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DNA methylation alterations in muscle of critically ill patients

Lisa Van Dyck[1], Fabian Güiza[2], Inge Derese[1], Lies Pauwels[1], Michaël P. Casaer[1,2], Greet Hermans[1,3], Pieter J. Wouters[1,2], Greet Van den Berghe[1,2†] & Ilse Vanhorebeek[1*†]

[1]*Laboratory of Intensive Care Medicine, Department of Cellular and Molecular Medicine, KU Leuven, Leuven, Belgium;* [2]*Clinical Division of Intensive Care Medicine, Department of Cellular and Molecular Medicine, KU Leuven, Leuven, Belgium;* [3]*Medical Intensive Care Unit, Department of General Internal Medicine, University Hospitals Leuven, Leuven, Belgium*

Abstract

Background Intensive care unit (ICU)-acquired weakness can persist beyond ICU stay and has been associated with long-term functional impairment of ICU survivors. Recently, DNA methylation alterations were found in the blood of ICU patients, partially explaining long-term developmental impairment of critically ill children. As illness-induced aberrant DNA methylation theoretically could also be involved in long-term weakness, we investigated whether the DNA methylation signature in muscle of adult critically ill patients differs from that in muscle of healthy controls.

Methods Genome-wide methylation was determined (Infinium® HumanMethylationEPIC BeadChips) in DNA extracted from skeletal muscle biopsies that had been collected on Day 8 ± 1 in ICU from 172 EPaNIC-trial patients [66% male sex, median age 62.7 years, median body mass index (BMI) 25.9 kg/m^2] and 20 matched healthy controls (70% male sex, median age 58.0 years, median BMI 24.4 kg/m^2). Methylation status of individual cytosine–phosphate–guanine (CpG) sites of patients and controls was compared with *F*-tests, using the Benjamini–Hochberg false discovery rate to correct for multiple comparisons. Differential methylation of DNA regions was assessed with bump hunting, with 1000 permutations assessing uncertainty, expressed as family-wise error rate. Gene expression was investigated for 10 representative affected genes.

Results In DNA from ICU patients, 565 CpG sites, associated with 400 unique genes, were differentially methylated as compared with controls (average difference 3.2 ± 0.1% ranging up to 16.9%, $P < 0.00005$). Many of the associated genes appeared highly relevant for muscle structure and function/weakness, including genes involved in myogenesis, muscle regeneration, nerve/muscle membrane excitability, muscle denervation/re-innervation, axon guidance/myelination/degeneration/regeneration, synapse function, ion channelling with especially calcium signalling, metabolism (glucose, protein, and fat), insulin signalling, neuroendocrine hormone regulation, mitochondrial function, autophagy, apoptosis, oxidative stress, Wnt signalling, transcription regulation, muscle fat infiltration during regeneration, and fibrosis. In patients as compared with controls, we also identified two hypomethylated regions, spanning 18 and 3 CpG sites in the promoters of the HIC1 and NADK2 genes, respectively (average differences 5.8 ± 0.01% and 12.1 ± 0.04%, family-wise error rate <0.05). HIC1 and NADK2 play important roles in muscle regeneration and postsynaptic acetylcholine receptors and in mitochondrial processes, respectively. Nine of 10 investigated genes containing DNA methylation alterations were differentially expressed in patients as compared with controls ($P \leq 0.03$).

Conclusions Critically ill patients present with a different DNA methylation signature in skeletal muscle as compared with healthy controls, which in theory could provide a biological basis for long-term persistence of weakness in ICU survivors. Trial registration: ClinicalTrials.gov: NCT00512122, registered on 31 July 2007.

Keywords Critical illness; Intensive care unit-acquired muscle weakness; Muscle; DNA methylation; Epigenetics

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**Correspondence to: Prof. Ilse Vanhorebeek, Clinical Division and Laboratory of Intensive Care Medicine, Department of Cellular and Molecular Medicine, KU Leuven, Herestraat 49, 3000 Leuven, Belgium. Email: ilse.vanhorebeek@kuleuven.be*
† Both authors equally contributed to this study.

Introduction

Improved survival of critical illness has augmented awareness for the longer-term physical, mental, and cognitive sequelae.[1,2] In particular, patients can suffer from prominent muscle weakness well beyond the intensive care unit (ICU) stay, which hampers functionality and adversely affects quality of life.[3,4] The muscle weakness develops *de novo* in ICU, has been associated with increased mortality and prolonged need of intensive care in survivors, and does not recover swiftly and fully after ICU discharge.[4–8]

Most mechanistic research has focused on how weakness develops in ICU.[4,6,9] Mechanisms include muscle atrophy, suppressed autophagy, inflammation, mitochondrial dysfunction, muscle necrosis, and axonal degeneration, among others. The search for mechanisms explaining incomplete recovery of weakness has only begun recently. A small study reported that markers of muscle tissue proteolysis, autophagy, inflammation, and mitochondrial structure normalized within 6 months after ICU discharge, without recovery of strength.[10] Hence, other mechanisms must be involved. Lower satellite cell content and aberrant expression of genes involved in muscle regeneration and extracellular matrix deposition suggested a role for impaired muscle regeneration.[10,11]

