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Differentially methylated gene patterns between age-matched sarcopenic and non-sarcopenic women

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Abstract

Background Sarcopenia is characterized by progressive decreases in muscle mass, muscle strength, and muscle function with ageing. Although many studies have investigated the mechanisms of sarcopenia, its connection with epigenetic factors, such as DNA methylation, still remains poorly understood. The aim of this study was to explore sarcopenia-related DNA methylation differences in blood samples between age-matched sarcopenic and non-sarcopenic older women.

Methods A sarcopenic group (n = 24) was identified and selected from a set of 247 older Caucasian women (aged 65–80 years) based on cut-off points of skeletal muscle index at 6.75 kg/m^2 and grip strength at 26 kg (the lower quintile of grip strength in the set). A non-sarcopenic group (n = 24) was created with a similar age distribution as that of the sarcopenic group. DNA methylation patterns of whole blood samples from both groups were analysed using Infinium MethylationEPIC BeadChip arrays. Differentially methylated cytosin–phosphate–guanine sites (dmCpGs) were identified at a P value threshold of 0.01 by comparing methylation levels between the sarcopenic and non-sarcopenic groups at each CpG site. dmCpG-related genes were annotated based on Homo sapiens hg19 genome build. The functions of these genes were further examined by GO and KEGG pathway enrichment analysis.

Results The global methylation level of all analysed CpG sites (n = 788 074) showed no significant difference between the sarcopenic and non-sarcopenic groups (0.812), while the average methylation level of dmCpGs (n = 6258) was significantly lower in the sarcopenic group (0.004). The sarcopenic group had significantly higher methylation levels in TSS200 (the region from transcription start site to 200 nucleotides upstream of the site) and lower methylation levels in gene body and 3'UTR regions. In respect of CpG regions, CpG islands in promoters and some intragenic regions showed greater levels of methylation in the sarcopenic group. dmCpG-related KEGG pathways were mainly associated with muscle function, actin cytoskeleton regulation, and energy metabolism. Seven genes (*HSPB1*, *PBX4*, *CNKSR3*, *ORMDL3*, *MIR10A*, *ZNF619*, and *CRADD*) were found with the same methylation direction as previous studies of blood sample methylation during ageing. Fifty-four genes were shared with previous studies of resistance training.

Conclusions Our results improve understanding of epigenetic mechanisms of sarcopenia by identifying sarcopenia-related DNA methylation differences in blood samples of older women. These methylation differences suggest underlying alterations of gene expression and pathway function, which can partially explain sarcopenia-related muscular changes.

Keywords Sarcopenia; Older women; DNA methylation; Differentially methylated CpG sites; Pathway analysis

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Introduction

DNA methylation is a mechanism of regulation of gene expression without alterating the original gene sequences.[1] In mammals, cytosine is the most common base where methylation takes place.[2] Methylation of cytosine involves the attachment of a methyl group to the 5' position of cytosine and can be found in 57–85% of cytosin–phosphate–guanine (CpG) sites.[3] Most CpG sites scatter in mammal genomes; yet, there are regions with clustered CpG sites, known as CpG islands,[4] which can be found in 72% of gene promoters.[5] The dynamic change of DNA methylation is connected to the regulation of gene expression during development and differentiation.[6] Methylated CpG islands in gene promoters have been associated with long-term gene silencing.[7] Moreover, methylated CpG islands of intragenic regions have been found to influence various functions; for example, intragenic DNA methylation in transcriptionally active genes can impede gene expression by reducing elongation efficiency of RNA polymerase II.[8] Methylation in intragenic regions might also prevent gene bodies from spurious transcriptions,[9] and the activities of some methylated intragenic CpG islands are possibly regulated by other CpG islands acting as initiators of transcription.[10] Besides CpG islands, the methylation of CpG shores (sequences within 2 kb distance from CpG islands[11]) is also found in the regulation of gene expression.[12] DNA methylation patterns can be modified by many factors such as age, air polution, lifestyle, nutrition, and training.[13–18] The association between ageing and DNA methylation has been studied in various tissues such as saliva,[19] blood,[20,21] muscle,[22] skin,[23] and brain.[24] *In vitro* myoblast cultivation demonstrated that an acute early proliferative lifespan tumour necrosis factor-α exposure induced a long-term maintenance of elevated myoD methylation, indicating an underlying epigenetic regulation that might be related to muscle loss in later life.[25] Zykovich *et al*. identified 500 ageing-related CpGs as possible predictors of chronological/biological age by comparing DNA methylation patterns in skeletal muscle biopsies between old and young adults.[22] Besides ageing studies, DNA methylation changes have also been related to many disease conditions such as breast cancer,[12] rhabdomyosarcoma,[26] and juvenile dermatomyositis.[27]

Sarcopenia has been recognized as a muscle disease, which is characterized by progressive decreases in muscle mass and muscle function.[28] Although ageing is the primary factor, other factors, such as disuse and malnutrition, have also been identified as covariates of sarcopenia.[29] Considering that these factors are also reported to be associated with methylation changes, a possible relationship between sarcopenia and DNA methylation is suggested. Notably, sarcopenia-related changes are not restricted to the muscle itself, as endocrine disorders are also linked to sarcopenia.[30] Inflammatory cytokines such as tumour necrosis factor-α and interleukin-6 increase muscle loss and impair muscle regenerating capacity during the ageing process.[31] Serum levels of interleukin-6, secreted protein acidic and rich in cysteine, and macrophage migration inhibitory factor were found to be higher in those with sarcopenia compared with controls while insulin-like growth factor 1 level was significantly lower in sarcopenics.[32] A combined score on these serum levels could be used as a biomarker for sarcopenia[32]; therefore, a specific focus on DNA methylation in blood could add to the knowledge of more systemic factors contributing to sarcopenia. If DNA methylation in blood (partially) overlaps with those from muscle biopsy-based DNA, the more easily accessible venous blood samples can be studied in further research and (biomarker) applications.

