition 1 (SMI_A), Definition II (% SMM), Definition III (EWGSOP) and a novel Definition IV (Z-score). The previous definitions failed to fully discriminate the characteristics of sarcopenic elderly on the basis of

neuromuscular phenotypes; while a novel approach based on Z-score differentiated the two groups in all the functional characteristics investigated in the present thesis (biceps brachii thickness, VL_{ACSA}, MVC_{EF}, MVC_{KE} and OLST). These phenotypes of low muscle strength and low muscle mass have been previously associated with the risk of high mortality (Janssen et al., 2000b), falls (Landi et al., 2012b), frailty (Clegg et al., 2013) and reduced ability to reach functional thresholds for activities of daily living (Shiozu et al., 2015). Using Z-score definition based on HGS and SMI, a definition of sarcopenia was established for subsequent analysis within the thesis.

7.1.2 Association of SNPs with sarcopenia and skeletal muscle phenotypes

As sarcopenia is multifactorial in nature and genetics has a high contribution for muscle mass and muscle strength, the association of sarcopenia with gene variants selected in the current thesis were investigated in Chapter 4. 24 SNPs were selected based on the rationale presented in chapter 1, and associations were assessed between sarcopenic and non-sarcopenic elderly women. This thesis identified *ACE* rs4341 CC and *HIF1A* rs11549465 CC as risk genotypes for being in the sarcopenia group. The possible reasons for association of *ACE* rs4341 and *HIF1A* rs11549465 genotypes with sarcopenia are discussed later in this chapter. With the identification of new gene variants (*ACE* and *HIF1A*) that are associated with sarcopenia, it might be beneficial for the screening for risk genotypes of elderly population that are prone to develop sarcopenia at their old age. Previous studies have investigated *ACTN3, IL6* and *VDR* polymorphisms association with sarcopenia; however, *IL6* rs1800795 was not found to be associated with sarcopenia (Tasar, 2018); which is in agreement with the present thesis. Although previous studies have found association of *VDR FokI* and *ACTN*3 R577X with sarcopenia (Roth et al., 2004; Cho et al., 2017), the present study did not find any such association. The probable reason could be the inclusion of low muscle mass only as the definition for defining sarcopenia in the previous studies, while the present study used low SMI and low HGS in combination.

As sarcopenia is not a clinical definition to distinguish people's ability to perform daily activities, the other skeletal muscle phenotypes that might affect an individual's independence were analysed for association with SNPs in Chapter 5. The main finding of this thesis (Chapter 5) was the identification of twelve gene variants associated with investigated skeletal muscle phenotypes. In doing so, it replicated the findings of a previous study for one gene variant; and identified 11 other novel gene variants with one or more skeletal muscle phenotypes; muscle strength (HGS, MVC_{EF} and MVC_{KE}), and muscle size (biceps brachii thickness, VL-thickness and VL_{ACSA}).

The gene and muscle phenotype associations in chapter 4 and 5 are consistent with previous studies that have found either similar SNPs-phenotype associations, or have presented favourable allele/genotypes in a range of populations. These previous associations are presented below (Table 7.1), after which, mechanisms for the most prominent SNPs associations observed in Chapters 4-5 are discussed.

Table 7.1 Associations between genotype and skeletal muscle phenotypes in present elderly population and suggesting similar associations in previous studies

Gene variants	Present thesis favourable genotypes	Present thesis phenotypes	Previous supporting findings
***TRHR* rs7832552**	CT+TT Vs CC	VL-thickness	Lean body mass (Liu et al., 2009)
***HIF1A* rs11549465**	CT+TT Vs CC CC+CT Vs TT	VL_{ACSA}/HGS VL thickness Sarcopenia	T-allele overrepresentation in weightlifters and wrestlers (Gabbasov et al., 2013) and power-oriented athletes (Cięszczyk et al., 2011)
***PTK2* rs7460**	AT+TT Vs AA	MVC_{EF} HGS	Exceptional longevity (Garatachea et al., 2014)
***PTK2* rs7843014**	AC+AA Vs CC	HGS	Exceptional longevity (Garatachea et al., 2014)
***ACVR1B* rs10783485**	GT+TT Vs GG	VL_{ACSA} Biceps brachii thickness	Knee strength (Windelinckx et al., 2011)
***ACVR1B* rs2854464**	AG+GG Vs AA	HGS	Knee strength (He et al., 2018)
***FTO* rs9939609**	AA+AT Vs TT	VL_{ACSA}	Fat mass and lean body mass (Sonestedt et al., 2011)
***NOS3* rs1799983**	TT+GT Vs GG	MVC_{KE}	T allele as favourable allele in power oriented athletes (Gómez-Gallego et al., 2009); and long distance swimmers (Zmijewski et al., 2018).
***CNTF* rs1800169**	AA+AG Vs GG	MVC_{KE}	Handgrip strength (Arking et al., 2006), gain in absolute strength after training (Walsh et al., 2009), lower HGS in middle aged female with AA genotype (De Mars et al., 2007b).
***ACE* rs4341**	GC+GG Vs CC	Biceps brachii thickness Sarcopenia	quadriceps muscle volume (Charbonneau et al., 2008)), better athletic performance (Costa et al., 2009a),

			higher muscle strength (Williams et al., 2005)
***COL1A1* rs1800012**	AA+AC Vs CC	HGS	Less likely for soft tissue injury in professional soccer players with AA genotype (Wang et al., 2017a)
***MTHFR1* rs1801131**	GT+GG Vs TT	MVC_{EF}	CC genotypes exhibiting higher Homocysteine plasma concentration (Castro et al., 2004); and that has been linked with reduced physical activity (Dankner et al., 2007) and reduced quadriceps strength (Kuo et al., 2007)