Epigenetic changes may be another mechanism explaining the long-term persistence of weakness, as such changes have been implicated in permanent effects of transient environmental hazardous exposures on health.[12,13] DNA methylation alterations can persist for years and have been associated with long-lasting alterations in gene expression. Muscle is sensitive to DNA methylation alterations. For example, underfeeding during critical developmental phases affects muscle DNA methylation in later life, coinciding with an increased risk of metabolic syndrome, and altered DNA methylation has been associated with age-related muscle weakness.[14–17] DNA methylation is also involved in regeneration of muscle tissue from satellite cells and in autophagy, both factors implicated in the development or maintenance of ICU-acquired weakness.[10,18–21] Furthermore, in an endotoxin-induced rhabdomyolysis rat model, administering a DNA methyltransferase inhibitor alleviated muscle injury.[22]

Rapid *de novo* DNA methylation alterations during critical illness were recently found in leukocytes of critically ill children, which statistically explained part of the adverse legacy of critical illness and its management on their long-term physical and neurocognitive development.[23,24] Also, differences in methylation have been observed in DNA from the blood of adult patients with vs. without sepsis.[25] The impact of critical illness on DNA methylation in muscle has not been investigated yet.

We hypothesized that aberrant DNA methylation also occurs in muscle during critical illness, which could be a biological basis for long-term persistence of muscle weakness.[3,7,8] Therefore, we compared the genome-wide methylation profile, at individual cytosine–phosphate–guanine (CpG)-site level and in regions spanning multiple CpG sites, of DNA extracted from skeletal muscle biopsies of ICU patients and matched control subjects. For CpG sites and regions identified as differentially methylated in patients and controls, we searched literature for corresponding gene functions and related pathways that could be relevant for muscle structure and/or weakness. Lastly, we explored the impact of the DNA methylation alterations on gene expression for a representative selection of affected genes.

Methods

Study design and participants

This is a hypothesis-generating pre-planned secondary analysis of the multicentre EPaNIC trial (ClinicalTrials.gov—NCT00512122).[26] 4640 adult ICU patients were randomly assigned to initiation of parenteral nutrition (PN) to supplement insufficient enteral nutrition (EN) within 48 h after ICU admission (Early-PN), or to withholding supplemental PN for 1 week, thereby accepting any macronutrient deficit resulting from EN intolerance (Late-PN). Both groups received EN as soon as possible, insulin infusions to maintain normoglycaemia, parenteral trace elements, minerals and vitamins, and physiotherapy.[21,26] The protocol and primary outcomes have been published.[21,26,27] Institutional review board approval was obtained (ML4190), and written informed consent was acquired from all patients or next of kin.

After (additional) written informed consent, *in vivo* needle biopsies were harvested percutaneously from the musculus vastus lateralis of the quadriceps femoris with the Bergström technique at mid-thigh level, after local anaesthesia (lidocaine 2%). Biopsies were obtained from 188 patients (on ICU day 8 ± 1) not meeting any exclusion criterion (pre-existing neuromuscular diseases, coagulation disorders, or therapeutic anticoagulant drugs) and from 20 healthy volunteers (ML4190). Healthy controls were matched for age, gender, and body mass index (BMI) but were never admitted to an ICU prior to the biopsy. Muscle strength was assessed with the Medical Research Council (MRC) sum score after ensuring that patients were awake and cooperative (median Day 11, inter-quartile range 9–15 in ICU). An MRC sum score <48 was used to diagnose clinically relevant muscle weakness.

DNA extraction and preparation

Genomic DNA was extracted using proteinase K tissue digestion and isopropanol DNA precipitation. The Qubit® 3.0 fluo-

rometer (Thermo Fisher Scientific, Waltham, MA) was used to quantify DNA concentrations. DNA (400 ng) was bisulfite converted (EZ-96 DNA Methylation-Direct® Kit, Zymo Research, Irvine, CA). DNA methylation analysis was performed with Infinium HumanMethylationEPIC BeadChips (Illumina Inc., San Diego, CA) interrogating more than 850 000 CpG sites, spanning over 99% of the genes in the RefSeq database (Genomics Core, KU Leuven, Leuven, Belgium). Samples were randomly distributed over 25 chips from the same production lot.