The present study aimed to analyse whole blood-based methylation differences between sarcopenic and non-sarcopenic populations. The results of this study will enrich our understanding of sarcopenia by identifying differentially methylated CpG (dmCpG) sites and possible alterations in related gene expression and corresponding signalling pathways.

Methods

Participants

A set of 247 older, independently living, Caucasian women (aged 65–80 years) were recruited from the local area and provided written informed consent following local ethics approval (Manchester Metropolitan University, Crewe, UK). Of the initial 247, 168 provided 5 mL venous blood samples. These were subsequently categorized into sarcopenic (n = 25) and non-sarcopenic (n = 138) groups using cut-off points of skeletal muscle index[33] (SMI, calculated using skeletal muscle mass divided by height squared) at 6.75 kg/m^2 and hand grip strength (HGS) at 26 kg (the lower quintile of HGS in the recruited set). Through a process of further selection including age matching,[34] completeness of data, rankings of SMI and HGS z score, and summed z score (Supporting Information, *Figure* S1), 24 participants (age of sarcopenic group 72.5 ± 4.2 years and non-sarcopenic group 70.5 ± 3.3 years) from each group were selected for DNA methylation analysis. In the sarcopenic group, 21 participants with negative z scores in SMI and HGS were selected first, with an additional three selected via an ascending sequence of summed z scores. Selection in the non-sarcopenic group was done in an opposite direction: 23 participants with positive z scores in SMI and HGS were selected first, with an additional participant with the highest summed z score selected from the remainder (Supporting Information, *Figure* S2).

Hand grip and skeletal muscle mass measurement

Hand grip strength was measured by digital handgrip dynamometer (Jamar Plus+, JLW Instruments, Chicago, IL, USA). Participants were asked to stand straight and to keep their testing arms straight out during the measurement. Verbal encouragement was given and three attempts were made on both hands. The highest value was kept for further analysis.

Electrical resistance of the body was measured by bioelectrical impedance analysis (BIA) (Bodystat 1500MDD, Bodystat Ltd, Douglas, UK). Before the test, participants were asked to remove any metal attachments and to lay in a supine position on a physiotherapy bed for 4 min. Electrodes were placed on the dorsum of the right hand and right foot according to manufacturer instructions. During the test, the participant was asked to stay quiet and relaxed. Skeletal muscle mass was estimated using the following equation that was developed by Janssen *et al.*[35]: skeletal muscle mass (kg) = ($Ht^2/R \times 0.401$) − age × 0.071 + 5.102 where Ht is height in cm; R is BIA resistance in ohms; and age is in years. This equation has a high coefficient of determination (r^2 = 0.86) and low bias (SEE = 2.7 kg) compared with MRI for skeletal muscle mass estimation across an age range of 18–86 years.[35] Whole body SMI was later calculated by dividing skeletal muscle mass by height squared.

DNA extraction and methylation measurement

DNA was extracted from venous blood samples by QIAamp® DNA Blood Mini Kit (Qiagen, Crawley, UK) following the instructions of the manual. DNA methylation was measured using Illumina® Infinium MethylationEPIC BeadChip arrays (Illumina Inc., San Diego, CA, USA) at the Genomics Core facility (Center for Human Genetics—UZ/KU Leuven—Herestraat 49 bus 602, B-3000 Leuven). Methylation files were read by R 'Minfi' package,[36] background signals were corrected by normal-exponential out-of-band ('Noob') method, and methylation values (β values, methylation percentages at measured probes) were normalized for blood cell composition by R 'FlowSorted.Blood.EPIC' package.[37] Probes were dropped under one of the three conditions: (i) probes with non-significant background signal levels ($P > 0.01$) at methylated and unmethylated channels; (ii) probes that contain either single nucleotide polymorphisms at the CpG interrogation or at the single nucleotide extension as suggested in the 'Minfi' package (reference array: 'IlluminaHumanMethylationEPIC', annotated by ilm10b4.hg19); and (iii) cross-active probes that were reported in the first supplementary table of Pidsley's study.[38] A final total of 788 074 probes were kept for further methylation analyses using Partek Genomics Suite V.7.0 (Partek Inc., St. Louis, MO, USA), in which CpG probes were annotated based on 'HumanMethylation850' reference, 'MethylationEPIC_v-1-0_B4' annotation file, 'Homo sapiens' species, and hg19 genome build. Notably, the DNA methylation analysis in Partek was based on the M value (log transformed methylation-to-unmethylation ratio at each CpG site, the default setting of the software) instead of the β value.

Statistics

t-tests were used to compare descriptive data [age, height, body mass, body mass index (BMI), SMI, and HGS] and methylation levels between the sarcopenic and non-sarcopenic groups with a significance of 0.05. Benjamini Hochberg method[39] was used for *P* value adjustment in the identification of dmCpG sites and pathway analysis. Because the *P* value of the methylation value comparison at each CpG site was greater than 0.05 after Benjamini Hochberg correction, an unadjusted *P* value threshold of 0.01 was used to define dmCpG sites. The average methylation level of analysed CpG sites, proportions of hypermethylated (defined as higher M values in the sarcopenic group than the non-sarcopenic group) and hypomethylated (defined as lower M values in the sarcopenic group) dmCpG sites, and significant genes or dmCpGs that have been identified in previous ageing-related or muscle-related methylation studies were compared with the results of our study. Gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses (databases till May 2019) were also conducted and compared between studies based on annotated 'gene symbols' of dmCpG sites using Partek.

Results

Descriptive data of the sarcopenic and non-sarcopenic groups

Consistent with the classification criteria, participants in the non-sarcopenic group had significantly higher SMI ($P < 0.001$) and HGS ($P < 0.001$) than the sarcopenic group while there was no significant age difference between the two groups (P = 0.070). Moreover, body mass (P = 0.003) and BMI (P = 0.006) were also significantly larger in the non-sarcopenic group in comparison with the sarcopenic group (Table 1).