Grey shaded genotype groups denote the favourable genotypes for skeletal muscle phenotypes investigated. Abbreviations: MVC_{EF}, Isometric Maximum Voluntary Contraction- Elbow flexion; MVC_{KE}, Isometric Maximum Voluntary Contraction-Knee extension; HGS, Handgrip strength; $VL_{ACSA,}$ Vastus lateralis anatomical cross sectional area

PTK2 rs7460 TT and *PTK2* rs7843014

PTK2 rs7843014 and *PTK2* rs7460 were investigated for association with sarcopenia (Chapter 4) and skeletal muscle phenotypes investigated (Chapter 5) in the current thesis. Although there was no association of *PTK2* polymorphisms with sarcopenia (Chapter 4), in Chapter 5 for the first time associations of *PTK2* rs7843014 and *PTK2* rs7460 were found with upper limb strength; *PTK2* rs7460 T-allele carriers for MVC_{EF} and HGS and *PTK2* rs7843014 A-allele carriers with HGS only. These SNPs were previously investigated with VL specific force (Stebbings et al., 2017) and exceptional longevity (Garatachea et al., 2014). The *PTK2* rs7843014 A-allele and *PTK2* rs7460 T-allele have previously been associated with exceptional longevity in an elderly Spanish population and lower Focal Adhesion Kinase (FAK) in luciferase gene assay (Garatachea et al., 2014). The authors speculated the low FAK could be associated with normal cell division and thus associated with longevity (Garatachea et al., 2014) as higher FAK has been previously associated with cancer and metastasis (Sood et al., 2004; Lark et al., 2005). Similarly, a lower muscle strength has been linked with early mortality, potentially reflecting this association between FAK, *PTK2* polymorphisms and longevity (Metter et al., 2002). Hence, the identification of novel associations of these SNPs with skeletal muscle phenotypes may be due to lower FAK activity in the present elderly participants who are *PTK2* rs7460 T-allele carriers and *PTK2* rs7843014 A-allele carriers.

CNTF rs1800169

The present thesis did not find any association of *CNTF* rs1800169 with sarcopenia (Chapter 4); while it found that elderly women with *CNTF* rs1800169 GG genotype had higher MVC_{KE}. The biological role of CNTF could explain the observed association in the present thesis. Myogenesis process upregulates and atrophy mediators downregulates with the CNTF treatment (Tsompanidis et al., 2016). CNTF level decreases with ageing and exogenous administration of CNTF in older rats has shown to improve muscle strength (Guillet et al., 1999). The non-functional protein has previously been associated with *CNTF* 1800169 AA genotype (Takahashi et al., 1994). It is therefore reasonable to assume that individuals producing the

functioning CNTF protein, GG homozygotes, would exhibit a higher MVC_{KE}. The present thesis finding is consistent with previous reports that showed higher handgrip strength in G-allele carriers amongst elderly women aged 70-79 years (Arking et al., 2006), greater gain in absolute strength in women post training with GG genotypes (Walsh et al., 2009) and lower HGS in middle aged females with AA genotype (De Mars et al., 2007b).

HIF1A rs11549465

To the best of the author's knowledge, this is the first study reporting an association of *HIF1A* rs11549465 with sarcopenia (Chapter 4), and associations with HGS, VL-thickness and VL_{ACSA} (Chapter 5) in an elderly population. The present thesis found *HIF1A* rs11549465 T-allele carriers had larger VL_{ACSA} and HGS than CC homozygotes, while TT homozygotes were found to be associated with thicker VL-thickness than C-allele carriers (Chapter 5). The biological role of HIF1A and the functional efficacy of *HIF1A* rs11549465 could explain the possible reason for this association. The *HIF1A* rs11549465 T-allele has previously been shown to enhance the transactivation capacity of HIF1A (Tanimoto et al., 2003). Although HIF1A is primarily associated with a hypoxic upregulation of glycolytic muscle metabolism in fast twitch muscle, there have been studies that have found that T-allele is overrepresented in weightlifters and wrestlers (Gabbasov et al., 2013) and power-oriented athletes (Cięszczyk et al., 2011). This overrepresentation of *HIF1A* rs11549465 T-allele within sports requiring a high level of strength and muscle mass is consistent with those elderly women who were not-sarcopenic, and had higher strength and muscle size phenotypes. Whist it is well established that the transactivation capacity increases with the T allele, the underlying mechanism/s responsible for the observed association with sarcopenia (Chapter 4) and HGS, VL_{ACSA} and VL-thickness (Chapter 5) remains unclear and warrants further research.

ACE rs4341

In the present thesis, *ACE* rs4341 has been investigated for the association with sarcopenia (Chapter 4) and skeletal muscle phenotypes (Chapter 5). This polymorphism has been reported to be in linkage disequilibrium with *ACE* I/D polymorphism, with the C allele behaving as I allele. Individuals with CC genotype were more likely to be sarcopenic and have thinner biceps brachii thickness (Chapter 5). These novel associations of *ACE* rs4341 with sarcopenia and biceps brachii thickness could be explained by the biological and function role of the ACE enzyme and its altered activity with I/D allele. The *ACE* D allele has been previously associated with higher ACE activity (Rigat et al., 1990) and thus helps in effective conversion of Angiotensin I to Angiotensin II, which is known to modulate skeletal muscle hypertrophy (Gordon et al., 2001). The current thesis finding is in agreement with previous studies that have shown the association of D allele with higher muscle mass and muscle strength phenotypes; such as higher quadriceps muscle volume (Charbonneau et al., 2008), better athletic performance (Costa et al., 2009b) and higher muscle strength (Hopkinson et al., 2004; Williams et al., 2005). Future research should extend these observations to different muscle and population samples to ascertain if the findings of Chapter 4 and Chapter 5 are specific to biceps brachii of elderly women or if these findings can be replicated in male elderly or younger female/male and other ethnic groups.