DNA methylation data preprocessing

Microarray data were pre-processed in R (Version 3.6.1, R Foundation for Statistical Computing), using the *minfi* package (Version 1.30.0).[28,29] Sample quality assessment was performed using detection *P*-values, signal intensities, density plots, and principal component analysis (Supporting Information, *Figures* S1–S5). Raw intensities were normalized with stratified quantile normalization.[28,30] Probes with mean detection $P > 0.01$ in at least 50% of samples (indicating poor detection of signal above noise) and probes spanning known single nucleotide polymorphisms or located on sex chromosomes (n = 49 780, *IlluminaHumanMethylationEPICanno.ilm10b4.hg19* annotation file, Version 0.6.0) were excluded. Residual batch effects were corrected with a parametric empirical Bayes method (ComBat, *sva* package, Version 3.32.1),[31,32] taking into account age, BMI, patient–control status, randomization to Early-PN vs. Late-PN, and presence of muscle weakness to preserve associated biological variation (*Figures* S4 and S5).

Gene expression

As proof of concept, gene expression was analysed for 10 genes with differential DNA methylation. Genes were selected based on magnitude of the DNA methylation alterations, to cover different combinations of position within the gene and hypomethylation or hypermethylation in patients vs. controls and to cover a range of representative functions associated with the identified genes. RNA extraction and real-time PCR are described in the Methods section of the Supporting Information and *Table* S1.

Statistical analysis

Demographics, baseline characteristics, and gene expression data are reported as number (frequency), or median (interquartile range), and were compared with Fisher's exact test or Mann–Whitney *U* test.

Differential methylation of CpG sites between patients and controls was assessed with *F*-tests (*dmpFinder*, *minfi* package, Version 1.30.0) on the batch effect-corrected *M*-values. False discovery rate (FDR) according to Benjamini–Hochberg was used to correct *P*-values for multiple comparisons, yielding *q*-values.[33] Associated genes, gene sections, and positions relative to CpG islands were extracted from the Illumina EPIC annotation file (*IlluminaHumanMethylationEPICanno.ilm10b4.hg19* package, Version 0.6.0). Promoter regions were defined as 1500 bp upstream of the transcription start site. Gene-associated biological processes, cellular components, and molecular functions over-represented among resulting genes were analysed with over-representation enrichment analysis in WebGestalt 2019.[34]

Differential methylation of genomic regions, with expected more profound impact on gene expression than single CpG sites, was assessed with bump hunting (*bumphunter*, *minfi* package, Version 1.30.0), using the average difference among significant CpG sites as cut-off.[35] Permutation testing with 1000 permutations assessed uncertainty, expressed as family-wise error rate (FWER). Overlapping gene loci were annotated via the Illumina EPIC annotation file (*IlluminaHumanMethylationEPICanno.ilm10b4.hg19* package, Version 0.6.0).

Analyses of participants' demographics and gene expression were performed in JMP® Pro 14.0.0 (SAS Institute, Cary, NC). Microarray data were analysed in R (Version 3.6.1). Two-sided *P*-values, *q*-values (FDR-adjusted *P*-values), or FWER < 0.05 were considered statistically significant.

Results

Participants

Sufficient DNA could be extracted from 173 patients and all healthy control muscle biopsies (*Figure* 1). Unrepairable damage to one IDAT data file forced exclusion of another patient. All the remaining samples passed the quality controls (*Figures* S1–S5) and were included in downstream analyses. Baseline characteristics of patients and controls are reported in *Table* 1.

Cytosine–phosphate–guanine sites differentially methylated between intensive care unit patients and healthy control subjects

The methylation status of 565 CpG sites differed for patients and healthy controls ($q < 0.05$, corresponding to $P < 0.00005$). Of these, 427 (75.6%) were hypomethylated and 138 (24.4%) hypermethylated in patients as compared

