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Citation	Güiza F, Vanhorebeek I, (2020), **Effect of early parenteral nutrition during paediatric critical illness on DNA methylation as a potential mediator of impaired neurocognitive development: a pre-planned secondary analysis of the PEPaNIC international randomised controlled trial.** Lancet Respir Med. 2020 Mar;8(3):288-303
Archived version	Author manuscript: the content is identical to the content of the published paper, but without the final typesetting by the publisher
Published version	http://dx.doi.org/10.1016/S2213-2600(20)30046-1
Journal homepage	Lancet Respiratory Medicine
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IR	https://lirias2.kuleuven.be/viewobject.html?cid=1&id=2959582

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Effect of Early Parenteral Nutrition during Paediatric Critical Illness on DNA Methylation as a Potential Mediator of Impaired Neurocognitive Development:

A pre-planned secondary analysis of the PEPaNIC international, randomised, controlled trial

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Key words: paediatric intensive care unit, PICU, epigenetics, DNA methylation, critical illness, nutrition, parenteral nutrition, children, long-term, outcome, development, cognition

Word count abstract: 333/300; Word count text: 4191/3500

Abstract (333/300)

Background: Early use of parenteral nutrition (early-PN) in the paediatric intensive care unit (PICU) has shown to negatively affect development of executive functions, externalising behaviour and visual-motor integration 2-years later, as compared with omitting PN until PICU day 8 (late-PN). The molecular basis is uncertain. We tested the *a priori* hypothesis that DNA methylation changes occur during critical illness and that early-PN, or a specific macronutrient component hereof, contributes to these changes, which could explain its negative impact on neurocognitive development.

Methods: A pre-planned secondary analysis of all patients with a last PICU-day blood sample (n=825, 0-17 years at PICU-admission) who were randomised (1:1) to early-PN or late-PN in the multicentre (Leuven-Rotterdam-Edmonton) PEPaNIC-trial (clinicaltrials.gov-NCT01536275, performed 2012-2018), as compared with 352 matched healthy children. Genome-wide peripheral blood leukocyte DNA methylation was determined with the Infinium® HumanMethylation EPIC-BeadChip for >850000 CpG-sites. Applying a false discovery rate <0·05, DNA methylation of patients on the last PICU-day was compared with healthy children, after excluding all CpG-sites differentially methylated upon PICU-admission, as these reflected pre-admission conditions and altered leukocyte composition. Bootstrapped multivariable linear/non-linear regression analyses assessed the impact of early-PN versus late-PN on illness-induced alterations in DNA methylation and to what extent differentially methylated CpG-sites explained impaired neurocognitive development 2 years later. Investigators were blinded to treatment allocation.

Findings: During PICU-stay, 159 CpG-sites in genes involved in brain development/plasticity/signalling, neuronal differentiation/migration/growth, metabolism, transcriptional regulation, physical development/locomotion, and neurodegenerative/neuropsychiatric diseases, became methylated differently than in healthy children, with mean±SD effect sizes of 2·6±2·5% ranging up to 21·6% (P<0·00002). Early-PN, in particular the dose of amino acids, independently contributed to differential methylation of 37 of these 159 CpG-sites (P from 0·0001 to 0·050) which, together, statistically explained its adverse impact on neurocognitive development at 2-years follow-up (R^2 0·61±0·01).

Interpretation: Early-PN during paediatric critical illness altered DNA methylation which suggested a plausible molecular basis for its negative impact on long-term neurocognitive development. The early administration of amino acids was identified as main culprit, a finding that requires further investigation.

Funding: European-Research-Council,Methusalem,Flanders-Institute-for-Science-and-Technology,Research-Foundation-Flanders,Sophia-Foundation,Stichting-Agis-Zorginnovatie,Erasmus-Trustfonds,ESPEN.

Introduction

Critical illness in children has been associated with long-term impairment of physical and neurocognitive development and with medical problems that remain present years after hospital discharge.[1-5] The use of parenteral nutrition (PN) early during critical illness (early-PN) was recently shown to be one modifiable risk factor for part of the long-term neurocognitive legacy of paediatric critical illness.[4] Indeed, the PEPaNIC randomised controlled trial showed that the use of early-PN, as compared with withholding PN up to day 8 in the paediatric intensive care unit (PICU), (late-PN), not only induced more infections and delayed recovery from organ failure in the PICU but also worsened long-term neurocognitive development, in particular of executive functions that are important for many aspects of daily life.[4,6] The molecular basis of this finding remains unclear.

The methylation or demethylation of DNA is one potentially important underlying molecular mechanism. Via the attachment to or removal of a methyl group from a nucleotide, occurring almost exclusively at the 5' carbon in the cytosine residue of CpG-dinucleotides,[7] DNA methylation or demethylation alters gene expression and hereby affects diverse biological processes such as physical and neurocognitive development.[8,9] Alterations in DNA methylation have been implicated in the adverse long-term effects of various environmental toxicants.[10] Particularly in young children, DNA methylation changes may bring about long-term effects.[9] One could speculate that also critical illness and/or its management could alter the DNA methylation status of children, which could explain part of the long-term impairments. Both undernutrition and overfeeding in otherwise healthy children have shown to induce methylome alterations that are associated with long-term disturbances in physical and neurocognitive development.[11-14] It is therefore plausible that the currently still variable nutritional management in the PICU[15] alters DNA methylation.

We *a priori* hypothesised that *de novo* DNA methylation changes occur during critical illness and that the use of early-PN, as compared with late-PN, or a specific macronutrient component hereof, is a contributor to these changes. We further hypothesised that such changes could provide a biological basis for the negative impact of early-PN on neurocognitive development 2 years later.

Methods

Study design

This is a pre-planned secondary analysis of the randomised, controlled PEPaNIC trial on nutrition in PICU-patients performed in the University Hospitals Leuven (Belgium), the Erasmus Medical Centre-Sophia Children's Hospital in Rotterdam (The Netherlands) and the Stollery Children's Hospital in Edmonton (Canada) (n=1440, performed June 18, 2012, through January 19, 2018); full trial protocol accessible at www.kuleuven.be/licm), in comparison with healthy control children.[4,6,16] Institutional Review Board approval for participation of patients and healthy controls in this study was obtained in all centres [Leuven_ML8052-ML2586; Rotterdam_NL38772.000.12; Edmonton_Pro00038098], in accordance with the 1964 Declaration of Helsinki and its amendments.

Participants

The PEPaNIC study included children, from term newborns to children 17 years of age, for whom a stay of 24 hours or more in the ICU was expected, who had a score on the Screening Tool for Risk on Nutritional Status and Growth (STRONGkids) of 2 or more (with a score of 0 indicating low risk of malnutrition, a score of 1 to 3 indicating medium risk, and a score of 4 to 5 indicating high risk), and who did not meet any of the criteria for exclusion.[6] All PEPaNIC patients for whom a blood sample had been collected on the last day in PICU (n=825) were selected for this study, together with a demographically matched healthy control group of children who had never been admitted to a PICU and underwent blood sampling immediately after placement of an intravenous catheter prior to minor elective surgery (n=352), recruited between July 14, 2011 and September 21, 2015.[4,6,16] All parents/legal guardians and/or the children provided written informed consent.

Procedures, randomisation and masking

Patients had been randomly assigned (1:1) to early initiation of PN within 24 hours, supplementing insufficient enteral nutrition (EN) to reach age-specific caloric/macronutrient targets (early-PN), or to postponing any supplemental PN to beyond the first week in PICU (late-PN), the latter corresponding to no PN in the majority of children.[6] After 1 week, for both groups equally, PN could be administered if necessary. When EN covered ≥80% of calculated targets, supplemental PN was discontinued. For both groups, EN was initiated early and patients received intravenous micronutrients until fully enterally fed. Impact on short-term PICU outcome and on long-term outcome at 2-years' follow-up has been reported.[4,6] Clinical outcome assessors were physicians, research nurses, and experienced paediatric psychologists not involved in patient management during PICU-stay and, together with the assessors of genome-wide DNA methylation status, were strictly masked for allocation to late-PN or early-PN. Parents were not masked during PICU-stay of their child and were not actively informed about the initial PEPaNIC study results.

Outcomes

Prespecified outcomes were the abnormal *de novo* alterations in DNA methylation that occur during PICU stay and are brought about by early PN, and their link with developmental impairment as assessed two years after PICU stay.[4] The use of early-PN, as compared with late-PN, has shown to negatively affect development of inhibitory control, metacognition, working memory as well as the total executive functions score, of externalising behaviour and of visual-motor integration. Although only inhibitory control met the conservative Bonferroni correction significance threshold,[4] as neurocognitive functions are not independent, this correction is considered too strict and the six outcomes were retained as relevant for this study. For details of the developmental assessments, see appendix (Methods_S1). Throughout PICU-stay, repeated blood samples were collected from which cell pellets were stored at -80°C. Genomic DNA from all available last PICU-day samples (n=825), all available PICU-admission samples from those patients with a last PICU-day sample (n=695), and from the samples of healthy children (n=352) was extracted and bisulfite-converted (Methods_S2). For 9 last PICU-day samples, DNA yield was insufficient (Figure_1). A genome-wide DNA methylation analysis was performed with the Infinium® HumanMethylation EPIC BeadChip (Illumina Inc., San Diego, CA), interrogating >850.000 CpG-

sites and spanning >99% of genes in the Reference Sequence (RefSeq) database (Genomics Core, KU Leuven).[17] Investigators were blinded to participants' characteristics and randomisation.