NOS3 rs1799983

To the knowledge of the author, the current thesis (Chapter 5) is the first to associate higher MVC_{KE} and with T-allele carriers of *NOS3* rs1799983 in the elderly. This finding is consistent with previous studies that have reported the T allele as favourable in power oriented athletes (Gómez-Gallego et al., 2009) and long distance swimmers (Zmijewski et al., 2018). Biologically, the *NOS3* gene encodes the enzyme endothelial NOS (eNOS) that catalyses the synthesis of NO. NO has been identified as a determinant of individual variations in health and exercise related phenotypes (Bray et al., 2009), skeletal muscle fibre conversion (Martins et al., 2012), mitochondrial energy production (Brown, 2007) and normal muscle

hypertrophy (Smith et al., 2002). The *NOS3* rs1799983 T-allele has been associated with higher NO activity compared to the C-allele (Persu et al., 2002). This association of increased NO activity from previous studies and its role in muscle phenotypes provide a potential basis for the present observation that elderly women who carry the T-allele possess greater muscle strength.

MTHFR rs1801131

The current thesis (Chapter 4) did not find any association of *MTHFR* rs1801131 with sarcopenia, but a novel association between the *MTHFR* rs1801131 polymorphism and MVC_{EF} in elderly women was observed. It is possible that the *MTHFR* gene variant association with homocysteine and methylation may explain the observed association in the present thesis (Chapter 5). The *MTHFR* rs1801131 CC genotypes exhibit higher homocysteine plasma concentration (Castro et al., 2004). The hyperhomocysteinaemia has been linked with reduced physical activity (Dankner et al., 2007) and reduced quadriceps strength (Kuo et al., 2007) previously. Consistent with the lower strength in the present C-allele carriers, muscle strength might have been affected by the homocysteine-related protein damage due to homocysteinylation (Kuo et al., 2007).

Contrasting to this hypothesis, however, is the fact that the CC genotype is favourable in athletic populations (Zarebska et al., 2014), and it has been suggested that this genotype might be associated with hypo-methylation, and leads to favourable expression of genes involved in myogenesis (Zarebska et al., 2014). Although the finding is opposite to the current thesis, it is possible that methylation pattern will differ with age in *MTHFR* gene. It is thus suggested that the methylation pattern in *MTHFR* gene should be studied functionally with ageing to confirm the present study result.

TRHR rs7832552

TRHR rs7832552 was investigated for association with sarcopenia (Chapter 4) and skeletal muscle phenotypes (Chapter 5). Although the present thesis did not find association of TRHR genotype with sarcopenia, it was associated with muscle size measures, with T-allele carriers having a thicker VL and larger VL_{ACSA} than CC homozygotes. This finding is consistent with the report that T-allele carriers have a higher lean mass (Liu et al., 2009). The mechanism underlying higher muscle mass in T-allele carriers could be explained by raised luciferase activity, observed in C212 muscle cell lines. The higher luciferase could result in a higher thyroid hormone level known to preserve muscle strength (Salvatore et al., 2014). Despite higher muscle mass in T-allele carriers, there was no difference in strength in this population (Chapter 5). This is similar to the previous study that reported no association of *TRHR* genotype with muscle strength (Lunardi et al., 2013).

COL1A1 rs1800012

In the present thesis, *COL1A1* rs1800012 T-allele carriers had higher HGS (Chapter 5), although this SNP was not associated with sarcopenia (Chapter 4). There is a change in binding capacity of Sp1 transcription factor with G-T transition in *COL1A1* rs1800012 (Mann et al., 2001) and results in a higher proportion of alpha 1 than alpha 2 compared to their normal ratio of 2:1. The present study showed that T-allele carriers have greater strength than GG homozygotes, which is contrary to a previous study in an elderly male population (Van Pottelbergh et al., 2001). The present finding might be possible due to the extra production of collagen alpha 1 with G-T transition. T–alleles as favourable allele, reported as stronger in chapter 5, are previously found to have higher bone mineral density (Berg et al., 2000), also described as appear to experience lower risk for ligament and tendon sports related injury (Wang et al., 2017a). This might suggest there is some favourable expression of collagen in T-allele carriers, which presents as higher muscle strength in elderly women. As there is contrary in the observation with muscle strength and BMD, a

good predictor of muscle strength, the author urge to study with this polymorphism in other population.

FTO rs9939609

The present thesis investigated the association of the *FTO* rs9939609 polymorphism with sarcopenia (Chapter 4) and skeletal muscle phenotypes (Chapter 5). No association was observed between *FTO* rs9939609 and sarcopenia; for the first time, however, an association was found between a larger VL_{ACSA} and elderly *FTO* A-allele carriers. *FTO* has been widely studied with obesity-related phenotypes (Frayling et al., 2007). More recently, the role of FTO in skeletal muscle development has been investigated. FTO-deficient mice have impaired skeletal muscle development, and increased levels of FTO during myogenic differentiation and silencing lead to myogenic suppression in cell lines (Wang et al., 2017c). The *FTO* rs9939609 A-allele has been linked with higher fat mass and lean body mass (Sonestedt et al., 2011). It is possible that the *FTO* A-allele favourably influences lean mass accretion, positively influencing body composition and thus is associated with VL_{ACSA}.

ACVR1B rs2854464 and *ACVR1B* rs10783485

Other novel associations in chapter 5 were that of the *ACVR1B* rs2854464 G-allele with HGS and *ACVR1B* rs10783485 GG genotype with thicker biceps brachii and larger VL_{ACSA}. *ACVR1B* gene encodes the activin A receptor type 1b protein, which is a member of TGF beta family and known to be involved in molecular pathways regulating myostatin and activin signalling - signalling pathways identified as a negative regulator of muscle growth (McPherron et al., 1997). The findings of the current study (Chapter 5) is similar to the previous studies reporting a *ACVR1B* rs10783485 G-allele association with knee strength in 20-90 years old Caucasian males (Windelinckx et al., 2011) and that the *ACVR1B* rs2854464 G-allele (He et al., 2018) was favourable for knee strength in a 60-83 years old elderly population. It is

therefore possible that the G-allele of the two *ACVR1B* SNPs in Chapter 5 might show less antagonist nature in muscle signalling pathways.