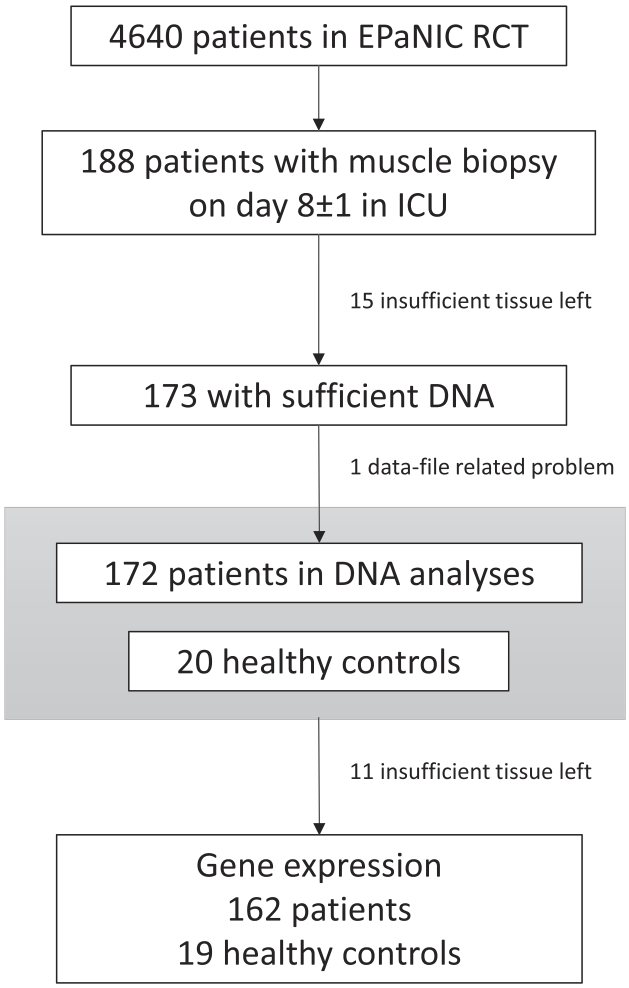

Figure 1 Consort diagram. Data file-related problem refers to unrepairable damage to one of the IDAT data files, which became unreadable. DNA, deoxyribonucleic acid; EPaNIC, Early Parenteral Nutrition Completing Enteral Nutrition in Adult Critically Ill Patients; ICU, intensive care unit; RCT, randomized controlled trial.

with controls (*Table* S2). Absolute differences in mean DNA methylation were 3.2% on average (SEM 0.07%), ranging up to 16.9%.

The 565 CpG sites were distributed over all autosomes (*Figure* 2A), and 396 (70.1%) of them were associated with a known gene transcript. Of these 396 CpG sites, 111 (28.0%) were located within a gene promoter, 267 within a gene body (67.4%), and 79 (19.9%) and 13 (3.3%) within the 5′ and 3′ untranslated region (UTR) of genes, respectively (*Figure* 2B and *Table* S2). Fifty-nine of the 565 CpG sites (10.4%) were located within known CpG islands, whereas the majority were located within open-sea regions (352/565 CpG sites, 62.3%), CpG shores (119/565, 21.1%), or CpG shelves (35/565, 6.2%) (*Figure* 2C).

Function and over-representation analysis of genes with differentially methylated cytosine–phosphate–guanine sites

The 565 differentially methylated CpG sites were associated with 400 unique genes (*Table* S2). These 400 genes have various functions, among which many are highly relevant for muscle structure and/or weakness. Indeed, these genes are involved in myogenesis, muscle regeneration, nerve/muscle membrane excitability, muscle denervation/re-innervation, axon guidance/myelination/degeneration/regeneration, synapse function, ion channelling with especially calcium signalling, metabolism (glucose, protein, and fat), insulin signalling, neuroendocrine hormone regulation, mitochondrial function, autophagy, apoptosis, oxidative stress, Wnt signalling, transcription regulation, muscle fat infiltration during regeneration, and fibrosis. Furthermore, several genes have been associated with muscle ageing, myopathies, and neurological diseases. Others have not been associated with muscle or nerve functioning yet, or their function remains to be identified. Intriguingly, several affected CpG sites are located in genes involved in epigenetic regulation, such as DNA methyltransferase 1, histone modifiers, and non-coding RNAs (miRNAs and lncRNAs).

Over-representation of biological processes, cellular components, and molecular functions among the 400 genes is shown in *Figure* S6 and *Table* S3. Twenty-four biological processes were identified as over-represented, which were related to adhesion, development and morphogenesis, and regulation of body core temperature. Seven over-represented cellular components related to the plasma membrane, cell interaction, the I band, and the actin cytoskeleton. Calcium ion binding was the only over-represented molecular function.

Differentially methylated regions and gene function

Two DNA regions were significantly differentially methylated (FWER < 0.05) between patients and controls. One spanned 635 nucleotides, containing 18 CpG sites (*Figure* 3A) hypomethylated in patients (average difference in beta-values 5.8%, SEM 0.01%). The region lies in a CpG island in the promoter of the hypermethylated in cancer 1 (HIC1) gene. HIC1 is a growth regulator and tumour-suppressor gene that regulates muscle regeneration and affects the level of postsynaptic acetylcholine receptors.[36] The second region spanned 34 nucleotides, containing three CpG sites hypomethylated in patients (average difference 12.1%, SEM 0.04%) (*Figure* 3B). This region lies in a CpG island in the promoter of the NAD kinase 2 (NADK2) gene. NADK2 encodes a

Table 1 Demographics, baseline characteristics, and muscle weakness of participants