Statistical analyses

The stepwise concept, rationale and design of the study are summarised in Figure_2.[18-21]

For univariable statistical analysis of the differences in genome-wide DNA methylation between groups, methylation data were pre-processed using Partek Genomics Suite® 7.0 (Partek, St. Louis, MO). Methylation β-values, ranging from 0 (no methylation) to 1 (full methylation), and M-values, which are the log2 ratios of the intensities of methylated probes versus unmethylated probes, were obtained after functional normalisation of the raw intensities. The data were subjected to several quality assessments at sample and probe level (Methods_S3).

Partek Genomics Suite® 7.0 was used to compare M-values between groups with ANOVA, applying a false discovery rate (FDR) <0·05 according to the Benjamini & Hochberg "Step-up" procedure to account for multiple testing inherent to epigenome-wide analyses.[22-25] Based on the RefSeq annotations, CpG-sites were assigned to corresponding genes and relative location to gene transcripts. Promoter sequences were defined as 1500 base pairs upstream of the transcription start site. Pathway analyses were performed based on the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database with Partek Pathway® (Partek, St. Louis, MO) and gene ontology analyses (Methods_S3).

To investigate whether DNA methylation was altered between PICU-admission and discharge it was first necessary -to avoid bias- to exclude from all further analyses all CpG-sites that were differentially methylated in patients upon PICU-admission as compared with healthy children, as these differences reflect pre-morbid conditions and illness-induced alterations in leukocyte composition.[18-20] To maximally avoid such bias, two “patient versus healthy control” cohorts were selected (Figure_1). For details on the criteria used to select PICU patients and healthy controls, see appendix (Methods_S4). All CpG-sites differentially methylated between patients and controls in one or both of these comparisons, with FDR<0·05 to adjust for multiple testing, were

discarded from further analyses (Figure_2A, step_1). To subsequently investigate whether *de novo* DNA methylation changes occurred during critical illness, the degree of DNA methylation in the remaining CpG-sites was compared with ANOVA and FDR<0·05, between the last PICU-day patient samples and samples from healthy children with similar age and gender distribution (Figure_2A, step_2).

To investigate to what extent parenteral nutrition (early-PN versus late-PN) plays a direct role in bringing about *de novo* (de)methylated CpG-sites on the last PICU-day in patients (Figure_2A, step_3), multivariable linear regression analyses were performed adjusting for baseline risk factors [age, centre, race, gender, geographic origin, language, history of malignancy, diabetes, predefined "syndrome" (Methods_S5), diagnosis and severity of illness (PIM3 and PeLOD), and risk of malnutrition (STRONGkids)], and further for the acute effects of early-PN, versus late-PN, on infections, duration of PICU-stay, mechanical ventilatory support and hypoglycaemia. To robustly identify those CpG-sites differentially methylated by early-PN, the analysis was repeated in 100 bootstrap samples and the result declared "robust" if the association (P<0.05) was present in at least 50.[26,27] Heatmaps were constructed to summarise these findings. Additional multivariable linear regression analyses were performed to investigate which macronutrient best explains the differential methylation of CpG-sites by early-PN, (appendix Methods_S6).[28]

To investigate any potential role of altered DNA methylation evoked by critical illness in statistically explaining impaired long-term outcomes (Figure_2A, step_4), multivariable linear and logistic regression analyses were performed adjusting for baseline risk factors, with stepwise backward-forward variable selection according to Rubin's method,[29] as described in the appendix (Methods_S7). To account for potential non-linear interactions between the differentially methylated CpG-sites on the last PICU-day and the long-term outcomes, analyses with random forest machine learning models[30] adjusted for baseline risk factors were also performed, with covariate significance levels obtained via permutation importance.[31] All analyses were evaluated in 100 bootstrapped replicates which allowed to quantify optimism-corrected R^2, a robust performance metric.[32] To quantify any additional explanatory power provided by the differentially methylated CpG-sites on the last PICU-day, the fold-change in optimism-corrected R^2 between models with and without addition of the CpG-sites as covariates was

calculated. The differentially methylated CpG-sites and the outcomes were then hierarchically clustered based on the frequency with which a CpG-site was found to be independently and significantly (P<0·05) associated with the outcomes in the 100 bootstrapped replicated analyses. Heatmaps and dendrograms with CpG-sites assigned to functional classes were used to visualise and summarise these findings.

To investigate any potential role of altered DNA methylation evoked by early-PN, as compared with late-PN, in statistically explaining the adverse impact of early-PN on neurocognitive development at 2-years follow-up (Figure_2A, step_5, Figure_2B), multivariable linear and non-linear regression analyses were performed, adjusting for baseline risk factors and further for any CpG-sites differentially methylated by early-PN. Stepwise backward-forward variable selection according to Rubin's method (Methods_S7)[29] was used for the linear case and covariate significance levels obtained via permutation importance were used for the non-linear case.[31] Again, the robustness of these results was evaluated by 100 bootstrapped replicates and the fold-change in optimism-corrected R^2 between models with and without addition of the CpG-sites differentially methylated by early-PN as compared with late-PN as covariates was calculated.[32] Heatmaps with dendrograms were used to visualise and summarise the findings.

To investigate correspondence between blood and brain for the CpG-sites differentially methylated on the last PICU-day, the BECon tool for interpreting DNA methylation findings from blood in the context of brain and the Brain Epigenomics blood brain DNA methylation comparison tool were used. For details on the cross-tissue comparisons, see appendix (Methods_S8).

Data are presented as numbers (proportions), medians (interquartile ranges) or means±SD. Univariable comparisons were performed with Chi-square (Fisher-Exact) testing and with Wilcoxon rank-sum test. Besides the above-mentioned analyses performed with the Partek software, statistical analyses were performed with JMP®13.0.0 (SAS Institute, Inc, Cary, NC), R 3.5.3 and additional packages MICE 3.4.0, miceadds 3.4.17, ranger 0.11.2, caret 6.0.84, parallel 3.5.3, boot 1.3.20 and simpleboot 1.1.7. For the genome-wide comparison of DNA

methylation differences between patients and healthy children, significance was set at FDR<0·05 to adjust for multiple testing.[25] For all other analyses, two-sided P-values <0·05 were considered statistically significant.

The trial is registered at clinicaltrials.gov-NCT01536275.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, writing of the report, or the decision to submit for publication. The corresponding author had full access to all data and had final responsibility for the decision to submit for publication.

Results

In a first step, we identified bias-inducing differences in DNA methylation present upon PICU-admission in patients as compared with healthy children, for subsequent exclusion from the analyses. Upon PICU admission, 374106 CpG-sites were identified as methylated differently in patients as compared with controls and were excluded from all further analyses to avoid bias. Gene ontology and corresponding pathway analyses revealed that these differences were related to cellular signalling and inflammation pathways (data not shown). DNA methylation quality control is shown in appendix Figures_S1-2.

In a second step, we documented *de novo* DNA methylation changes occurring in patients during PICU-stay. From last PICU-day blood samples, quality-approved DNA was available for 814 patients to compare with 119 healthy children of similar age and gender (Figure_1). Patient characteristics are shown in Table_1. DNA methylation quality control upon PICU discharge is shown in appendix Figure_S3. On the last PICU-day, 159 CpG-sites were identified, with FDR<0·05 (equivalent to P<0·00002), as methylated differently in patients than in matched controls (Figure_3,Table_S1). On average, the absolute mean±SD differences in DNA methylation β-values for these CpG-sites were 2·6±2·5%, ranging up to 21·6%, with mostly hypomethylation (126/159 CpG-sites, 79·2%) in patients (Figure_3,Table_S1). Of the differentially methylated CpG-sites, only 14/159 (8·8%) were located in gene-promoter regions, 79/159 (49·7%) were located in gene bodies of which 57/79 (72·2%) were hypomethylated in patients, 8/159 (5·0%) were in non-protein-coding regions and 58/159 (36·5%) were in intergenic regions. Genes containing the differentially methylated CpG-sites are involved in a variety of relevant molecular functions, developmental processes and diseases. These include, amongst others, brain development/plasticity/signalling, neuronal differentiation/migration/growth, metabolism, transcriptional regulation, other cellular processes, physical development/locomotion and several neurodegenerative/neuropsychiatric diseases. For further details on the CpG-sited identified, see appendix (Table_S1).