In contrast, however, studies have reported *ACVR1B* rs2854464 AA genotype as favourable for knee strength in young adults (Windelinckx et al., 2011) and to be over-represented in Caucasian sprint/power athletes (Voisin et al., 2016). As the real mechanism behind the association is not functionally tested, the author urges functional study and validation of this finding in other populations.

7.1.3 Polygenic profiling

Since both muscle mass and muscle strength are multifactorial in nature and there is high heritability for muscle mass and muscle strength, there was the possibility that numerous SNPs would be associated with skeletal muscle phenotypes. In Chapter 6, it was found that there was involvement of subsets of SNPs for the observed phenotypes when multiple SNPs were considered at a time. The GPS in Chapter 6 were calculated based on the limited subset of significant SNPs (GPS_{dd} approach) which were able to explain 5.0%, 2.2%, 2.2% and 8.2% variance of HGS, MVC_{KE}, biceps brachii thickness and VL_{ACSA} respectively, in the present elderly Caucasian females. Although the amount of explained variance for muscle size and strength measures in this thesis are rather small compared to the heritability estimates from twin studies phenotypes (Tiainen et al., 2004; Silventoinen et al., 2008; Zempo et al., 2017). Heritability estimates are however based on genome wide influences, in contrast in the present thesis, the variance of muscle size and muscle strength measures when accounted by only 24 SNPs. There are numerous genes identified that affect the skeletal muscle phenotypes (Roth et al., 2012; Garatachea and Lucía, 2013; Matteini et al., 2016; Tikkanen et al., 2017; Willems et al., 2017). Given the multitude of external factors that can influence muscle phenotypes as people age, the associated variances in the present thesis represent a step forward in the understanding of the genetics of sarcopenia and the ageing muscle. The lower explained variance in the present thesis suggests the likelihood

of involvement of many SNPs (more than considered in the present thesis) and other environmental factors that could explain the observed muscle phenotypes. The fact remains however, that as this is the first GPS study of muscle phenotypes in elderly women, with the uncovering of more associated SNPs, the strength of the GPS association can only improve in future research.

7.2 Methodological considerations and limitations

The findings of the current thesis have a wide range of implications in disease related to muscle wasting. With the identification of new gene variants that are associated with skeletal muscle phenotypes important for the maintenance of individual independence, the finding can be implemented as the new target for the treatment of sarcopenia and thus generating new directions for future research. Considering sarcopenia can affect both upper and lower limb muscle mass and muscle strength (Candow and Chilibeck, 2005), vastus lateralis and biceps brachii muscles were chosen as suitable muscles for assessing the size measures in the present thesis. Similarly, elbow flexion and handgrip strength were selected for the measurement of upper limb strength and knee extension muscle strength for assessing lower limb strength. As ageing results in deterioration in physical performance; hence the standing balance test was considered for the study.

Knee extensor strength is important for various functional activities such as ambulation (Fukagawa et al., 1995), jumping (Yamauchi and Ishii, 2007) and squatting (Fujita et al., 2016). Lower muscle strength in the elderly, has been shown to contribute specifically to a decrease in walking speed (LaRoche et al., 2011), a decrease in gait speed (Alqahtani et al., 2017) and a decline in quality of life (Trombetti et al., 2016). While it is simple to assess the whole quadriceps femoris (QF) function via the use of dynamometer (Chapter 2), it is less convenient to assess morphological characteristics of each constituent muscle (Blazevich et al., 2006). Therefore, the VL muscle was selected as a representative of QF for more detailed assessment as consistent with previous studies (Trappe et al., 2001; Reeves et al.,

2004). As there are differences in CSA between the muscles of the lower limb, more studies are required to confirm if the implications made in the current thesis in directing developments for sarcopenia are equally comparable between different muscle groups.

In terms of the upper limb strength phenotypes, decreased handgrip strength in the elderly has been linked with health decline, specifically its association with functional disability (Giampaoli et al., 1999) and mortality (Al Snih et al., 2002). Throughout the thesis, based on the relationship between knee extensor strength/HGS and functional measures such as activities of daily living, implications are made about the impact of having unfavourable alleles/genotypes, and the potential impact on broader daily life. During the conception of the phenotype assessment, it was determined that (due to time constraints), identifying the neuromuscular phenotype in the first instance was the first step in understanding the genetics of sarcopenia. Subsequent research should include direct measures of daily life tasks, including gait analysis, stair climbing and more functional measures. As mentioned, however, the neuromuscular phenotypes presented throughout this thesis are considered essential as the determinants of those functional tasks.

In the present thesis, the body composition of participants was measured using Bioelectrical Impedance Analysis (BIA). SMM was estimated using the cross-validated predictive equation (Janssen et al., 2000a). BIA has been described as a reliable and valid tool when compared with MRI (Janssen et al., 2000a). It should be acknowledged that the estimation of FFM is affected by several factors such as temperature (8% change in resistance with 8.4 °C change in skin temperature) (Gudivaka et al., 1996), hydration status (Lukaski et al., 1986) and body position while testing (Deurenberg et al., 1998). The standard error of the estimate (SEE) is reported between 0.81-2.7 kg when compared to appendicular lean mass or skeletal muscle mass measured with MRI/DEXA (Janssen et al., 2000a). It is also affected by sex, age, disease state, or ethnicity (Rush et al., 2006). In the present thesis, participants were tested during the test regardless if they were hydrated or not

before the test; hence, the result may have been affected. The implications are reduced somewhat, particularly in the sarcopenia chapter as a) classification was based on the Z-score rather than the absolute SMM, and b) there were no genetic differences or associations with any of the BIA measures.