Characteristic	Patients (N = 172)	Controls (N = 20)	P-value
Male sex, *n* (%)	114 (66.3)	14 (70.0)	0.80
Age (years), median [IQR]	62.7 [50.3–73.0]	58.0 [52.5–66.5]	0.31
BMI (kg/m^2), median [IQR]	25.9 [23.4–29.0]	24.4 [22.7–29.3]	0.43
Diabetes mellitus, *n* (%)	27 (15.7)		
History of malignancy, *n* (%)	46 (26.7)		
NRS score ≥5, *n* (%)	58 (33.7)		
APACHE II score upon admission, median [IQR]	34 [27–40]		
Emergency admission, *n* (%)	154 (89.5)		
Diagnostic category at admission, *n* (%)			
Cardiac surgery	16 (9.3)		
Elective other surgery	7 (4.1)		
Emergency other surgery	97 (56.4)		
Medical disease	52 (30.2)		
No enteral feeding instructed by surgeon, *n* (%)	32 (18.6)		
Infection upon admission, *n* (%)	100 (58.1)		
Sepsis upon admission, *n* (%)	96 (55.8)		
Randomization to Late-PN, *n* (%)	82 (47.7)		
Muscle weakness, *n* (%)			
Weak (MRC < 48)	45 (26.2)		
Not weak (MRC ≥ 48)	39 (22.7)		
Not assessable	88 (51.2)		

APACHE, Acute Physiology And Chronic Health Evaluation; BMI, body mass index; IQR, inter-quartile range; MRC, Medical Research Council; NRS, Nutritional Risk Screening; PN, parenteral nutrition.

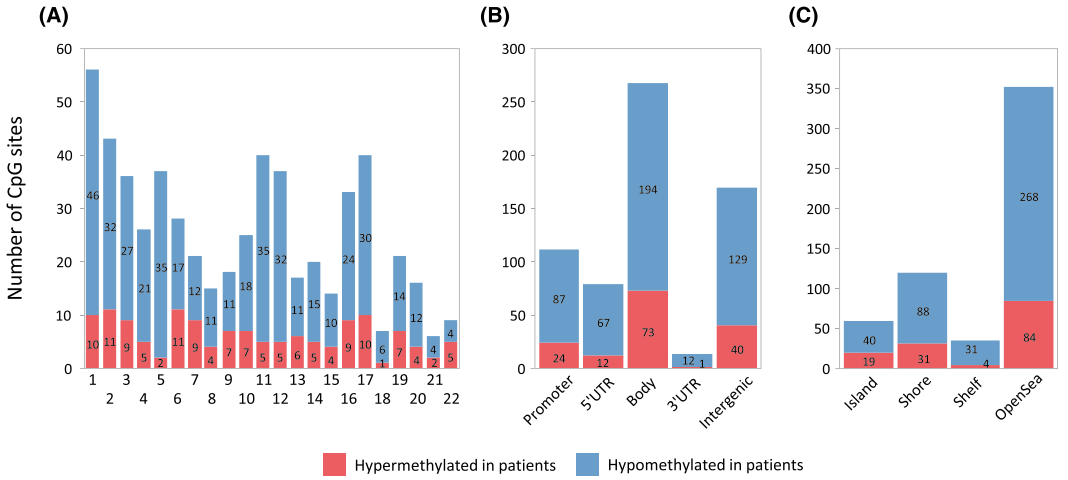

Figure 2 Location of differentially methylated cytosine–phosphate–guanine (CpG) sites. (A) Distribution over the autosomes, (B) association with known gene transcripts, and (C) relative position to CpG islands are shown for the 565 CpG sites that were significantly differentially methylated in critically ill patients as compared with healthy controls. Gene transcript regions are divided into the promotor region, where proteins bind to initiate transcription of the gene; the 5′ untranslated region (5′-UTR) region, which regulates translation; the gene body, which actually encodes the protein; the 3′ untranslated region (3′-UTR) region, which is a regulatory unit; and the intergenic region, which is not related to a known gene transcript. In relation to CpG islands, the regions are divided into islands, which are regions with a high frequency of CpG sites; shores, which immediately flank the islands (within 2000 bp); shelves, which flank the shores (within 4000 bp from a CpG island); and open-sea regions, which are not related to a CpG island. The height of the bars indicates the total number of CpG sites. Red and blue bars indicate the number of CpG sites that were respectively hypermethylated and hypomethylated in patients as compared with controls. In relation to gene transcripts, the 78 CpG sites that were associated with more than one gene section type are included for each of these associations.

mitochondrial kinase that catalyzes phosphorylation of NAD to NADP and plays a role in the metabolism of polyunsaturated fatty acids and lysine and various other mitochondrial processes.[37,38]

Gene expression analysis

Among the 10 studied genes, which included the HIC1 and NADK2 genes and other genes involved in metabolism,