In a third step, we investigated the impact of early-PN, versus late-PN, on DNA methylation alterations occurring during PICU-stay. Baseline characteristics of patients in the early-PN and late-PN groups are reported in Table_1 and caloric intake in the appendix (Figure_S4). Adjusted for baseline risk factors, and after 100 bootstrapped replicated analyses for robustness, early-PN, as compared with late-PN, was found to contribute to abnormal methylation of 37 of the 159 CpG-sites (P ranging from 0·0001 to 0·050) (Figure_4,Table_S2). This adverse impact of early-PN, versus late-PN, on DNA methylation was not mediated by its acute effects on infections, duration of PICU-stay or mechanical ventilatory support, nor was it affected by hypoglycaemia, as indicated by further adjusting for these effects. For results of these additional bootstrapped multivariable linear regression analyses, see appendix (Figure_S5,Table_S3). The impact of early-PN was mostly hypomethylation (27/37 CpG-sites, 73·0%) (Figure_4,Table_S1). Of these 37 CpG-sites, 22/37 (59·5%) were located within gene bodies of which 15/22 (68·2%) were hypomethylated by early-PN (Table_S1). For 27 of these 37 CpG-sites (73·0%), specifically the dose of amino acids, rather than the dose of glucose or lipids, explained the differential methylation evoked by early-PN. A schematic overview of these analyses is provided in the appendix (Figure_S6) and illustrations for 6 representative CpG-sites are provided in Figure_5.

In a fourth step, we investigated whether the differentially methylated CpG-sites evoked by critical illness could play a role in explaining impaired long-term outcome at 2-years follow-up. Of the patients who were included for DNA methylation analysis, 473 were evaluated functionally at 2 years' follow-up, with similar baseline characteristics but different caloric intake for early-PN and late-PN patients (Table_1,Figure_1),Figure_S4). Adding the differentially methylated CpG-sites on the last PICU-day to the multivariable regression models of the continuous long-term outcomes substantially improved the explanatory power of the models as compared with the models adjusted for baseline risk factors only, with a mean±SD optimism-corrected R^2 fold-increase of 4·09±5·00 and of 1·91±0·04 for the linear and non-linear models, respectively. With a mean±SD optimism-corrected R^2 of 0·62±0·01 the non-linear models explained an additional mean±SD 42·5±10·9% of the outcome variance as compared to the linear case. For details on the added explanatory power for each of the long-term outcomes provided by adding the differentially methylated CpG-sites, and by the non-linear models, see appendix (Table_S4). Based on the frequency of the independent association between CpG-sites and outcomes in the

bootstrapped analyses, the outcomes clustered into meaningful functional groups for the linear (Figure_S7) and non-linear (Figure_6) models, such as anthropometric measures, measures of intelligence, motor coordination, visual-motor integration, and executive and behavioural functions. For each group of outcomes, Figure_6 and Figure_S7 clearly reveal distinct patterns of association with the differentially methylated CpG-sites. The classication of CpG-sites according to function (derived from Table_S1) is reported in the appendix (Table_S5).

In a fifth step, we investigated whether altered DNA methylation evoked by early-PN, as compared with late-PN, could play a role in explaining the adverse impact of early-PN on neurocognitive development at 2-years follow-up. The neurocognitive long-term outcomes adversely affected by early-PN were development of executive functions (inhibitory control, metacognition, working memory and total executive function score), externalising behaviour and visual-motor integration. As compared with the multivariable regression models for these 6 long-term outcomes adjusted for baseline risk factors only, adding the 37 CpG-sites differentially methylated by early-PN as covariates improved the explanatory power (optimism-corrected R^2), with mean±SD fold-increases of 1·24±0·16 and 1·94±0·09 for the linear and non-linear models, respectively. With a mean±SD optimism-corrected R^2 of 0·61±0·01 the non-linear models explained an additional mean±SD 52·1±7·3% of the outcome variance as compared to the linear case. For details on the added explanatory power for the six long-term outcomes provided by the 37 differentially methylated CpG-sites, and by the non-linear models, see appendix (Table_S6). The hierarchical clustering revealed that distinct groups of CpG-sites were explanatory for the negative impact of early-PN on the 4 executive functions, externalising behaviour, and visual-motor integration in the linear (Figure_S8) and non-linear models (Figure_7).

Finally, we performed a web-based cross-tissue comparison. Given that tools for querying cross-tissue comparisons have been developed on the smaller Illumina 450K Human Methylation Array, cross-tissue comparison was only possible for about 30% of the affected CpG-sites in the current study that used the 850K Array. Among these, significant correlation between methylation status in blood and brain was present for 80% of the CpG-sites differentially methylated by early-PN.

Discussion

This study showed that critical illness in children altered DNA methylation, mostly towards hypomethylation, which statistically partially explained the disturbed long-term physical/neurocognitive development assessed 2 years later. Rather than in gene promoters, altered DNA methylation mainly occurred in non-protein-coding DNA, in intergenic DNA regions and in gene bodies of protein-coding genes, in particular those involved in growth and brain development, with average effect sizes of 2·6% ranging up to 21·6%. The use of early-PN, as compared with not using PN for 1 week in PICU, independently contributed to approximately a quarter of these *de novo* changes in DNA methylation, a direct effect best explained by the dose of amino acids, which suggested a plausible molecular basis for its negative impact on development of executive functions, externalising behaviour and visual-motor integration.

De novo alterations in DNA methylation of 159 CpG-sites occurred during PICU-stay which were statistically linked with the long-term physical/neurocognitive legacy of paediatric critical illness, providing evidence for a possible molecular basis hereof. This possibility was further supported by the finding that differentially methylated CpG-sites occurred in genes involved in brain development/plasticity/signalling, neuronal differentiation/migration/growth, metabolism, transcriptional regulation, physical development/locomotion, and several neurodegenerative/neuropsychiatric diseases. The impact on DNA methylation was quite large as compared with that of other early-life stressors.[33,34] In the context of the randomised controlled PEPaNIC trial, the use of early-PN could be identified as a modifiable, directly causal factor for approximately a quarter of the observed changes in DNA methylation. The main culprit appeared to be the early administration of amino acids rather than glucose or lipids. This finding was consistent with the previously identified role of amino acids in explaining the negative effect of early-PN on infections and intensive care dependency.[28] Differential DNA methylation evoked by early-PN statistically explained its negative impact on neurocognitive development. Earlier research supports a direct impact of nutrition on chromatin regulation via products of intermediary metabolism.[12] Although most studies focused on underfeeding, also overfeeding affects DNA methylation.[11,13,14]

It is well known that DNA methylation is an important controller of brain development and functioning, including neuronal stem cell maintenance and differentiation, and synaptic plasticity underlying memory and learning,[8] and

plays a role in the pathogenesis of neurodegenerative and psychiatric diseases.[8,35] Furthermore, disruption of epigenetic networks is a key pathogenic mechanism of intellectual and developmental disability,[36] corroborated by the increased explanatory power observed here when including non-linear interactions. Interestingly, although research on DNA methylation traditionally focuses on gene promoters, we here observed that the majority of the differentially methylated CpG-sites were located outside promoter regions, with hypomethylation in patients as compared with healthy children. Aberrant methylation patterns in non-promoter regions have previously been related with psychiatric problems in children.[37] While promoter methylation mostly represses gene expression, intragenic DNA methylation upregulates gene expression.[7] Active DNA demethylation is known to occur in somatic tissues such as the brain.[38] A recent epigenome-wide study showed intragenic hypomethylation induced by obesity.[39] Hypomethylation has been shown to impair learning and memory and to negatively affect plasticity of the hippocampal CA1 region.[40] Other studies revealed associations between hypomethylation and neurological diseases.[41] Hence, it is plausible that the observed hypomethylation within gene bodies evoked by critical illness, and by the early use of PN in the PICU, provides a molecular basis for the adverse impact on long-term neurocognitive development, as suggested by our statistical models. Altered methylation that affected long-term outcomes also occurred in non-protein coding DNA regions, perhaps not surprising given that non-protein-coding DNA drastically outnumbers protein-coding DNA in the human genome. Altered methylation of non-protein-coding DNA could exert its effect via short- and long non-coding RNAs, known to play key roles in neuronal development, homeostasis and plasticity.[8,42]