DNA methylation levels

We compared methylation values at each of the analysed 788 074 CpG sites between the sarcopenic and non-sarcopenic groups but no significant CpG sites were found after Benjamini Hochberg false discovery rate (FDR) control at a level of 0.05. Therefore, CpG sites with unadjusted *P* values

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Table 1 Descriptive data of participants by groups

Group	Age (year)	Body mass (kg)	Height (m)	BMI (kg/m^2)	SMI (kg/m^2)	HGS (kg)
Non-sarcopenic	70.5 ± 3.3	71.7 ± 12.8	1.60 ± 0.05	27.9 ± 4.9	7.45 ± 0.67	36.0 ± 3.7
Sarcopenic	72.5 ± 4.2	61.5 ± 9.4	1.56 ± 0.11	24.4 ± 3.4	6.00 ± 0.47	23.2 ± 2.5
P value	0.070	0.003*	0.154	0.006*	<0.001*	<0.001*

*Significant difference between the sarcopenic and the non-sarcopenic groups.

<0.01 were identified as dmCpG sites (*n* = 6258) (*Figure* 1A) (Supporting Information, *Table* S1A).

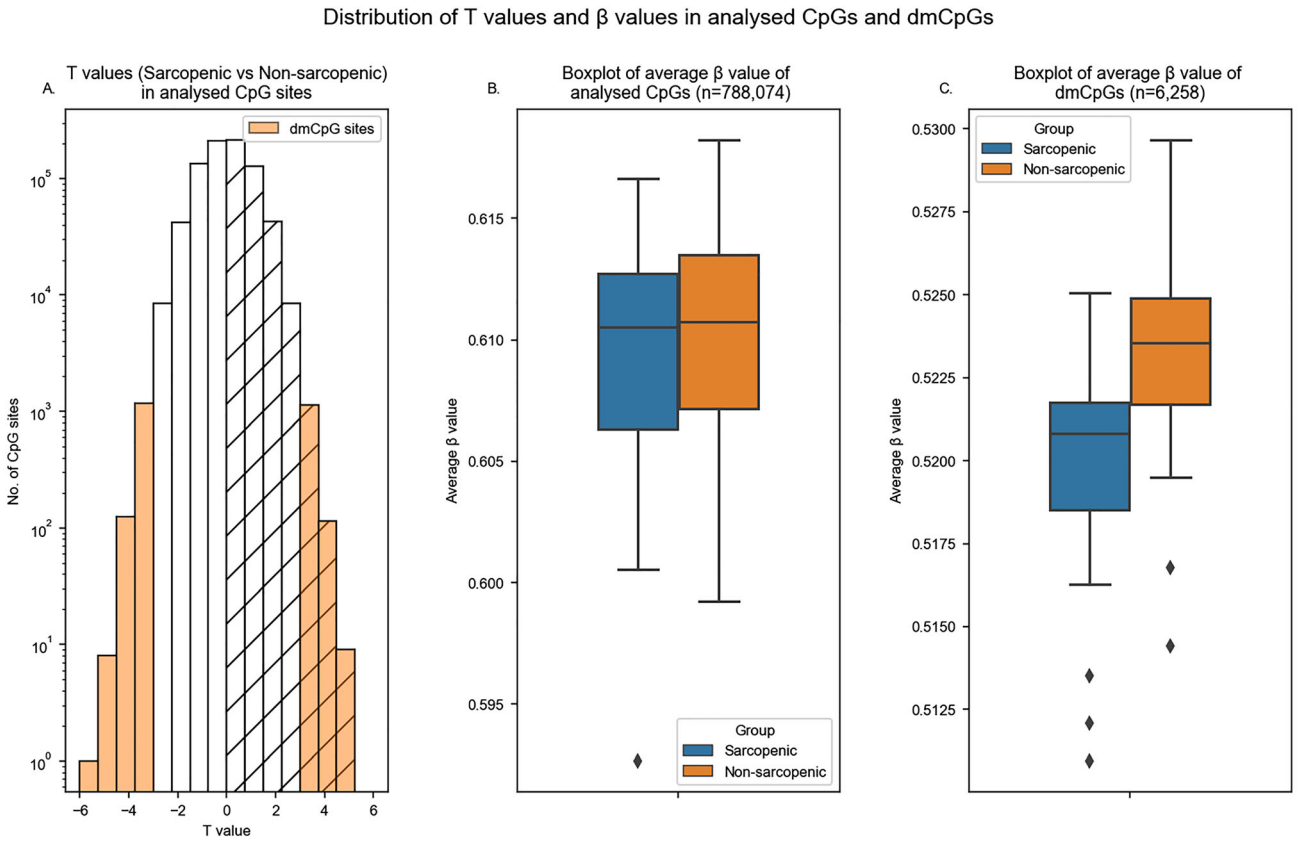
In our study, the total methylation level, represented by the mean methylation value of all analysed CpG sites, showed no significant difference between the sarcopenic and non-sarcopenic groups (*P* = 0.812, Supporting Information, *Table* S1B and *Figure* 1B), while the average methylation value of dmCpGs was significantly lower in the sarcopenic group (*P* = 0.004, Supporting Information, *Table* S1B and *Figure* 1C). When comparing average methylation values of dmCpGs by gene regions, the sarcopenic group had significantly higher methylation levels in gene promoters (TSS200) and lower methylation levels in gene body and 3'UTR regions (Supporting Information, *Table* S1B and *Figure* S3). Moreover, in the sarcopenic group, a greater level of methylation at CpG islands was not only found in promoter regions but also in some intragenic regions, such as Exon 1, 3'UTR, and 5'UTR (Supporting Information, *Table* S1C).

Among those identified dmCpG sites, 51.2% (*n* = 3205) were hypermethylated and the remaining 48.8% (*n* = 3053) were hypomethylated (Supporting Information, *Table* S1D and *Figure* S4. dmCpG methylation value-based unsupervised clustering showed that the majority of participants were clustered by corresponding groups (Supporting Information, *Figure* S5).