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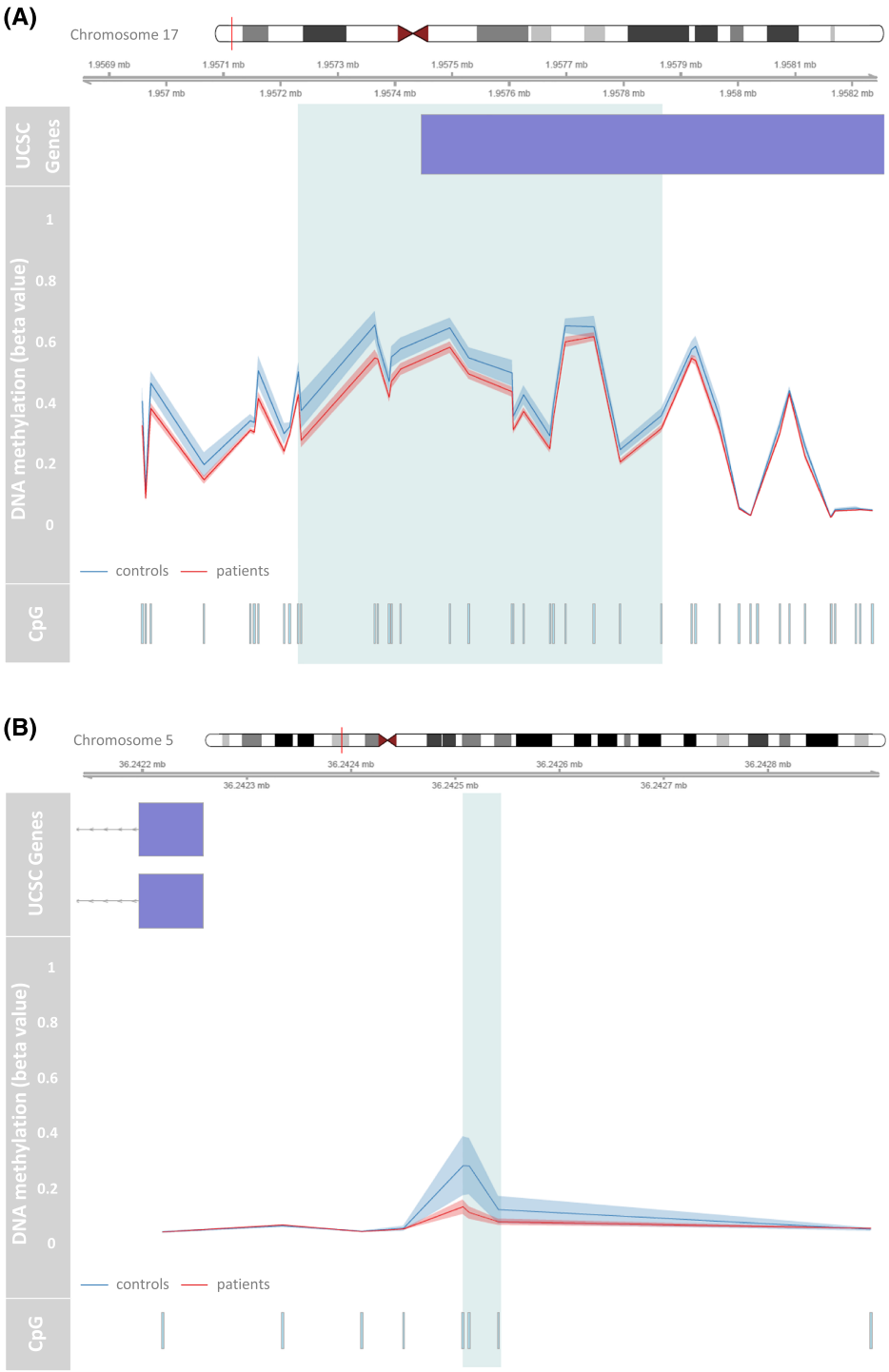

Figure 3 Visualisation of the differentially methylated regions in patients vs. controls. Regions that are differentially methylated in muscle of critically ill patients as compared with healthy controls are visualised. The red line on the chromosome ideogram (top line of each panel) indicates the position of the differentially methylated region (DMR) relative to the chromosome. The light blue highlighted area shows the DMR. In blue and red, average cytosine–phosphate–guanine (CpG) methylation (beta-value) and its confidence interval are shown for healthy controls and patients, respectively. At the bottom of each panel, the location of CpG sites evaluated by the microarray is indicated. Purple areas show locations of exons of known gene transcripts in this region. (A) The first DMR, which is located on chromosome 17 and spans 636 nucleotides containing 18 CpG sites. All 18 CpG sites were hypomethylated in patients as compared with controls. This region spans the hypermethylated in cancer 1 (HIC1) gene. (B) The second DMR, which is located on chromosome 5, spans 34 nucleotides, and contains three CpG sites that were hypomethylated in patients as compared with controls. This region spans the NAD kinase 2 (NADK2) gene.

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myogenesis, muscle regeneration, calcium channelling, mitochondrial stress, apoptosis, and histone modification, 9 were significantly differentially expressed between patients and controls, with effect sizes ranging from 18.3% to 77.5% (*Figure* 4 and *Table* S4).