The strengths of this study comprise the randomised controlled study design of the PEPaNIC trial with the multicentric inclusion supporting external validity, the large groups of healthy and critically ill children, the genome-wide DNA methylation analysis interrogating >850.000 CpG-sites and the paired measurements per patient. The latter allowed to exclude confounding by pre-admission conditions and altered leukocyte composition and thus allowed to reliably document *de novo* changes occurring during PICU-stay. Another strength is the use of a bootstrap methodology to minimise risk of spurious associations. The study is limited by the fact that epigenetic changes can occur in a tissue-specific manner and thus it is unclear whether the methylation changes that we observed in DNA extracted from leukocytes are also present in the brain. However, human postmortem studies have reported overlapping DNA methylation changes occurring in brain and leukocytes,[43,44] further

supported by evidence from animal models.[45] Importantly, of the differentially methylated CpG-sites evoked by early-PN, for which cross-tissue comparison was possible,[43,44] 80% showed a significant correlation between blood and brain. We did not assess the impact of changes in DNA methylation on corresponding gene expression. However, our finding that DNA methylation changes in genes highly relevant for neurocognitive development and functioning were found to be statistically robustly linked to the studied neurocognitive outcomes supports the concept that DNA methylation changes could indeed mediate parts of the poor neurognitive development of critically ill children. All CpG-sites differentially methylated upon PICU-admission were excluded to avoid bias from pre-morbid conditions and illness-induced alterations in leukocyte composition. This likely underestimated, rather than overestimated, the number of CpG-sites affected by critical illness. We also may have underestimated the number of hypomethylated CpG-sites, as the bisulfite conversion procedure does not discriminate between 5-methyl-cytosine or 5-hydroxymethyl-cytosine, which is a first step towards demethylation. Five-hydroxymethyl-cytosine may also exert bona-fide regulatory functions, particularly in the brain,[46,47] which we could not investigate. Whether the observed DNA methylation changes are permanent or reversible, or correlate with corresponding gene expression, is unclear. However, neither scenario excludes a potential role in mediating adverse long-term outcome. Further, even though we identified different epigenetic changes in the two randomised nutritional groups, we cannot exclude any role of post-randomisation exposure, which remains subject for future studies. Additionally, although we have previously shown that infants younger than 1 year are particularly vulnerable to the neurocognitive harm evoked by early-PN,[4] it remains to be investigated whether specific age groups are particularly vulnerable for DNA methylation changes. Another limitation is the smaller sample size than that of the original study cohort, of patients who were assessed for altered DNA methylation during PICU-stay and were tested for neurocognitive development at 2-years follow-up. However, this reduced, rather than inflated, the odds of attributing a role for altered DNA methylation as a plausible mediator of impaired neurocognitive development. Finally, the observational nature of the further analyses that identified the dose of amino acids as the main culprit is a limitation.

In conclusion, functionally relevant *de novo* alterations in DNA methylation occurred during critical illness in children. The early use of PN statistically contributed significantly to this altered DNA methylation which suggested a plausible molecular basis for its negative impact on long-term neurocognitive development. This

effect appeared to be mostly explained by the dose of amino acids, rather than that of glucose or lipids, a finding that requires further investigation.

Research in context

Evidence before this study

The randomised Paediatric Early versus Late Parenteral Nutrition In Critical Illness (PEPaNIC) study showed that early use of parenteral nutrition (early-PN) to supplement insufficient enteral nutrition in critically ill children treated in the paediatric intensive care unit (PICU) negatively affected short-term outcomes, increasing the risk of acquiring infections and delaying recovery as compared with not using PN before PICU day 8 (late-PN). Clinicians remained concerned about potential adverse longer-term consequences of accepting large macronutrient deficits with late-PN for growth, health status, and neurocognitive development. However, in a 2-year follow-up study of these children, such harm was not observed. Instead, the use of early-PN in the PICU proved harmful for the longer-term development of executive functions, externalising behaviour and visual-motor integration of these children. The molecular basis of this finding was unknown. DNA methylation or demethylation affects diverse biological processes, such as physical and neurocognitive development and, particularly in young children, may bring about long-term effects. Both undernutrition and overfeeding in otherwise healthy children have been associated with methylome alterations in relation to long-term disturbances of physical and neurocognitive development. It is therefore plausible that the use of early-PN in critically ill children alters DNA methylation, which could explain part of its negative impact on long-term neurocognitive development.

We searched PubMed up to May, 2019, with no date limits set, and without language restrictions, with different combinations of the search terms “nutritional support”, “parenteral nutrition or feeding”, “intravenous nutrition or feeding”; “intensive care unit”, “critical care”, “critical illness”, “intensive care”, “ICU”, “PICU”; “long-term”, “neurocognitive development”; and “DNA methylation”, “5-methyl-cytosine”, “5mC”, or “epigenetic*”. We did not find any published studies on the impact of paediatric critical illness or of the timing of parenteral nutrition in PICU on (genome-wide) DNA methylation status in relation to the long-term development of critically ill children.

Added value of this study

This study showed that critical illness in children altered intergenic DNA methylation and DNA methylation in genes involved in brain development/plasticity/signalling, neuronal differentiation/migration/growth, metabolism, transcriptional regulation, physical development/locomotion, and neurodegenerative/neuropsychiatric diseases. These changes in DNA methylation were mostly directed towards hypomethylation and mainly occurred in non-protein-coding DNA, in intergenic DNA regions and in gene bodies of protein-coding genes rather than in gene promoters, with effect sizes that were quite large as compared with those of other early-life stressors. Together, these DNA methylation changes statistically explained part of the disturbed long-term physical and neurocognitive development that was documented 2 years after PICU admission. The use of early-PN, as compared with not using PN throughout the first week in PICU, independently contributed directly to approximately a quarter of these *de novo* changes in DNA methylation which, together, statistically explained part of the adverse impact of early-PN on long-term neurocognitive development. The main culprit appeared to be the dose of amino acids rather than glucose or lipids, a finding that requires further investigation.

Implications of all the available evidence

Functionally relevant *de novo* alterations in DNA methylation in critically ill children provided a plausible molecular basis for the negative impact of the early use of PN on long-term neurocognitive development, particularly of executive functions, externalising behaviour and visual-motor integration. The observation that nutrition during critical illness can modify DNA methylation status opens perspectives for identifying other causal factors, such as drugs used in the PICU, and for investigating the potential of pharmacologically targeting DNA methylation to improve long-term outcome.

Contributions

Greet Van den Berghe, FG, IV, and SV, designed the study. FG, IV, SV, IVe, ID, CI, KD, SCV, GGG, KFJ and PJW gathered data. FG, IV, SV, IVe and Greet Van den Berghe analysed the data and wrote the manuscript, which was reviewed and approved by all authors. All authors jointly decided to publish. Greet Van den Berghe had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Declaration of interests

We declare no competing interests.

Data sharing

Data sharing is offered under the format of collaborative projects. Proposals can be directed to the corresponding author.

Acknowledgements

This work was supported by the Research Foundation-Flanders (FWO), Belgium (FWO fellowship to Sören Verstraete); by the Methusalem program of the Flemish government (through the University of Leuven to Greet Van den Berghe and Ilse Vanhorebeek, METH14/06); by European Research Council Advanced Grants (AdvG-2012-321670 and AdvG-2017-785809) to Greet Van den Berghe; and by the Institute for Science and Technology, Flanders, Belgium (through the University of Leuven to Greet Van den Berghe, IWT/110685/TBM and IWT/150181/TBM); by the Sophia Research Foundation (SSWO) to Sascha Verbruggen; by the "Stichting Agis Zorginnovatie" to Sascha Verbruggen; and by a European Society for Clinical Nutrition and Metabolism (ESPEN) research grant to Sascha Verbruggen.

The authors acknowledge the psychologists and research team members involved in the study for their help with the neurocognitive testing, and for the technical and administrative support. Furthermore, they thank the children and their parents for their willingness to participate in the study.

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Figure legends

Figure 1: CONSORT diagram of study participants

All PEPaNIC patients from whom a white blood cell sample had been collected at the last day in the PICU (n=825) were eligible for this study. DNA was extracted from these samples and from the corresponding white blood cell samples collected upon PICU admission if available (n=695), as well as from white blood cells collected from a cohort of healthy children (n=352). From the healthy controls and the patients with a DNA sample upon PICU admission that passed the quality control, two matched patient-control cohorts were selected for identifying CpG-sites that were already methylated differently in patients upon PICU admission as compared with healthy children (Figure_2), one with a similar age and gender distribution than the total patient group with upon PICU admission DNA available ("patient-control cohort 1") and one selected via propensity score matching with a somewhat older age than that of the total patient group ("patient-control cohort 2") (Methods_S4). The green box highlights the patients with high quality DNA (n=814) used for the main analyses identifying the CpG-sites that were differentially methylated at PICU discharge and association of these differentially methylated CpG-sites with functional outcomes as assessed two years after PICU admission for the patients for whom these data were available (n=473) (Figure_2).

Figure 2: Concept, rationale and design of the study

The main hypothesis of this study was that the use of early-PN, as compared with late-PN, during paediatric critical illness alters DNA methylation status of CpG-sites important for neurocognition, and that this effect explains part of its negative impact on neurocognitive development 2 years later.[4] In order to address this hypothesis, we first needed to identify which CpG-sites became differentially methylated during PICU stay as compared with matched healthy children, followed by determining which of those CpG-sites were affected by early-PN versus late-PN. This allowed a statistical mediation analysis in a final step to assess the role of early-PN-induced DNA methylation changes as plausible mediator of long-term neurocognitive harm by early-PN.