Distribution of differentially methylated CpGs

Although a large amount of dmCpGs were located in CpG-poor areas (known as the Open Sea), CpG islands had the highest methylation rate among all the analysed CpG regions (1.19%, Supporting Information, *Figure* S6 and *Table* S1D). dmCpGs

Figure 1 (A) Distribution of T values of analysed CpGs and dmCpGs. (B) Boxplot of average β values of the CpGs between sarcopenic and non-sarcopenic women (p = 0.812). (C) Boxplot of average β values of dmCpGs between sarcopenic and non-sarcopenicwomen. The β values in the sarcopenic group are significantly lower than thenon-sarcopenic group (p = 0.004).



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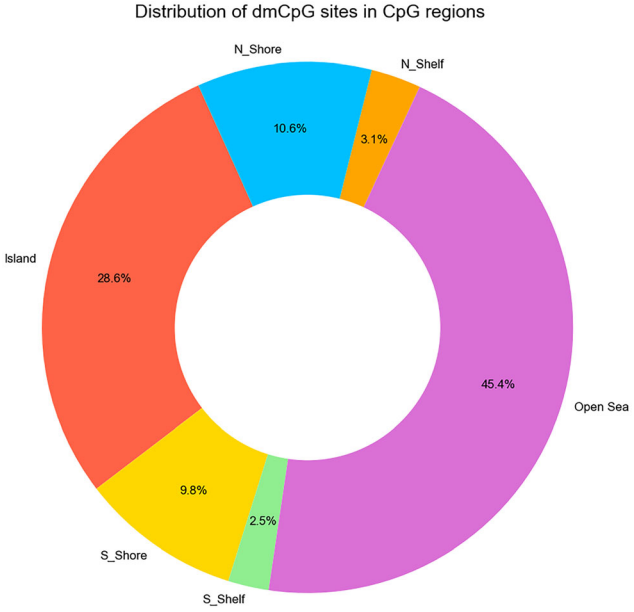
distribution included 28.6% located in CpG islands, 9.8–10.6% (totalling 20.4%) in CpG shores (within 2 kb of CpG islands[11]), and 2.5–3.1% (totalling 5.6%) in CpG shelves (within 2 kb of CpG shores[11]) (*Figure* 2). This indicated that the proportion of dmCpGs was negatively related to the distance away from the CpG island. Meanwhile, 83.8% of dmCpG sites located in CpG islands were hypermethylated while CpG south shelf (S_Shelf) had the largest hypomethylated proportion of 80% (*Figure* 3).

Besides analyses on CpG regions, we also analysed the distribution of dmCpGs by chromosomes. The largest amount of dmCpG sites were found in chromosome 1 (Supporting Information, *Figure* S7A) while chromosome 19 and chromosome 18 had the largest (1.0%) and the smallest proportion (0.6%) of dmCpGs in analysed CpG sites, respectively (Supporting Information, *Table* S1D and *Figure* S7B). Furthermore, chromosome 19 had the highest percentage (1.1%) of hypermethylated dmCpG sites in analysed hypermethylated sites while chromosome X had the highest hypomethylated proportion (1.2%), followed by chromosome 16 (1.0%).

Genes and gene regions with altered methylation status

Among the identified 6258 dmCpG sites, 4840 dmCpGs were annotated by gene names and regions (based on the 'UCSC_RefGene_Name' and 'UCSC_RefGene_Group' columns in Supporting Information, *Table* S1A). In total, hypermethylation and hypomethylation were found in 2422 and 1913 genes, respectively (some CpG sites are annotated with multiple gene names and regions, Supporting Information, *Table* S2A). With the largest number of analysed CpG sites (n = 7572, Supporting Information, *Table* S2B) among all annotated genes, *PC* gene contributed the largest amount of dmCpGs (n = 71, Supporting Information, *Table* S2B), which were located in hypermethylated CpG islands of the gene body region (Supporting Information, *Table* S2M). Meanwhile, 279 genes were identified with both hypermethylation and hypomethylation (Supporting Information, *Table* S2B and *Figure* S8). Hypermethylation was more common than hypomethylation in promoter regions (TSS1500 and TSS200), 5'UTR, and Exon 1 (Supporting Information, *Table* S2 and *Figure* S9). Notably, we found that the methylation status of some genes was not identical across the same gene region. From our results, gene promoters of 16 genes and gene bodies of 97 genes were found with both hypermethylation and hypomethylation (Supporting Information, *Table* S2D).

Figure 2 Distribution of dmCpGs in CpG regions. dmCpGs distribution includes 28.6% located in CpG islands, 9.8-10.6% in CpG shores and 2.5-3.1% in CpG shelves. This indicates that the proportion of dmCpGs is negatively related to the distance away from the CpG island.