One Leg Standing balance test (OLST) was performed to access balance impairment of the elderly participants. This test has been described as a simple, predictive and inexpensive marker for screening the low functional level and frailty associated with ageing (Vellas et al., 1997b) and 30 s duration is considered the standard test (Bohannon et al., 1984). Although the normal ranges with eyes open condition for 60-69 years and 70-79 years are suggested as 22.5±8.6 s and 14.2±9.3 s respectively, and with eyes closed 10.2±8.6 s and 4.3±3.0 s respectively (Bohannon et al., 1984), over 60% of the participants in the current population passed the 30 s-eye open test easily (70.7±5.7 years, OLST 23.9±9.7s). Therefore, the test might not be predictive for checking the balance impairment in the present thesis. It is important to note, however, that despite these shortcomings OLST was differentiated by the sarcopenia definition between the two groups in the present study.

7.3 Conclusion

The findings of the current thesis add to the growing body of literature investigating a genetic influence on human skeletal muscle phenotypes, in particular to sarcopenia and skeletal muscle size and strength measures in elderly Caucasian female population. Additionally, the study also estimated the predictive power of the GPS approach to explain the variance in muscle size and muscle strength phenotypes observed in studied elderly women.

As there is no consensus in the clinical definition of sarcopenia, the present study identified the definition based on a Z-score could differentiate most of the neuromuscular characteristics of sarcopenic elderly and identified the *HIF1A* rs11549465 and *ACE* rs4341 as the gene variants associated with sarcopenia. In

addition, 12 novel gene associations were observed with skeletal muscle phenotypes investigated in the present thesis. The biological role of associated SNPs, specifically genes identified as encoding for structural protein, epigenetic regulator, transcriptional regulator, muscle growth inhibitor, body composition regulator or myotrophic factor could explain the observed associations. A GPS_{dd} approach was successful in explaining up to 14.5% and 17.2% variance of the observed skeletal muscle size and strength respectively in the women studied in the present thesis. The present findings could contribute to augmenting understanding of skeletal muscle disorders, which may have implications for how sarcopenia and cachexia or other muscle wasting condition are treated and/or prevented in future.

7.4 Directions for future research

The specific areas for future research have been explained in the subsequent chapters of the current thesis. Therefore, this section discusses the broader directions for future research, considering the findings of the current thesis, to add knowledge in the field of ageing genetics. Within the chapters included in the present thesis (Chapter 4, 5 and 6), SNPs were found to be associated with several functional and morphological skeletal muscle phenotypes and sarcopenia. Although the current thesis findings (Chapter 5) replicated the finding of *CNTF* rs1800012 in an independent elderly population and identified several novel gene associations with skeletal muscle phenotypes, several genes have been identified as potential candidates for associations with skeletal muscle phenotypes and athlete performance (Abe et al., 2018). Such findings should be replicated independently or investigated further using direct measurements of skeletal muscle mass and muscle strength (related phenotypes) with a similar approach to that employed in the current thesis. Hence, new research should bolster the field of ageing muscle genetics by strengthening the evidence of existing associations by independently replicating in other populations and identifying the new gene variants associated with skeletal muscle phenotypes with direct measurement of a plethora of muscles in a large and different population.

In the present elderly population, the finding demonstrated a somewhat unpredictable nature of candidate gene associations. For instance, *ACTN3* genotype, which is widely studied with multiple phenotypes, did not show any association with any of the phenotypes in the current study. On the other hand, some of the SNPs showed associations with HGS but not with MVC_{EF}. The findings of the current thesis therefore suggest the inclusion of broad, and where possible, rigorous measurements of phenotype.

It is well established that both skeletal muscle size and strength are polygenic phenotypes, in the present thesis 24 SNPs predicted 8.2% variance of muscle size measures and 5.0% for muscle strength measures. Therefore, the future research should focus on incorporating the SNPs reported in the present thesis and those reported in other studies.

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Appendices

Appendix 1. Conference and Symposium: Poster presentation

YLS 2017, 25th November, Musculoskeletal Ageing Research, Derby UK.

The assessment of previously established definitions of sarcopenia in elderly women.

Praval Khanal [1,3], Lingxiao He[1,3],Gladys L Onambélé-Pearson [1], Georgina K Stebbings [1], Hans Degens [2], Alun G Williams [1], Martine Thomis [3], Christopher I Morse[1]
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The gerontological syndrome, sarcopenia, has been defined using measures of skeletal muscle mass index (SMI) and handgrip strength (HGS) in the elderly [1]. Any definition of sarcopenia should be able to discriminate meaningful outcome measures between sarcopenic and non-sarcopenic (NS) elderly. Independence and activities of daily living in the elderly are determined by the decline in neuromuscular outcome measures such as muscle size, strength and balance performance [2]. The purpose of this study is to identify which commonly used standard definitions of sarcopenia in older Caucasian females discriminate meaningful outcome measures between sarcopenic and NS older women.

150 Caucasian participants (71±5 yrs, 1.59±0.06 m, 66.8±12.3 kg, mean±SD) were classified into two groups, sarcopenic and NS, using 5 previously established definitions based on measures of SMI, (Skeletal Muscle Mass (derived from bio-impedance measured impedance) divided by height^2) and HGS. Six neuromuscular parameters were measured to assess whether differences existed between sarcopenic and NS participants: Isometric Maximum Voluntary Contraction-Knee Extension (MVC_{KE}) and Isometric MVC_{EF} (Elbow Flexion) were measured by dynamometer. Biceps brachii muscle thickness and Vastus Lateralis Anatomical Cross Sectional Area (VL_{ACSA}) were measured with B-mode ultrasonography. Physiological Cross Sectional Area of Vastus Lateralis (VL_{PCSA}) was measured as volume/fascicle length. 30sec standing balance test was performed with eyes open (BT-EO) or eyes closed (BT-EC).