A. Stepwise approach to address the study hypothesis: In step 1, we compared DNA methylation status in critically ill children upon PICU-admission with that of healthy children matched for age and gender. CpG-sites differentially methylated between patients and controls at this time point reflect pre-morbid conditions and illness-

induced alterations in leukocyte composition.[18] As these differences can induce bias,[19,20] we discarded all these CpG-sites for further analyses. In step 2, we compared DNA methylation status of the remaining CpG-sites in critically ill children upon PICU-discharge with that of healthy children matched for age and gender. Remaining CpG-sites differentially methylated between patients and controls at this time point are those CpG-sites for which DNA methylation became altered between PICU-admission and PICU-discharge, representing "*de novo* DNA methylation changes". These *de novo* changes may have been induced by critical illness and/or intensive care. In step 3, we investigated the independent impact of early-PN versus late-PN on the "*de novo* DNA methylation changes" in the patients. Assessment of such independent impact of early-PN versus late-PN required adjustment for the context (*) of the patients, including the baseline risk factors age, centre, race, gender, geographic origin, language, history of malignancy, diabetes, predefined "syndrome" (Methods_S5), diagnosis and severity of illness, and risk of malnutrition. Further adjustment for the acute effects of early-PN, versus late-PN, on infections, duration of PICU-stay, mechanical ventilatory support and hypoglycaemia allowed establishing whether any effect of early-PN was direct or mediated via its effect on short-term outcome. In step 4, we investigated the potential role of the *de novo* DNA methylation changes developing during PICU stay in explaining part of the impaired physical and neurocognitive development of critically ill children as observed two years after PICU-admission, adjusted for the context of the patients as described in step 3. In final step 5, we investigated whether the altered DNA methylation evoked by early-PN versus late-PN could explain part of the negative impact of early-PN on neurocognitive development of critically ill children as observed two years after PICU-admission (more specifically on inhibitory control, metacognition, working memory, total executive function, externalising behaviour, and visual-motor integration), adjusted for the context of the patients as described in step 3. Numbers of the corresponding figures showing the results of each step are indicated.

B. Directed acyclic graph of the statistical mediation analyses.[21] a represents the association of the "intervention" (early-PN versus late-PN) with the hypothesised "mediator" (*de novo* DNA methylation during PICU stay) as assessed in step 3 of panel A, and b represents the association of the "mediator" with the "outcome" of interest (inhibitory control, metacognition, working memory, total executive function, externalising behaviour, and visual-motor integration evaluated two years after PICU-admission) as assessed in step 5 of panel A. The total effect of the "intervention" (c) equals the sum of any direct effect of early-PN represented by c' and the indirect effect of early-PN through pathway a-b.

Figure 3. *De novo* differences in DNA methylation between patients upon PICU discharge and healthy children.

This figure summarises the results obtained for step 2 in Figure_2A. Beta-values are shown for the 159 differentially methylated CpG-sites identified based on a false discovery rate <0·05. Box plots depict medians with interquartile ranges (IQR) and whiskers drawn to one time the IQR in each direction of the box. The healthy control group is indicated in blue, the patient group in red. CpG-sites are ordered left-to-right from most to least significantly different.

Figure 4. Independent role of early-PN, versus late-PN, as a contributor to DNA methylation alterations occurring during PICU say, adjusted for baseline risk factors.

Summary of the 100 bootstrapped multivariable linear regression analyses for identification of the robustly significant (with a P<0·05 in at least 50 replicated analyses), independent associations of the nutritional management strategy (early-PN versus late-PN) and baseline risk factors, with the methylation beta-values of the differentially methylated CpG-sites identified upon PICU discharge. This figure summarises the results obtained for step 3 in Figure_2A. Each row corresponds to 100 bootstrap replicates of linear regression models for the given CpG-site and columns correspond to the model covariates. A "+" in front of a CpG-site indicates more methylation in patients than in controls. A "-" in front of a CpG-site indicates less methylation in patients than in controls. Colored boxes indicate robust independent associations with CpG-sites. Green colored boxes indicate where the covariate alters DNA methylation towards healthy control values, suggesting a protective effect. Red colored boxes indicate where the covariate alters DNA methylation away from healthy control values, suggesting a harmful effect. PIM3: Paediatric Index of Mortality 3; PN: parenteral nutrition; PeLOD: Paediatric logistic organ dysfunction; STRONGkids: Screening Tool for Risk on Nutritional Status and Growth.

Figure 5. Association of average macronutrient doses with the DNA methylation status of 6 representative CpG-sites differentially methylated by early-PN

For each of the first 7 days in the PICU (the time window of the randomised intervention), associations of the average daily doses of each of the individual macronutrients up to that day with methylation status of a CpG-site

upon PICU discharge are shown as scaled estimates and 95% confidence intervals per 10% of reference doses added, with macronutrient doses simultaneously entered as continuous variables. These analyses were adjusted for treatment centre, age, race, gender, geographic origin, language, history of malignancy, diabetes, predefined "syndrome" (Methods_S5), type and severity of illness, and severity of malnutrition risk (Methods_S6). A positive scaled estimate indicates a higher degree of methylation of the corresponding CpG-site, whereas a negative scaled estimate indicates a lower degree of methylation. For CpG-sites hypomethylated by early-PN (cg14109551, cg05371584, cg00687889, cg24475272), the negative estimate for amino acid doses points to a contribution of the amino acids to the hypomethylation. For CpG-sites hypermethylated by early-PN (cg05174290, cg14071298), the positive estimate for amino acid doses likewise points to a contribution of the amino acids to the hypermethylation. *n* indicates the number of patients still in the PICU on the day of analysis.

Figure 6. Role of the 159 *de novo* differentially methylated CpG-sites evoked by critical illness in explaining impaired long-term outcome in non-linear models.

This figure summarises the results obtained for step 4 in Figure_2A. Each row corresponds to 100 bootstrap replicates of a multivariable non-linear model for the given outcome assessed at 2-years follow-up and columns correspond to the 159 differentially methylated CpG-sites. CpG-sites and outcomes in orange font correspond to those affected by the nutritional management strategy (early-PN versus late-PN). All models resulted from random forest machine learning, with covariate significance levels obtained via permutation importance,[30,31] including the baseline risk factors age, centre, race, gender, geographic origin, language, history of malignancy, a predefined "syndrome" (Methods_S5), diagnosis and severity of illness (PIM3 and PeLOD), risk of malnutrition (STRONGkids) and the 159 CpG-sites becoming differentially methylated in patients during PICU-stay as compared with matched healthy children. The robustness of these results was evaluated in 100 bootstrapped replicates.[32] Color intensity of the boxes reflects the frequency with which a CpG-site was found to be independently and significantly ($P<0{\cdot}05$) associated with the outcomes in the 100 bootstrapped replicated analyses, with darker blue colors corresponding to a higher frequency. Outcomes and CpG-sites were clustered based on these frequencies. Dendrograms show clustering hierarchy. The base of the column dendrogram is color-coded according to CpG-site functional classes "Cerebral/neuronal", "Peripheral neuronal," "Growth/Development/Locomotion", "Metabolism", "Gene expression/DNA regulation/Epigenetic regulation",

"Inflammation/Immune system", "Cell structure", "Intra/intercellular Signalling/Transport", "Non-coding RNA/pseudogene", "Oncogenic/Apoptosis", or "Intergenic" (Table_S5). For anthropometrics, particularly strong associations were observed for groups of CpG-sites in genes involved in growth, bone formation, myogenesis, locomotion, action potential initiation and propagation in nerve and muscle cells, neurodevelopment/neuronal functioning/neuropathy/neurodegeneration, apoptosis, and metabolic functions. For measures of intelligence, the strongest associations were observed for CpG-sites in genes involved in neurocognitive development/functioning (cognition/concentration/memory), psychomotor speed, memory, visual motor/fine motor function, emotional functioning, behaviour and speech, neuropsychiatric and neurodegenerative disorders, apoptosis, metabolism, and transcriptional regulation. For motor coordination the strongest associations were observed for CpG-sites involved in skeletal muscle and brain development, connectivity and signal transduction in brain, visual motor/fine motor function, muscle disorders/nerve degeneration, and apoptosis. For visual-motor integration the strongest associations were observed for CpG-sites in genes involved in neurodevelopment/neurotransmission, muscle development, locomotor activity, memory, visual learning, speed and flexibility, neurodegeneration, neuropsychiatric disorders, and transcription regulation/signal transduction. For the executive functions, the strongest associations were observed for CpG-sites in genes involved in brain development, learning and memory, neuromuscular development, visual motor/fine motor function, emotional functioning, behaviour, speech, neurological, neurodegenerative, neuropsychiatric and attention deficit disorders, muscle development, signal transduction in brain, and apoptosis. For behavioural problems, the strongest associations were observed for CpG-sites in genes involved in emotional functioning, behaviour, neuropsychiatric and attention deficit disorders, neurodevelopment, muscle disorders, nerve degeneration/neurodegeneration, cognitive function, visual learning, speed, flexibility, memory, signal transduction in the brain and transcriptional regulation, metabolism, and apoptosis. For alertness, the strongest associations were observed for CpG-sites in genes involved in neurodevelopment, visual motor/fine motor function, memory, speech, social and emotional function, behaviour, neurodegenerative, neuropsychiatric and attention deficit disorders, transcriptional regulation, and metabolism. Also several strong associations were observed between various outcomes and intergenic CpG-sites (Table_S1).