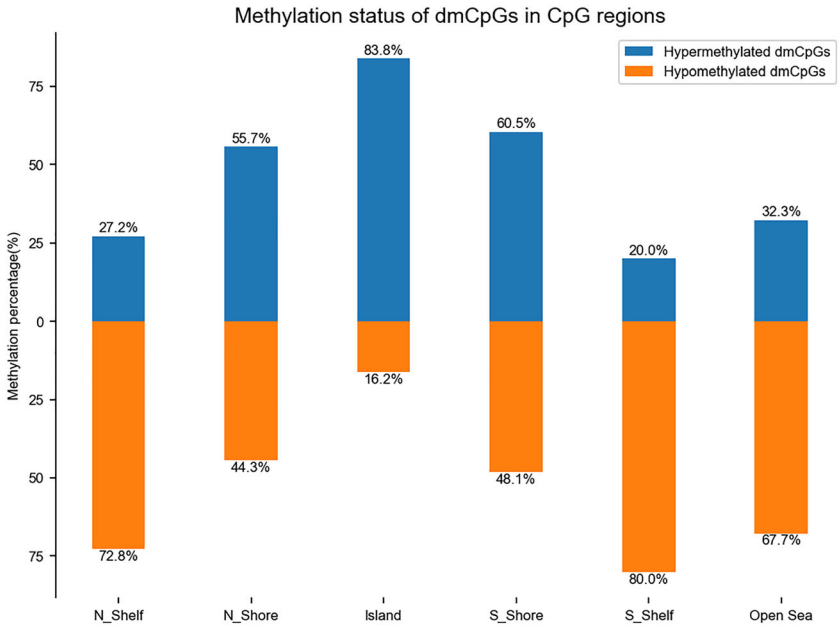


Enrichment and pathway analysis

Six hundred thirty-three terms in GO enrichment analysis were identified with significance after FDR control (q value <0.05) based on the 'gene symbols' of dmCpG sites (Supporting Information, *Table* S3A). The most significant GO term was 'protein binding' that included 1680 hypermethylated CpG sites (1488 genes) and 1356 hypomethylated CpG sites (1152 genes) (Supporting Information, *Table* S3C,E). Most hypermethylated CpGs were located in the CpG island (Supporting Information, *Table* S3D) while most hypomethylated CpGs were found in the Open Sea (Supporting Information, *Table* S3F). There were 197 genes with both hypermethylated and hypomethylated CpGs in the 'protein binding' term (Supporting Information, *Table* S3G). GO analysis based on hypermethylated and hypomethylated promoter regions (Supporting Information, *Table* S1E,F) showed that 291 GO terms were significantly related to hypermethylated promoter regions (q value <0.05) with the term 'intracellular part' as the most significant term while 10 GO terms were significantly connected to hypomethylated promoter regions (q value <0.05) with the term 'androgen receptor binding' as the most significant term (Supporting Information, *Table* S3H,I).

No significant KEGG pathways (q value < 0.05) were found after FDR control while 37 pathways showed unadjusted significance (P value < 0.05) (Supporting Information, *Table* S4A). These unadjusted significant terms covered many muscle-related aspects such as muscle function (e.g. apelin signalling, cGMP-PKG signalling, and insulin resistance), actin cytoskeleton regulation (e.g. phosphatidylinositol signalling, focal adhesion, and adherens junction), energy metabolism (e.g. thermogenesis, AMPK signalling, and glucagon

Figure 3 Methylation status of dmCpGs in CpG regions. dmCpGs with higher M values in the sarcopenic group than the non-sarcopenic group are defined as hypermethylated. dmCpGs with lower M values in the sarcopenic group are defined as hypomethylated.



signalling), neural control (e.g. axon guidance and GABAergic synapse), signal transduction (e.g. Wnt signalling, MAPK signalling, and cAMP signalling), blood pressure regulation (e.g. aldosterone), and cell regeneration (e.g. cell cycle and oxytocin signalling). As the most significant KEGG pathway, the 'apelin signalling pathway' included 56 dmCpGs, half of which were hypermethylated (Supporting Information, *Table* S4C,E, J). Most hypermethylated CpGs were located in the CpG island (Supporting Information, *Table* S4D) and the majority of hypomethylated CpGs were found in the Open Sea (Supporting Information, *Table* S4F). There were five genes with both hypermethylated and hypomethylated CpGs in the 'apelin signalling pathway' (Supporting Information, *Table* S4G). KEGG analysis based on hypermethylated and hypomethylated promoter regions showed that the 'cell cycle' and 'thermogenesis' pathways were the most significant terms associated with hypermethylated and hypomethylated promoter regions, respectively (Supporting Information, *Table* S4H,I).

Comparative analysis with previous studies

We first compared muscular phenotype-related genes, which have been reported in previous studies,[40–42] with the genes identified in our study (based on the 'UCSC_RefGene_Name' column, Supporting Information, *Table* S1A) and found 34 genes in common (Supporting Information, *Table* S5A). We further compared our dmCpGs, annotated genes, and significant KEGG pathways (unadjusted, *P* value <0.05) with those previously reported in DNA methylation studies on ageing,[20–22] muscle development,[43] muscle diseases,[26,27] and exercise.[14–18]

When compared with studies of blood sample methylation during ageing,[20,21] we found seven common CpGs in total and the CpGs showed consistent methylation direction in all studies (Supporting Information, *Table* S5B). Zykovich *et al*.[22] studied the skeletal muscle methylation difference between older and young participants and identified 5963 ageing-related dmCpGs, among which 35 dmCpGs were found in our dmCpGs and 11 CpGs had the same methylation direction in both studies (Supporting Information, *Table* S5B). Two dmCpGs (cg10093679 and cg19291355) in our study were found in the top 500 ageing-related dmCpGs suggested by Zykovich *et al*., and the methylation status was identical in both studies (Supporting Information, *Table* S5B). Moreover, Zykovich *et al*. located 17 ageing-related intragenic dmCpGs (with 16 CpGs being hypermethylated) in *NFATC1*, a gene closely associated with muscle function as it codes for a transcription factor promoting the expression of slow fibre types and is involved in neuromuscular signal conduction. In our results, three dmCpGs were located in intragenic regions of *NFATC1* and all were hypomethylated (Supporting Information, *Table* S5B). However, none of these CpGs were included as dmCpGs in Zykovich's study.

Four CpGs from three genes (*HOXD4*, *SEPT9*, and *MBP*) (Supporting Information, *Table* S5C) were differentially methylated in both our study and muscle inflammatory disease in children.[27] By comparing with the methylation study on rhabdomyosarcoma,[26] we found 360 genes in common. GO and

KEGG analysis on those genes showed that many of the significant terms were associated with muscle function, DNA transcription regulation, nervous system development, and signal transduction (Supporting Information, *Table* S5D,E).