Based on previous definitions of sarcopenia, the prevalence of sarcopenia in the present elderly ranged from 1.3-70%. The definition suggested by EWGSOP (2SD below young SMI and HGS, sarcopenic prevalence = 21%) shows the sarcopenic group to have lower Isometric MVC_{KE}, VL-PCSA, BT-EO, and BT-EC compared to NS (4/6 measured outcomes). Of the other sarcopenic definitions used; 2SD SMI below young, 2SD SMI below old, least quintile of HGS, cut-off from NHANES III study [3] sarcopenic prevalence was 36.7%, 1.3%, 20% and 70% respectively, and showed sarcopenic group differences in 1, 0, 2 and 0 of the neuromuscular outcome measures, respectively.

In conclusion, when considering definitions of sarcopenia, based on the ability to distinguish differences in neuromuscular parameters between sarcopenic and NS, the EWGSOP was the only definition to discriminate MVC_{KE}, and VL_{PCSA}, and showed group differences in a further 2 measures. Despite sarcopenia being classically defined as a decline in muscle mass, the only definition that was able to successfully discriminate MVC_{KE} and PCSA between sarcopenic and NS, included the measurement of handgrip strength and SMI together.

References. (1) Cruz-Jentoft, A.J., et al., *Sarcopenia: European consensus on definition and diagnosisReport of the European Working Group on Sarcopenia in Older People.* Age & ageing, 2010. **39**(4): p. 412-423. (2) Rantanen, T., et al., *Muscle strength as a predictor of onset of ADL dependence in people aged 75 years.* Aging clinical & experimental research, 2002. **14**(3 Suppl): p. 10-15. (3) Davison, K. K., et al., *Percentage of body fat and body mass index are associated with*

mobility limitations in people aged 70 and older from NHANES III. Journal of the American Geriatrics Society, 2002. **50**(11): 1802-1809.

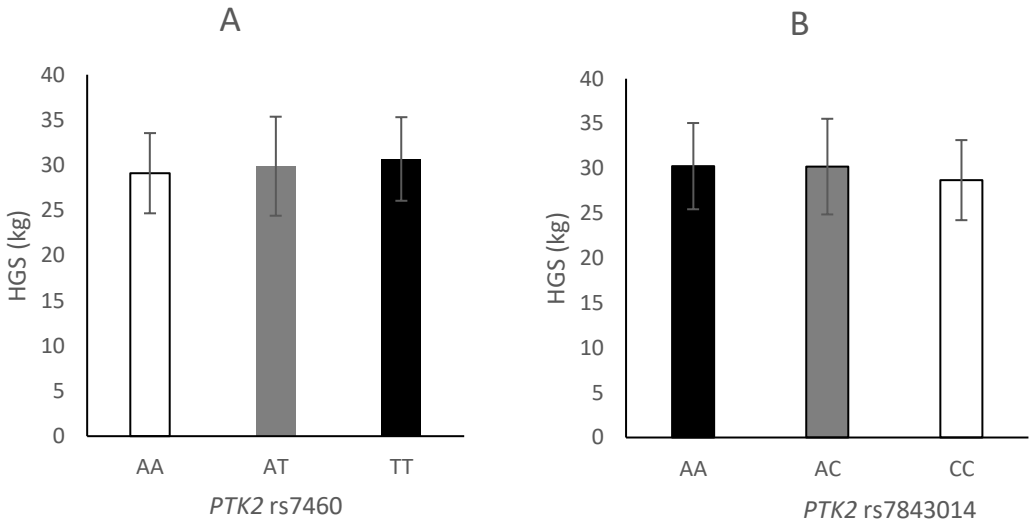
Appendix 2

Table: Polymorphisms used in genotyping, identification of allele-specific probes and when known the flanking primers and probes used for DNA amplification

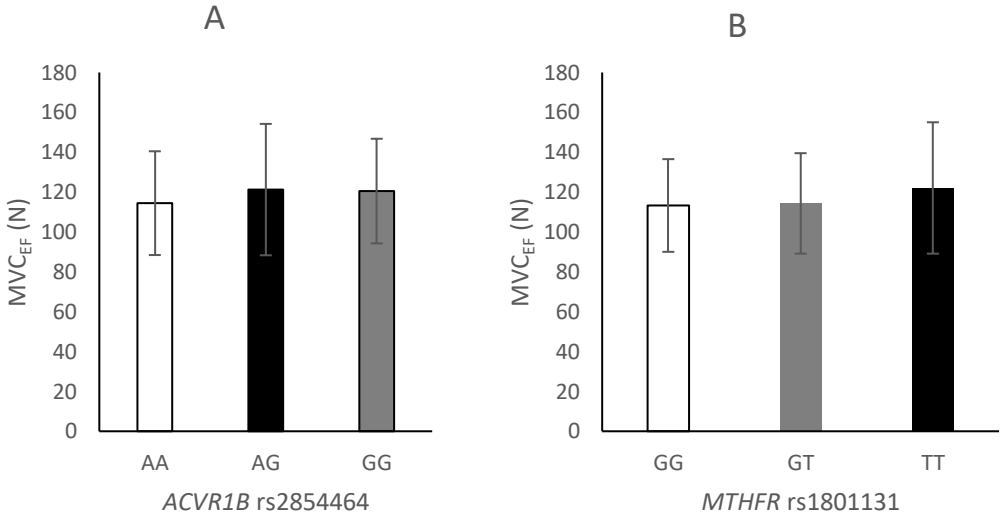
SNPs	**VIC®**	**FAM®**	**Primer (5'-3')**
ACTN3 rs1815739	T-allele	C-allele	
ACE rs4341	C-allele	G-allele	*ACE*111 CCCATCCTTTCTCCCATTTCTC *ACE*112 AGCTGGAATAAAATTGGCGAAAC *ACE*113 CCTCCCAAAGTGCTGGGATTA
CNTF rs1800169	A-allele	G- allele	
MSTN rs1805086	C-allele	T-allele	
COL1A1 rs1800012	A-allele	C-allele	
VDR rs2228570	A-allele	G-allele	
TRHR rs7832552	C-allele	T-allele	
PTK2 rs7843014	A-allele	C-allele	
PTK2 rs7460	A-allele	T-allele	
IGF1 rs35767	A-allele	G-allele	
IL6 rs1800795	C-allele	G-allele	
ACVR1B rs2854464	A-allele	G-allele	
ACVR1B rs10783485	G-allele	T-allele	
ESR1 rs1999805	A-allele	G-allele	
ESR1 rs4870044	C-allele	T-allele	
MTHFR rs1537516	A-allele	G-allele	
MTHFR rs17421511	A-allele	G-allele	
MTHFR rs1801131	G-allele	T-allele	