Figure 7. Role of the 37 differentially methylated CpG-sites evoked by early-PN in explaining its adverse impact on neurocognitive development in non-linear models.

This figure summarises the results obtained for step 5 in Figure_2A. Each row corresponds to 100 bootstrap replicates of multivariable non-linear models for the 6 neurocognitive outcomes negatively affected by early-PN, versus late-PN. Columns correspond to the 37 CpG-sites that were differentially methylated by early-PN. All models resulted from random forest machine learning, with covariate significance levels obtained via permutation importance,[30,31] including the baseline risk factors age, centre, race, gender, geographic origin, language, history of malignancy, a predefined "syndrome" (Methods_S5), diagnosis and severity of illness (PIM3 and PeLOD), risk of malnutrition (STRONGkids) and the 37 differentially methylated CpG-sites evoked by early-PN. The robustness of these results was evaluated in 100 bootstrapped replicates.[32] Color intensity of the boxes reflects the frequency with which a CpG-site was found to be independently and significantly ($P<0{\cdot}05$) associated with the outcomes in the 100 bootstrapped replicated analyses, with darker orange colors corresponding to a higher frequency. Outcomes and CpG-sites were clustered based on these frequencies. Dendrograms show clustering hierarchy. The base of the column dendrogram is color-coded according to CpG-site functional classes "Cerebral/neuronal", "Peripheral neuronal," "Growth/Development/Locomotion", "Metabolism", "Gene expression/DNA regulation/Epigenetic regulation", "Inflammation/Immune system", "Cell structure", "Intra/intercellular Signaling/Transport", "Non-coding RNA/pseudogene", "Oncogenic/Apoptosis", or "Intergenic" (Table_S5).

The 37 CpG-sites were found to be independently and significantly associated with any of the 6 outcomes affected by early-PN in at least 50 of the 100 bootstrapped replicated analyses, confirming robustness of all associations. The CpG-sites that were most often retained in the bootstrapped non-linear models as independently associated with either the 4 executive functions, the externalising behaviour or the visual-motor integration were cg26308668 in srGAP1 (involved in neuronal development and migration, linked to mental retardation), cg14364797 in FNBP1 (involved in neuronal network formation and information processing), cg11047783 in KAT6B (involved in cerebral cortex development, attention deficit hyperactivity disorder (ADHD) and intellectual disability), cg14109551 in CEP85L (involved in brain tumors, ADHD and bipolar disorder), cg22645359 in TCF7L2 (involved in Wnt signalling and related to neurodevelopment and plasticity of mature neurons), cg17522929 in PKM (involved in brain development and neuronal differentiation), cg14450616 in PLD3 (involved in neuronal development, survival and neurotransmission, visual learning, memory and flexibility), cg14172797 in PRKCA (involved in memory, mood regulation and behaviour), cg12928479 in NLRC5 (involved in

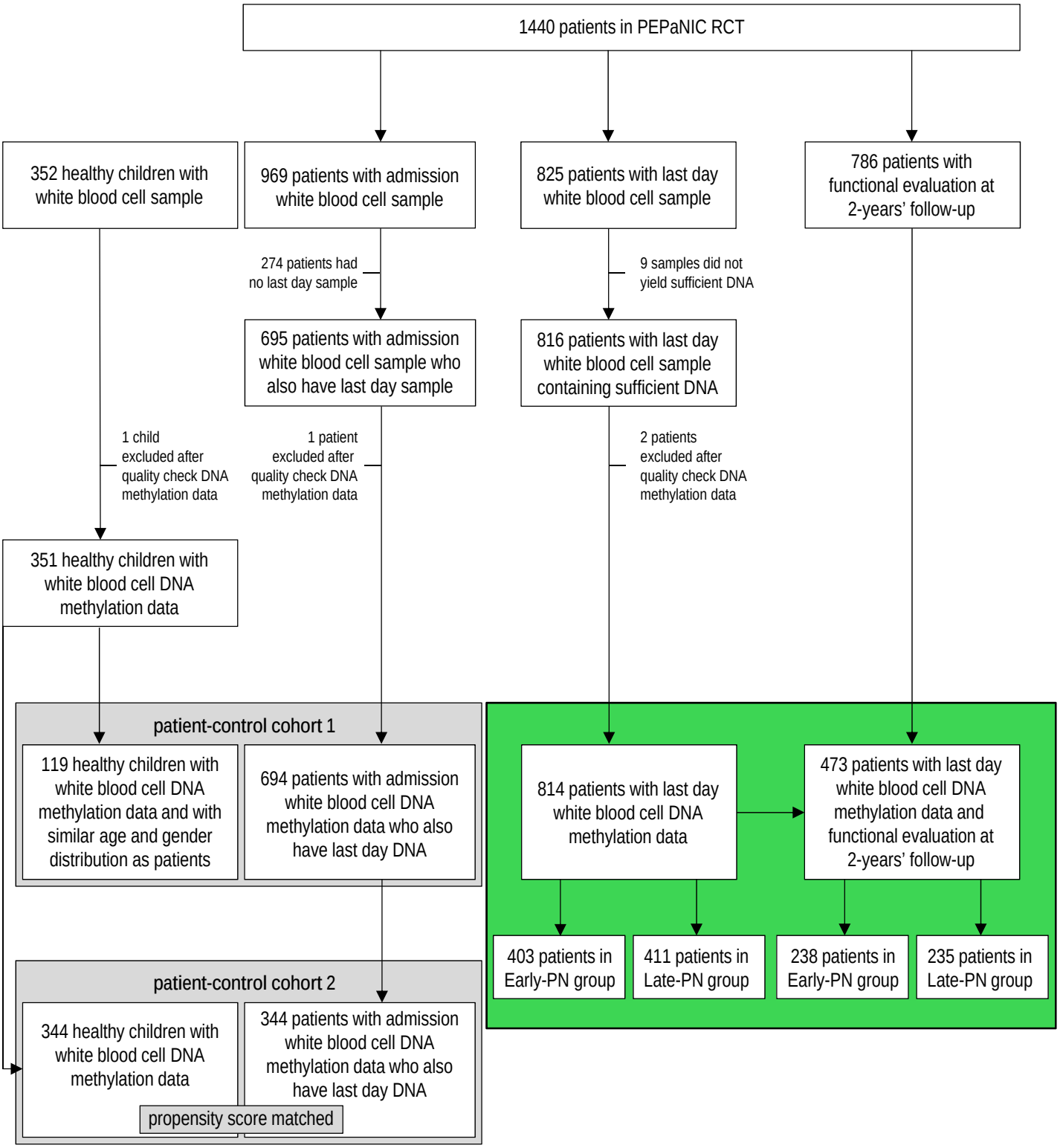
neuroimmune and neuroinflammatory processes), cg22076676 in THADA (involved in apoptosis, neuroinflammation and multiple sclerosis), cg01842756 in RNF217 (involved in apoptosis), cg16301196 in PLA2G15 (involved in lipid metabolism), cg24475272 in SETD7 (involved in histone methylation), and several intergenic and non-coding CpG-sites (Table_S1).