Seaborne *et al.*[16] identified several DNA methylation sites that were closely related to resistance training. When comparing our dmCpGs with the top 500 resistance training-related CpG sites in Seaborne's study, only two sites (cg00077516 and cg09739536) were found in common (Supporting Information, *Table* S5F). We found 17 common CpGs when comparing with loading-related CpGs and eight CpGs showed different fold change directions in our study and Seaborne's study (Supporting Information, *Table* S5F). We also identified nine common CpGs by comparing with unloading-related CpGs and only one CpG had the same direction in both studies (Supporting Information, *Table* S5F). Turner *et al.*[17] made a thorough analysis of gene expression and DNA methylation induced by acute and chronic resistance training. We shared two CpGs (cg08284143 and cg11692073) that showed hypermethylation after acute resistance training (based on Turner's study) and hypomethylation in sarcopenia (based on our study) (Supporting Information, *Table* S5G). Six common CpGs were found when comparing with hypomethylated CpGs after acute resistance training and all of them had the same fold change direction in both studies (Supporting Information, *Table* S5G). When comparing with dmCpGs related to chronic resistance training, we identified seven common CpGs, five of which had the same fold change direction in both studies (Supporting Information, *Table* S5G). We also found 19 genes (24 CpGs) out of 51 genes that had significant epigenetic and transcriptome changes under all acute/chronic training/detraining conditions reported in Turner's study (Supporting Information, *Table* S5G). Furthermore, the *SRGAP1*, *PLXNA2*, and *JOSD1* were found with increased gene expression after resistance training (based on Turner's study) while hypermethylated gene promoters were found in sarcopenia (based on our study) (Supporting Information, *Table* S5H). We compared unadjusted significant KEGG pathways (P value < 0.05) between our study and Turner's study and found three pathways (cGMP-PKG signalling pathway, human papillomavirus infection, and proteoglycans in cancer) in common (Supporting Information, *Table* S6A–K). Notably, the counts of hypermethylated and hypomethylated CpGs in the pathway 'cGMP-PKG signalling' and 'human papillomavirus infection' were very similar while the amount of hypomethylated CpGs was twice as many as the hypermethylated CpGs in the pathway 'proteoglycans in cancer'. Sailani *et al.*[18] identified 748 gene promoters with significant methylation difference between physically active and inactive older participants. By comparing with our hypermethylated and hypomethylated gene promoters (Supporting Information, *Table* S1E,F), we found 23 common genes with hypermethylated promoters and 369 genes with hypomethylated promoters (Supporting Information, *Table* S6L). The significant pathways based on these common genes were associated with energy metabolism, signal transduction, myogenesis, and actin cytoskeleton regulation (Supporting Information, *Table* S6M,N).

Discussion

DNA methylation patterns

The ageing-related DNA methylation pattern is characterized by globally decreased and regionally (CpG islands and shores) increased methylation levels.[6] By comparing the methylation status of blood CD4+ cells between newborns and centenarians, Heyn *et al.*[20] reported a decreased global methylation level in older participants. However, the study of Zykovich *et al.*[22] revealed a global trend of hypermethylation in ageing skeletal muscle. In our study, we found no significant difference in total methylation level of all analysed CpGs between the sarcopenic and the non-sarcopenic groups. The even distribution of hypermethylated and hypomethylated dmCpGs in our study also contradicted the findings of Bell *et al.*[21] (whole blood based) and Zykovich *et al.*[22] (skeletal muscle based) where more than 90% of ageing-related dmCpGs were hypermethylated. Such inconsistency in DNA methylation might be partly related to the relatively narrow range of age of our participants. Because our participants in both groups came from a similar age range (65–80 years) with no difference between them, the age effect on DNA methylation might be partially controlled. Nevertheless, the methylation condition of CpG islands in gene promoters was quite similar between sarcopenia-related and ageing-related methylation patterns. In our study, promoter CpG islands of the sarcopenic group were hypermethylated when compared with those of the non-sarcopenic counterpart. Similarly, a higher methylation level was also found in promoter CpG islands of centenarians in comparison with newborns.[20]

Hypermethylated CpG islands in gene promoters have been associated with the down-regulation of genes.[7] In our study, the sarcopenic group had a higher methylation of promoter CpG islands than the non-sarcopenic group, perhaps indicating that reduced function of corresponding genes accompanies sarcopenia. However, gene expression was not studied in the present manuscript and therefore would require further analysis to confirm this assumption. Unlike promoters, significant hypomethylation was found in gene bodies and 3'UTRs of the sarcopenic group. Gene bodies in the human genome are prevalently methylated.[44] Yet the connection between methylated gene

bodies and gene expressions remains debatable. Mendizabal *et al*.[45] studied nearly 2000 genes, the gene bodies of which were commonly hypomethylated in normal tissues, and found that those genes were prone to have significantly hypomethylated gene bodies in cancer samples. Such results suggested that hypomethylated gene bodies were more related to cancer-associated dysregulation, which is supported by Yang *et al*.,[46] who showed that demethylated gene bodies could cause down-regulation in gene expression. Contradictorily, by analysing cell lines, Jjingo *et al*.[47] reported a bell-shaped relationship between gene transcriptions and methylated gene bodies. Therefore, further measurements on gene expression are needed to evaluate the effect of methylated gene bodies on the activity of corresponding genes. Furthermore, although most of the genes identified in our study showed a single methylation pattern, we found some genes with both hypermethylation and hypomethylation in the same gene region, indicating that the sarcopenic condition might be related to the interaction between methylation and demethylation in these genes.

DmCpG-related GO terms and KEGG pathways

The 'protein binding' was the most significant GO term identified in our study (Supporting Information, *Table* S3A), indicating that signal transduction and cellular metabolism were closely related to our dmCpGs. The 'apelin signalling pathway' was the mostly enriched pathway in KEGG analysis (Supporting Information, *Table* S4A). Apelin is a peptide that can reduce arterial stiffness[48] and enhance muscle mitochondriogenesis[49] and protein synthesis.[50] The endogenous apelin level decreases with age while apelin treatment can induce muscle mass and reverse age-associated sarcopenia in mice.[50] Moreover, apelin can be up-regulated by insulin and the increased concentration of plasma apelin has been found in obese humans and mice.[51] Therefore, our finding of the 'apelin signalling pathway' not only suggests a close association between apelin and muscle degeneration but also indicates a possibility of sarcopenia-related alterations in energy metabolism and body composition that might result in sarcopenic obesity.[28]