Discussion

We demonstrated that the skeletal muscle DNA methylation signature was significantly different in critically ill patients as compared with matched healthy control subjects. Indeed, 565 CpG sites were differentially methylated, with an average difference in beta-values of 3.2%, and predominantly towards hypomethylation in patients. Many of the 400 genes associated with these CpG sites are important for muscle structure and function, and several have been associated with myopathies or neurological disorders. In addition, two DNA regions spanning multiple CpG sites were identified as hypomethylated in patients, located in the promoters of genes important for muscle regeneration, postsynaptic acetylcholine receptors, and mitochondrial processes. Differences in DNA methylation were associated with altered gene expression in 90% of the studied genes.

DNA methylation differences occurred throughout the genome, with 565 differentially methylated CpG sites spread over all autosomes and occurring both in known genes (70.1%) and in intergenic regions (29.9%). CpG sites within genes were predominantly located in gene bodies and also occurred in promoter regions and 5′-UTR and 3′-UTR. With an average difference of 3.2%, ranging up to 16.9%, the observed effect size of DNA methylation differences was similar to that before and after a 5 day high-fat diet in healthy men (average difference 3.5%, ranging up to 13%), but smaller than the impact of ageing (average difference 9.9% between healthy volunteers with mean age 73 vs. 21 years) or type 2 diabetes (average difference 5.0%).[16,39,40] However, ageing and type 2 diabetes represent chronic conditions, allowing much more time for the changes to develop. Also, the small sample sizes in those studies may have required larger differences to reach statistical significance. Especially when compared with ageing muscle, which shows hypermethylation in 92% of the affected CpG sites,[16] the DNA methylation signature in ICU patients was strikingly different, revealing predominantly hypomethylation as compared with controls. However, critical illness had a comparable impact on methylation in leukocyte DNA in children, with predominantly hypomethylation and an average effect size of 2.6% ranging up to 21.6%, but in different genes.[23]

The majority of the 400 differentially methylated genes have functions expected to be important for muscle homeostasis and to interfere with normal muscle structure or function when dysregulated. Several functions have already been implicated in the development of ICU-acquired weakness.[9] As proof of concept, we observed differences in gene expression in 9/10 studied genes when comparing patients with controls, suggesting that the differences in DNA methylation alterations go hand in hand with differences in gene expression, further supported by substantial overlap of the differen-

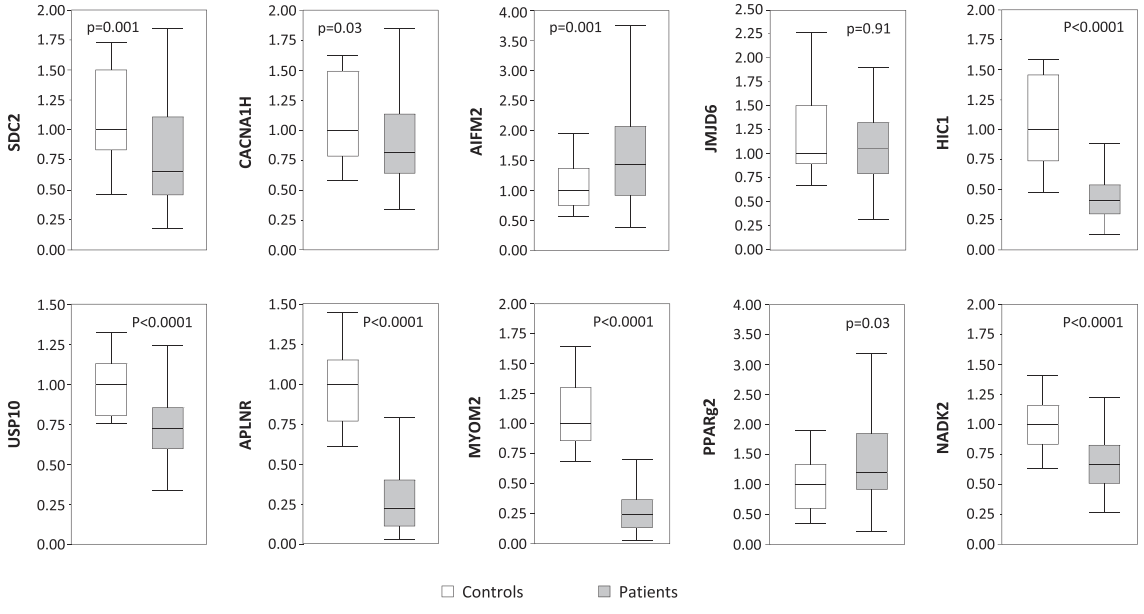

Figure 4 Gene expression. mRNA expression of selected genes affected by DNA methylation alterations is shown for controls (n = 19) and patients (n = 162). Results are expressed relatively to the median of the healthy controls, with cancer susceptibility candidate gene 3 (CASC3) used as housekeeping gene. Boxes represent medians and inter-quartile ranges, and whiskers are drawn to the furthest point within 1.5 × inter-quartile range from the box. *P*-values for comparison of expression between patients and controls are shown for each gene.