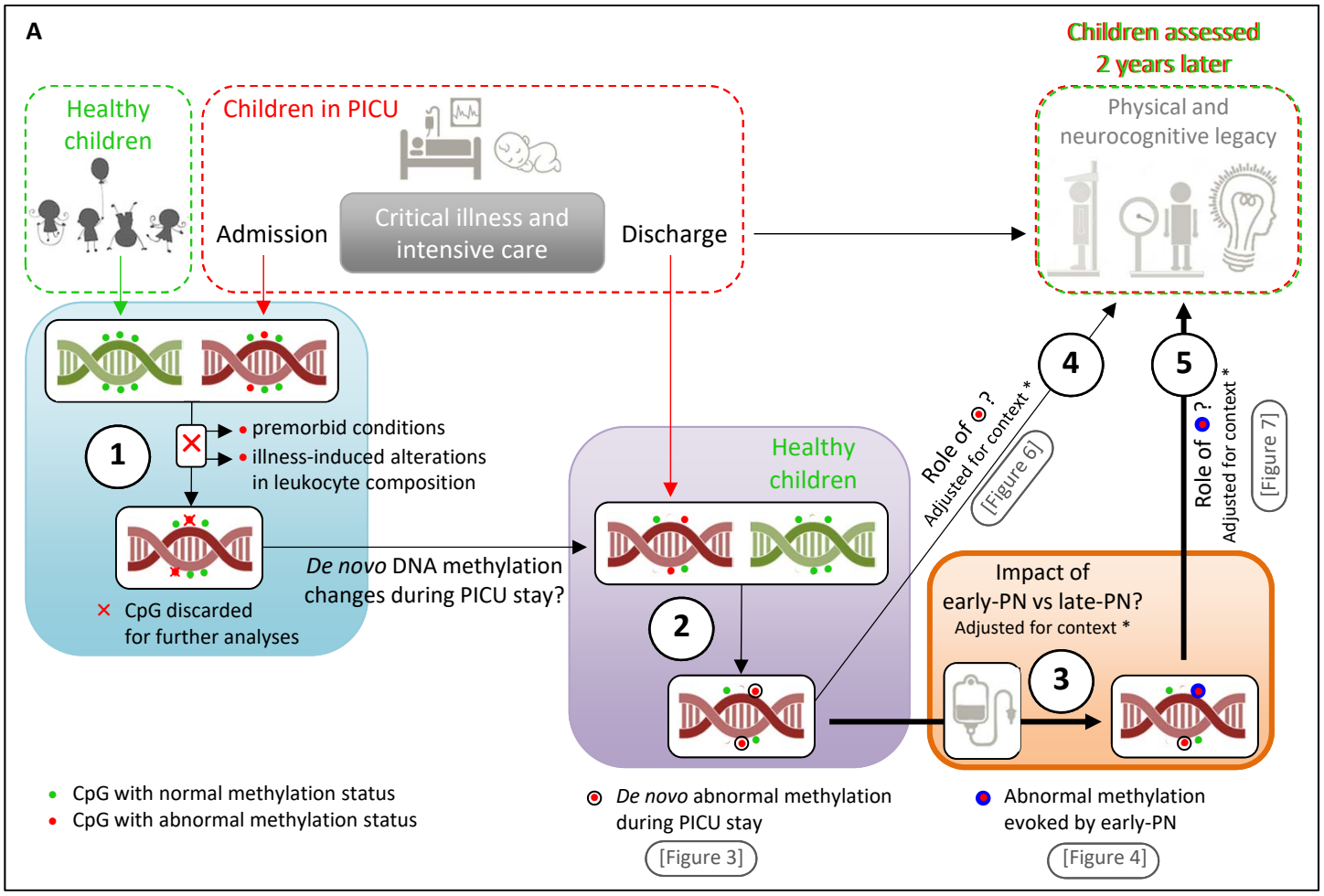
Table 1. Characteristics of patients assessed for DNA methylation upon PICU discharge and for long-term outcomes

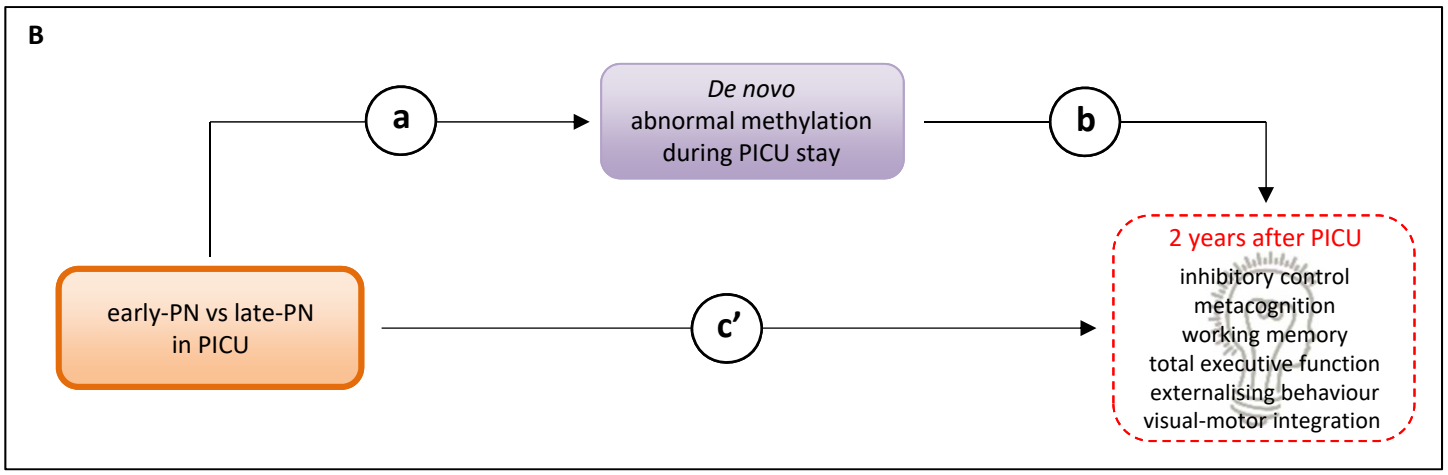
	Healthy children	PICU patients assessed for DNA methylation [a]			PICU patients assessed for DNA methylation and long-term outcome		
		Total	Early-PN	Late-PN	Total	Early-PN	Late-PN
	N=119	N=814	N=403	N=411	N= 473	N=238	N=235
Demographics							
Male sex - no. (%)	68 (57·1)	462 (56·8)	223 (55·3)	239 (58·2)	275 (58·1)	139 (58·4)	136 (57·9)
Age (mean±SD) - years	4·2±4·7	4·5±5·0	4·2±4·8	4·7±5·2	4·0±4·6	4·0±4·6	3·9±4·6
Infant (age<1y) - no. (%)	43 (36·1)	316 (38·8)	154 (38·1)	162 (39·4)	183 (38·7)	87 (36·6)	96 (40·9)
Known non-Caucasian race - no. (%)[b]	NA	33 (4·1)	20 (5·0)	13 (3·2)	22 (4·7)	14 (5·9)	8 (3·4)
Known non-European origin - no. (%)[b]	NA	166 (20·4)	92 (22·8)	74 (18·0)	87 (18·4)	52 (21·8)	35 (14·9)
Known not exclusive Dutch or English language - no. (%)	NA	142 (17·4)	79 (19·6)	63 (15·3)	117 (24·7)	63 (26·5)	54 (23·0)
Patient characteristics upon PICU admission							
STRONGkids risk level - no. (%)[c]							
Medium	NA	764 (93·9)	386 (95·8)	378 (92·0)	447 (94·5)	229 (96·2)	218 (92·8)
High	NA	50 (6·1)	17 (4·2)	33 (8·0)	26 (5·5)	9 (3·8)	17 (7·2)
PeLOD score (mean±SD)[d]	NA	23·6±10·8	23·8±10·8	23·5±10·9	23·9±10·2	24·1±10·0	23·7±10·4
PIM3 score (mean±SD)[e]	NA	-3·4±1·6	-3·3±1·6	-3·4±1·6	-3·6±1·2	-3·6±1·2	-3·7±1·1
Diagnostic category - no. (%)							
Surgical							
Abdominal	NA	25 (3·1)	11 (2·7)	14 (3·4)	12 (2·5)	6 (2·5)	6 (2·5)
Burns	NA	2 (0·3)	1 (0·3)	1 (0·2)	0·0 (0·0)	0·0 (0·0)	0·0 (0·0)
Cardiac	NA	430 (52·8)	217 (53·9)	213 (51·8)	280 (59·2)	144 (60·5)	136 (57·9)
Neurosurgery-traumatic brain injury	NA	71 (8·7)	38 (9·4)	33 (8·0)	43 (9·0)	23 (9·7)	20 (8·5)
Thoracic	NA	33 (4·1)	17 (4·2)	16 (3·9)	22 (4·7)	10 (4·2)	12 (5·1)
Transplantation	NA	11 (1·4)	2 (0·5)	9 (2·2)	7 (1·5)	1 (0·4)	6 (2·5)
Orthopedic surgery-trauma	NA	44 (5·4)	21 (5·2)	23 (5·6)	19 (4·0)	12 (5·0)	7 (3·0)
Other	NA	23 (2·8)	9 (2·3)	14 (3·4)	13 (2·7)	5 (2·1)	8 (3·4)
Medical							
Cardiac	NA	26 (3·2)	13 (3·2)	13 (3·2)	11 (2·3)	5 (2·1)	6 (2·6)
Gastrointestinal-hepatic	NA	5 (0·6)	2 (0·5)	3 (0·7)	3 (0·6)	1 (0·4)	2 (0·8)
Oncologic-haematologic	NA	3 (0·4)	1 (0·3)	2 (0·5)	2 (0·4)	1 (0·4)	1 (0·4)
Neurologic	NA	51 (6·3)	27 (6·7)	24 (5·8)	22 (4·7)	11 (4·6)	11 (4·7)
Respiratory	NA	50 (6·1)	25 (6·2)	25 (6·1)	19 (4·0)	9 (3·8)	10 (4·3)