Because hypermethylated gene promoters are related to repression of gene expression,[7] we conducted GO analysis based on genes with hypermethylation/hypomethylation in promoter regions to explore possible biological processes that might be different between the sarcopenic and non-sarcopenic groups. From GO results, many metabolism-related processes and cellular components were significantly related to genes with hypermethylated promoter regions (Supporting Information, *Table* S3H), indicating that cellular metabolism might be down-regulated in the sarcopenic group when compared with the non-sarcopenic group. Meanwhile, based on genes with hypomethylation in promoter regions, several terms related to negative regulations of the insulin signalling pathway have been identified (Supporting Information, *Table* S3I). Such findings suggest that negative regulation of insulin signalling might be strengthened in the sarcopenic group, possibly resulting in restricted glucose uptake in skeletal muscles[52] and reduced protein synthesis via down-regulated activations of insulin receptor and PI3K.[53] Moreover, the associations between hormone receptor bindings terms (e.g. androgen and steroid hormone) and hypomethylated promoters (Supporting Information, *Table* S3I) also indicate an overexpression of hormone receptors in sarcopenia to compensate decreased plasma hormone levels that are important in maintaining muscle and bone mass.[54] However, hormone measurements are required for further confirmation.

The 'cell cycle' and 'thermogenesis' pathways identified by genes with hypermethylated and hypomethylated promoter regions (Supporting Information, *Table* S4H,I) suggest possibly decreased cell regeneration ability and increased heat production associated with sarcopenia. Because the sarcopenic group has lower body mass and BMI than the non-sarcopenic group, the sarcopenic group might have less fat for heat preservation, resulting in elevated heat production for body temperature maintenance. There is also a possibility that the sarcopenic group has less body fat because of increased heat production.

Gene, dmCpGs, and pathway comparisons with previous studies

The current study highlights several muscle-related genes that have been identified in previous muscle mass-related or muscle strength-related studies,[40–42] among which is the gene *VDR* (Supporting Information, *Table* S5A). *VDR* encodes vitamin D receptor, a protein widely known for regulation of calcium and phosphate homeostasis.[55] *VDR* gene knockout mice have impaired bone and mineral metabolism and rickets after weaning.[56] Many studies of ageing have also connected decreased *VDR* expression to osteoporosis,[57] frailty,[58] low muscle strength,[59] and low muscle mass.[42] In our study, one hypermethylated CpG island was indeed in the *VDR* promoter region. This indicates a possible reduced expression of *VDR* in the sarcopenic group and could partially explain the decline in muscle function and increased risk of frailty associated with sarcopenia.[28]

We also identified 42 genes (Supporting Information, *Table* S5B) that were reported in previous ageing-related methylation studies.[20–22] Many of these genes are also closely muscle related. For example, we found five genes (*HSPB1*, *PBX4*, *FZD5*, *HMGA1*, and *AARS2*) with sarcopenia-related hypermethylation in CpG islands (Supporting Information, *Table* S5B). *HSPB1* encodes heat shock protein beta-1, which is a member of the small heat shock protein family that plays an important role in muscle development, differentiation, and protection

against heat and mechanical stress.[60] Mutations of *HSPB1* have been related to distal hereditary motor neuropathy, muscle weakness, and fat infiltration.[61] The protein Pre-B-cell leukemia transcription factor 4 (encoded by *PBX4*) is a member of the Pbx family, which facilitates binding of MyoD to gene regulatory regions to initiate skeletal muscle differentiation.[62] In zebrafish, Pbx helps regulate the development of fast-twitch skeletal muscle[63] that undergoes preferential atrophy during ageing in humans.[64] *AARS2* encodes mitochondrial alanyl-tRNA ligase, a member of the aminoacyl-tRNA synthetase family that plays an important role in mRNA translation. Mutations of *AARS2* have been related to neurodegenerations[65] and cardiomyopathy.[66] The hypermethylated CpG island of the *AARS2* promoter region in our study suggests a down-regulation of this gene's activity in sarcopenia. *FZD5* encodes receptors for the Wnt5A ligand, which is connected with muscle mass regulation via the mTOR pathway.[67] *HMGA1* is involved in multiple cellular processes such as DNA repair, transcriptional regulation, and cell cycle regulation. Overexpression of *HMGA1* has been found in cancer, indicating the association of *HMGA1* with cell regeneration.[68] The *SRGAP1* promoter region was hypomethylated after resistance training[17] and a hypermethylated promoter region was found in our study (Supporting Information, *Table* S5H). *SRGAP1* encodes a GTPase activator that regulates cell regeneration and axon guidance and knockdown of *SRGAP1* suppresses cell proliferation by inhibiting the Wnt/β-catenin pathway.[69] These comparisons indicate a possible connection between *SRGAP1* and exercise-induced muscle growth as well as muscle degeneration during ageing.