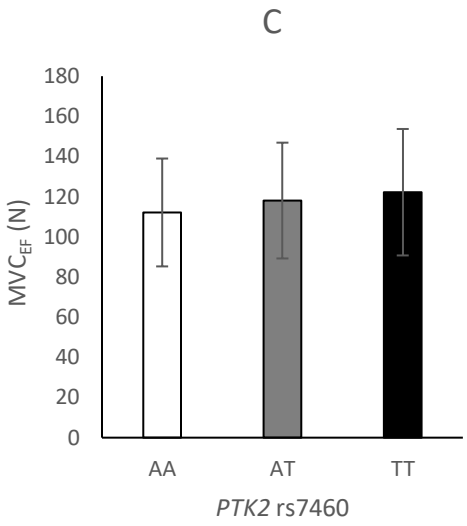
HIF1A rs11549465	C-allele	T-allele
ID3 rs11574	C-allele	T-allele
NOS3 rs1799983	G-allele	T-allele
FT0 rs9939609	A-allele	T-allele
CNTFR rs2070802	A-allele	T-allele
TTN rs10497520	C-allele	T-allele

Appendix 3

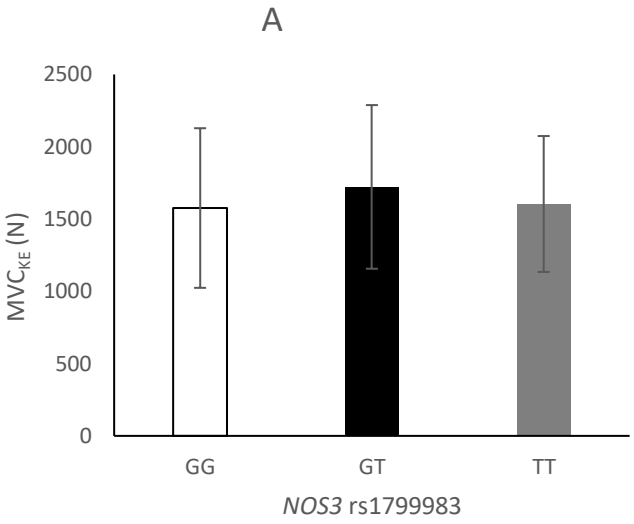


Association of SNPs and Handgrip strength (HGS). Comparison of HGS between genotype groups for *PTK2* rs7843014 (AA=105, AC=142 Vs CC=57) and *PTK2* rs7460 (AA=72, AT=153 and TT=80) polymorphisms. Black color denotes group with highest mean followed by grey and white color.

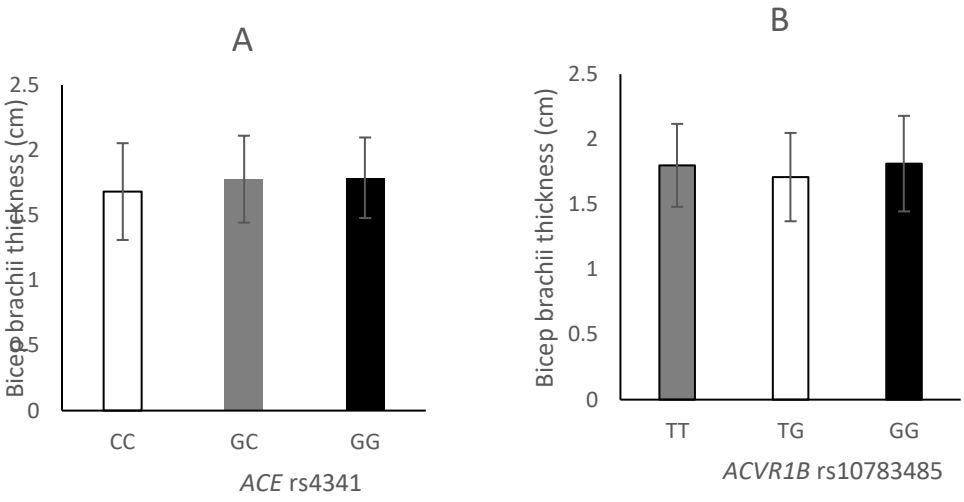




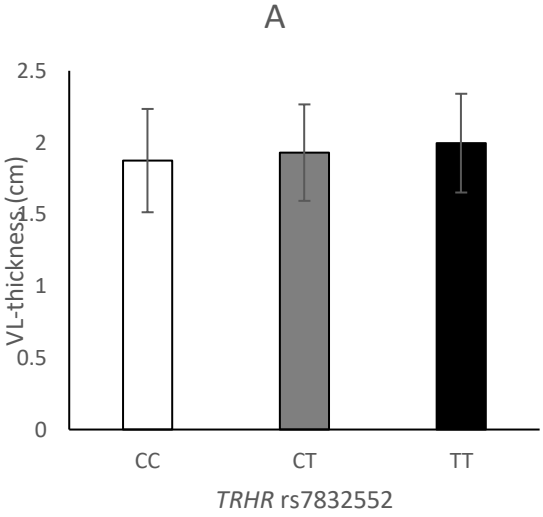
Association of SNPs and Isometric elbow flexion maximum voluntary contraction (MVC_{EF}). Comparison of MVC_{EF} between genotype groups for ACVR1B rs2854464 (AA=153, AG=127, GG=24), PTK2 rs7460 (TT=80, AT=153 and AA=71) and MTHFR rs1801131 (GG=2, GT=132, TT=144). Black color denotes group with highest mean followed by grey and white color.