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tially methylated genes with genes found to be differentially expressed in critical illness in transcriptome analyses.[41,42] Whether the differences in DNA methylation remain present after ICU discharge and have a lasting impact on gene and protein expression, and functions, remains to be investigated, but may be supported by overlap with genes shown to be differentially expressed post-ICU[10,11] in a small study of patients with persisting weakness 6 months after ICU discharge. Strikingly, this study suggested a role for impaired long-term muscle regeneration, known to be regulated by DNA methylation and now found among the functions associated with differential methylation and gene expression in our study.[10,11,43] Defective regeneration after sepsis due to muscle stem cell malfunction only improved after engrafting healthy stem cells, suggesting a rather permanent effect.[44] Together with our findings, it is tempting to hypothesize that DNA methylation alterations may be responsible for long-term muscle dysfunction after critical illness through an impact on regeneration.

Interestingly, several affected genes play a role in epigenetic regulation itself, with altered DNA methylation in genes encoding DNA methyltransferase 1, histone modifiers, and non-coding RNAs such as miRNAs and long non-coding RNAs. Roles for histone modification and miRNAs have been proposed in development but not yet in persistence of ICU-acquired weakness.[9,45,46] Our findings further suggest a complex crosstalk between epigenetic regulators during critical illness, as in other diseases,[47] and may indicate that the DNA methylation signature of critical illness was not fully developed yet on Day 8 ± 1 in ICU.

Differential methylation of genomic regions can be expected to have a more profound impact on gene expression than single differentially methylated CpG sites. We found hypomethylated regions in HIC1 and NADK2 associated with altered gene expression. Effect sizes were large, with 5.8% and 12.1% average differences in beta-values over the regions. HIC1 functions in muscle regeneration,[36] which plays a role in persistence of weakness after ICU discharge. HIC1 also affects the level of postsynaptic acetylcholine receptors, of which reduced levels compromised muscle excitability in animals with sepsis.[9,48] NADK2 plays a role in fatty acid and lysine metabolism, and various other mitochondrial processes.[37,38] Derangement of several metabolic pathways and mitochondrial dysfunction have been described in the context of ICU-acquired weakness.[9] For both genes, however, gene and/or protein expression have not yet been studied directly in ICU-acquired weakness or its persistence. Our findings suggest that they form interesting targets for further investigation.

Strengths of this study include the genome-wide DNA methylation analysis through a very large number of CpG sites selected by 'epigenetics' experts, spread over all autosomes and spanning most RefSeq database genes. This allows to expand knowledge beyond gene functions that had already been implicated in ICU-acquired weakness. Next, stringent corrections for multiple comparisons ensured that the observed differences in DNA methylation were not findings by chance but rather remain a tip-of-the-iceberg observation.

Our study also has some limitations. First, the control group was relatively small, possibly underestimating differences between patients and controls. Second, muscle biopsies were obtained on ICU day 8 ± 1, precluding any conclusions with regard to DNA methylation differences upon ICU admission and at ICU discharge. Hence, we cannot rule out that underlying diseases or pre-admission functional status, or potential differences in lifestyle (e.g. diet or smoking) among the patients and controls, may have affected DNA methylation prior to ICU admission.[49] Fully ruling out such confounding can only be performed by obtaining an additional muscle biopsy just prior to the development of critical illness in the same patients, which is precluded by the unpredictable nature of human disease.[50] In addition, it hereby also remains unclear which DNA methylation differences remained present throughout the ICU stay, while DNA methylation alterations at ICU discharge may be expected to have a higher chance of being involved in long-term weakness. Third, we could not directly link the documented DNA methylation differences to long-term persistence of ICU-acquired weakness, because of lack of data on the persistence of weakness after ICU discharge in these specific patients. Fourth, heterogeneity within muscle tissue, as well as contamination with blood, may have affected our findings. However, there was virtually no overlap with CpG sites documented to be differentially methylated between type 1 and type 2 myofibres,[51] or in leukocytes of critically ill children,[23] suggesting that this theoretical issue cannot fully explain our findings. Finally, gene expression analysis was only performed on a limited selection of affected genes, because of scarcity of the remaining muscle tissue.

In conclusion, DNA methylation in muscle of critically ill adults differed from that in matched healthy subjects. These differences appeared functionally relevant as they occurred in genes with important functions in muscle tissue homeostasis and related to myopathies and neurological diseases, and coincided with differences in gene expression. Future research should focus on the impact of such differences on long-term gene and protein expression, and their association with persistent ICU-acquired weakness.

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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.

Conflict of interest

None declared.

All authors certify that they comply with the ethical guidelines for authorship and publishing in the Journal of Cachexia, Sarcopenia and Muscle.[52] Institutional review board approval was obtained (ML4190). The study was performed in accordance with the 1964 Declaration of Helsinki and its later amendments. Written informed consent was acquired from all patients or their next of kin.

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