Although the pattern of DNA methylation is mostly tissue specific,[70] there are still some overlaps between our dmCpGs and those that have been previously reported in muscle samples. In a recent study on muscle DNA methylation changes induced by resistance training and detraining, Seaborne *et al*.[16] reported a significantly decreased methylation in dmCpG site cg09739536 from gene *ZFP2* after a 7 weeks of resistance training. This finding is consistent with our observation that the same CpG site was significantly hypermethylated in the sarcopenic group and that less methylation of *ZFP2* is associated with greater skeletal muscle mass and strength. Similarly, six genes (*SNHG5*, *FUCA2*, *MUC5B*, *SLC30A1*, *CCR3*, and *SMEK2*) were found in the loading phase of Seaborne's study as having different fold change directions from our study, and the genes *SNHG5*, *FUCA2*, and *CCR3* had methylation changes in promoter regions (Supporting Information, *Table* S5F), suggesting that the changes in expression of these three genes might be associated with both muscle degeneration and regeneration. Despite some similarity in methylated genes and CpG sites that we shared with previous muscle biopsy-based studies,[16–18,22,26,27] the methylation patterns were not always consistent. For instance, the dmCpG site cg00077516 from gene *MRPS27* was hypomethylated during detraining-induced muscle changes in one study[16] but hypermethylated in the sarcopenic group in our study. A possible explanation for this discrepancy might be the difference in physical condition of participants. Our study was based on older women susceptible to age-related muscle degeneration while the detraining phase in participants of Seaborne's study can be considered as a return to pre-training levels after a prolonged training load stimulus. It is possible that different mechanisms are involved in age-related atrophy and detraining-related muscular changes. Among the 35 common CpGs shared with the study of ageing by Zykovich *et al*.,[22] 24 CpGs showed a different methylation direction (Supporting Information, *Table* S5B). *Wang et al*.[27] identified hypomethylated *HOXD4* gene in juvenile dermatomyositis patients, while we only found one hypermethylated dmCpG located in the gene body of *HOXD4*. Similar inconsistent methylation patterns were also found in comparison with the study of Mahoney *et al*.[26] on rhabdomyosarcoma (Supporting Information, *Table* S5C). Moreover, we only identified two common CpGs from the top 500 ageing/exercise-related dmCpGs (skeletal muscle based)[16,22] (Supporting Information, *Table* S5B,F). On the other hand, all CpGs that were shared with previous blood sample-based methylation studies had the same methylation direction (Supporting Information, *Table* S5B). These methylation findings confirm tissue-specific methylation differences, and therefore, it is difficult to determine sarcopenia-related methylation differences in skeletal muscles of our participants based on differentially methylated genes identified from blood samples.

In respect of pathway comparative analysis, we identified three common pathways (cGMP-PKG signalling pathway, human papillomavirus infection, and proteoglycans in cancer), which are associated with cell proliferation and development from previous exercise-related methylation studies[17] (Supporting Information, *Table* S6E). Activation of the cGMP-PKG signalling pathway is related to the inhibition of cell proliferation.[71] Increased activation of phosphodiesterase 5, an inhibitor of the cGMP-PKG signalling pathway, has been reported in tumour cell lines.[72] Proteoglycans play an important role in regulating muscle development. Heparan sulphate proteoglycans are essential for signal transduction in several muscle growth or differentiation pathways, such as FGF-2 and HGF.[73] The increases of biglycan and decorin found in muscle dystrophy also suggest an involvement of proteoglycans in response to myofibre damage.[74]

Limitations

Several parameters have been suggested to diagnose sarcopenia, while cut-off points vary with measurement techniques and populations.[29] Recently, cut-off points for sarcopenia tests have been advised by the European

Working Group on Sarcopenia in Older People (EWGSOP).[28] In our study, however, although the assessment methods were the same (SMI and HGS), none of these recommended values were used. We did not use the advised HGS because the value given by EWGSOP was derived from the study of Dodds *et al.*,[75] who summarized the grip strength across 12 British studies that used different brands of dynamometers and test protocols from our study. Because we also did not have a healthy young group as a reference,[29] we used the lower quintile of HGS from our participants as one of the cut-off points to define sarcopenia, an approach having been previously used in several studies to classify sarcopenia.[76,77] Moreover, EWGSOP only provided values for appendicular SMI and not whole body SMI.[28] Considering that our SMI was calculated using whole body skeletal muscle mass estimated by BIA, to identify a second cut-off point to define sarcopenia, we followed Janssen *et al.*,[33] who previously defined sarcopenia cut-off points using estimated whole body skeletal muscle mass in a larger sample of comparably aged participants. Besides the definition of sarcopenia, we also acknowledge that our study involves a limited sample size and the methylation value at each CpG site was compared without correction for covariates such as age and physical activity. Moreover, our assumptions on gene function changes are based on methylation status; gene expression was not studied. Therefore, further studies with a larger sample size, adjusted methylation values, and gene transcriptome analysis might be more informative.

Conclusions

In the present study, we compared blood DNA methylation patterns between age-matched sarcopenic and non-sarcopenic older women. We identified 6258 dmCpG sites and found that the sarcopenic group had significantly less total methylation at these sites. Pathway analyses showed that these sarcopenia-related CpG sites are involved in many muscle-related aspects, such as muscle differentiation, muscle function, and energy metabolism. Moreover, sarcopenia-related hypermethylation was typically found in gene promoters and hypomethylation was found more often in gene body and 3'UTR regions. Our study has therefore enriched the understanding of DNA methylation differences associated with sarcopenia.

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Online supplementary material

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

Figure S1. Flowchart of this study.
Figure S2. Distribution of Z scores in participant screening for DNA methylation analysis.
Figure S3. Comparison of average β values in different gene regions between sarcopenic and non-sarcopenic women. The sarcopenic group has significantly higher methylation levels in TSS200 (p = 0.009) and lower methylation levels in gene body and 3'UTR regions ($p < 0.001$ and p = 0.021, respectively).
Figure S4. Scatter plot of average β values of CpGs in sarcopenic versus non-sarcopenic women with hypermethylated dmCpGs in red and hypomethylated dmCpGs in green.
Figure S5. Hierarchical clustering of dmCpGs.
Figure S6. Distribution of analysed CpGs and dmCpGs by CpG regions.
Figure S7A. Distribution of dmCpGs over chromosomes.
Figure S7B. Chromosomal distribution of relative dmCpGs proportions. Chromosome 19 has the largest relative dmCpG methylation proportion (1.0%) while chromosome 18 has the smallest relative dmCpG methylation proportion (0.6%).
Figure S8. Venn diagram of genes annotated by dmCpGs. 2,143 genes are found only with hypermethylated dmCpGs and 1,634 genes are found only with hypomethylated dmGpGs. There are 279 genes with both hyper- and hypomethylated dmCpGs.
Figure S9. Distribution of differentially methylated genes by gene regions.
Table S1. Supporting Information
Table S2. Supporting Information
Table S3. Supporting Information
Table S4. Supporting Information
Table S5. Supporting Information
Table S6. Supporting Information

Conflict of interest

The authors declare no conflicts of interest.

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