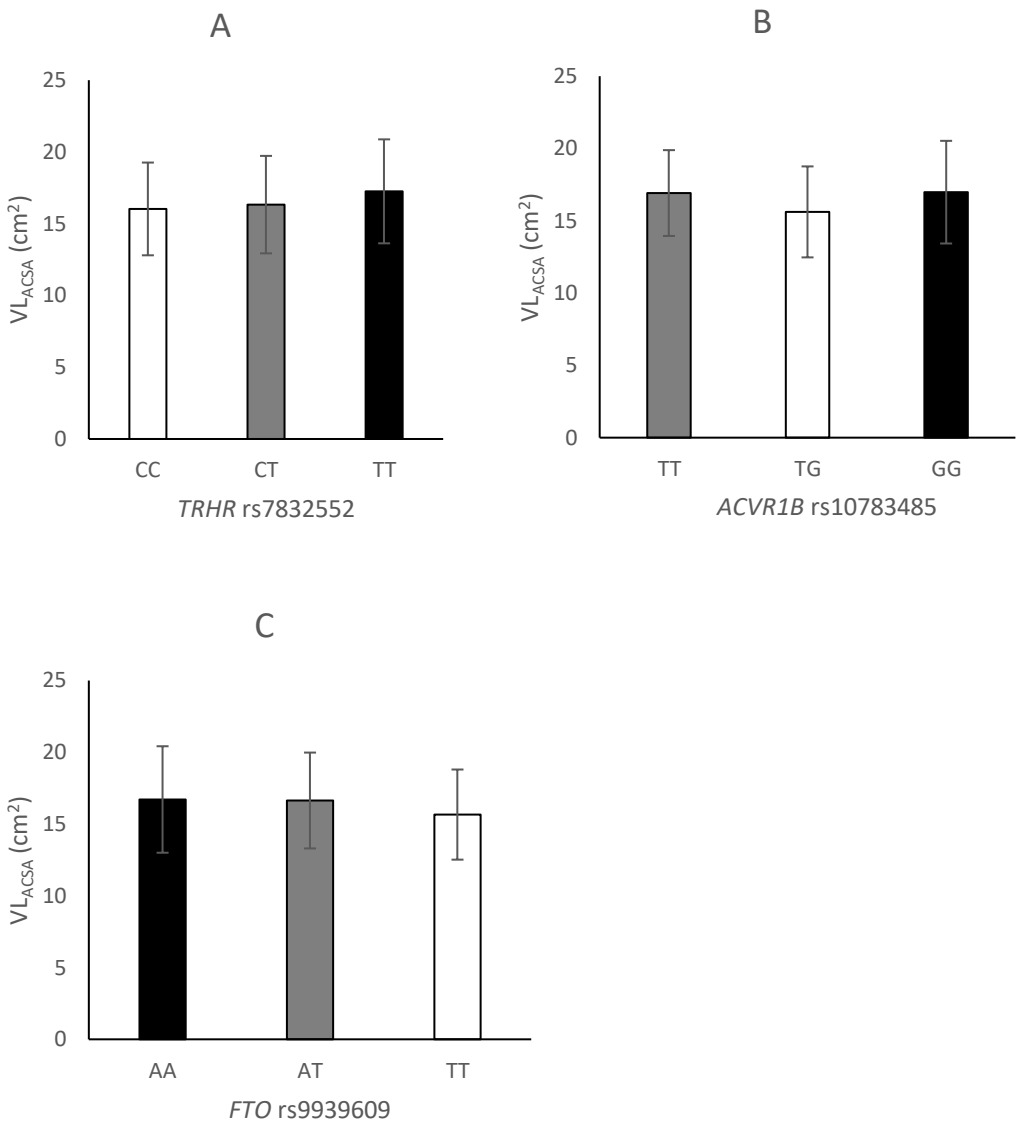
Association of SNPs and Isometric Knee Extension Maximum Voluntary contraction (MVC_{KE}). Comparison of MVC_{KE} between genotype groups for NOS3 rs1799983 (TT=43, GT= 142 and GG= 117) polymorphisms. Black color denotes group with highest mean followed by grey and white color.



Association of SNPs and bicep brachii thickness. Comparison of biceps brachii thickness between genotype groups for ACE rs4341 (CC=61, GC=135, GG=96) and ACVR1B rs10783485 (TT=26, TG=141, GG=124) polymorphisms. Black color denotes group with highest mean followed by grey and white color.



Association of SNPs and Vastus lateralis thickness. Comparison of VL thickness between genotype groups for TRHR rs7832552 (TT=37, CT=122 and CC=130). Black color denotes group with highest mean followed by grey and white color.



Association of SNPs and Vastus Lateralis Anatomical Cross sectional area (VL_{ACSA}). Comparison of VL_{ACSA} between genotype groups for *TRHR* rs7832552 (TT=37, CT=122 and CC=130), *ACVR1B* rs10783485 (TT=28, GT=140 and GG=121) and *FTO* rs9939609 (AA=45, AT=143 and TT=102) polymorphisms. Black color denotes group with highest mean followed by grey and white color.

Figure: Association of SNPs with skeletal muscle phenotypes