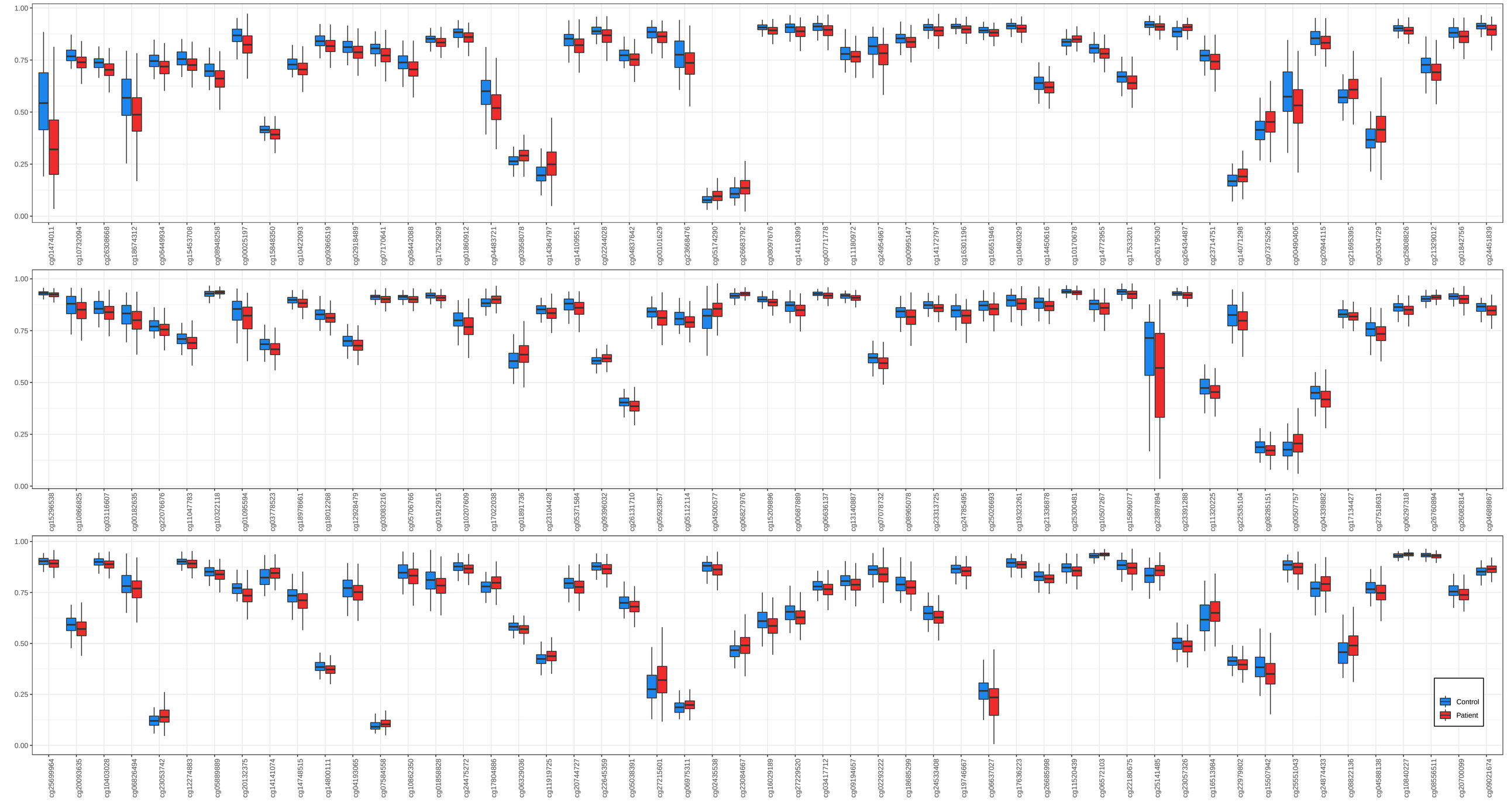
Other	NA	40 (4·9)	19 (4·7)	21 (5·1)	20 (4·2)	10 (4·2)	10 (4·3)
Patient characteristics upon PICU admission							
Malignancy - no. (%)	NA	51 (6·3)	29 (7·2)	22 (5·4)	28 (5·9)	16 (6·7)	12 (5·1)
Diabetes - no. (%)	NA	2 (0·3)	2 (0·5)	0 (0·0)	0·0 (0·0)	0·0 (0·0)	0·0 (0·0)
Syndrome - no. (%)[f]	NA	139 (17·1)	67 (16·6)	72 (17·5)	50 (10·6)	22 (9·2)	28 (11·9)
Acute effects of randomisation							
Duration of stay in the PICU (mean±SD) - days	NA	4·6±7·0	5·2±8·9	4·1±4·6	4·3±8·0	5·1±10·7	3·5±3·5
Patients who acquired a new infection in PICU - no. (%)	NA	70 (8·6)	44 (9·2)	26 (6·3)	35 (7·4)	26 (10·9)	9 (3·8)
Duration of mechanical ventilatory support (mean±SD) - days	NA	2·9±4·4	3·1±4·8	2·8±4·0	2·5±4·1	2·8±5·2	2·2±2·6
Number of days with hypoglycaemia <40mg/dl (mean±SD) - days	NA	0·1±0·5	0·1±0·5	0·2±0·4	0·1±0·5	0·1±0·5	0·2±0·5

[a] There were no significant differences in gender, age, and number of infants between subgroups of patients and healthy control children.
[b] Participants were classified according to race and geographical origin by the investigators. These classifications were performed to capture ethnical and regional differences in the frequency of consanguinity, which may adversely affect cognitive performance.
[c] Scores on the Screening Tool for Risk on Nutritional Status and Growth (STRONGkids) range from 0 to 5, with a score of 0 indicating a low risk of malnutrition, a score of 1 to 3 indicating medium risk, and a score of 4 to 5 indicating high risk.
[d] Paediatric Logistic Organ Dysfunction (PeLOD) scores for the first 24h range from 0 to 71, with higher scores indicating more severe illness.
[e] Paediatric Index of Mortality 3 (PIM3) scores, with higher scores indicating a higher risk of mortality.
[f] A prerandomisation syndrome or illness a priori defined as affecting or possibly affecting neurocognitive development (eMethods_5).
Abbreviations: PeLOD, paediatric logistic organ dysfunction score; PICU, paediatric intensive care unit; PIM3, paediatric index of mortality 3 score; PN, parenteral nutrition; SEM, standard error of the mean.


1440 patients in PEPaNIC RCT
352 healthy children with white blood cell sample
969 patients with admission white blood cell sample
825 patients with last day white blood cell sample
786 patients with functional evaluation at 2-years' follow-up
274 patients had no last day sample
9 samples did not yield sufficient DNA
695 patients with admission white blood cell sample who also have last day sample
816 patients with last day white blood cell sample containing sufficient DNA
1 child excluded after quality check DNA methylation data
1 patient excluded after quality check DNA methylation data
2 patients excluded after quality check DNA methylation data
351 healthy children with white blood cell DNA methylation data
patient-control cohort 1
119 healthy children with white blood cell DNA methylation data and with similar age and gender distribution as patients
694 patients with admission white blood cell DNA methylation data who also have last day DNA
814 patients with last day white blood cell DNA methylation data
473 patients with last day white blood cell DNA methylation data and functional evaluation at 2-years' follow-up
403 patients in Early-PN group
411 patients in Late-PN group
238 patients in Early-PN group
235 patients in Late-PN group
patient-control cohort 2
344 healthy children with white blood cell DNA methylation data
344 patients with admission white blood cell DNA methylation data who also have last day DNA
propensity score matched


A
Children assessed
2 years later
Healthy
children
Children in PICU
Admission
Critical illness and
intensive care
Discharge
Physical and
neurocognitive legacy
1
premorbid conditions
illness-induced alterations
in leukocyte composition
CpG discarded
for further analyses
De novo DNA methylation
changes during PICU stay?
Healthy
children
2
4
5
Role of ?
Adjusted for context *
[Figure 6]
Role of ?
Adjusted for context *
[Figure 7]
Impact of
early-PN vs late-PN?
Adjusted for context *
3
CpG with normal methylation status
CpG with abnormal methylation status
De novo abnormal methylation
during PICU stay
[Figure 3]
Abnormal methylation
evoked by early-PN
[Figure 4]


B
a
De novo
abnormal methylation
during PICU stay
b
early-PN vs late-PN
in PICU
c'
2 years after PICU
inhibitory control
metacognition
working memory
total executive function
externalising behaviour
visual-motor integration


1.00
0.75
0.50
0.25
0.00
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cg08948258
cg00025197
cg15848350
cg10422093
cg09366519
cg02918489
cg07170641
cg08442088
cg17522929
cg01860912
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cg17533201
cg26179530
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cg23714751
cg14071298
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cg20944115
cg21695395
cg05304729
cg25808826
cg21329012
cg01842756
cg24451839
cg15296538
cg10866825
cg03116607
cg00182635
cg22076676
cg11047783
cg10322118
cg01095594
cg03778523
cg18978661
cg18012268
cg12928479
cg03083216
cg05706766
cg01912915
cg10207609
cg17022038
cg01891736
cg23104428
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cg26131710
cg05923857
cg05112114
cg04500577
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cg11919725
cg20744727
cg22645359
cg05038391
cg27215601
cg06975311
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cg24533408
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cg06637027
cg17636223
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cg11520439
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cg15507942
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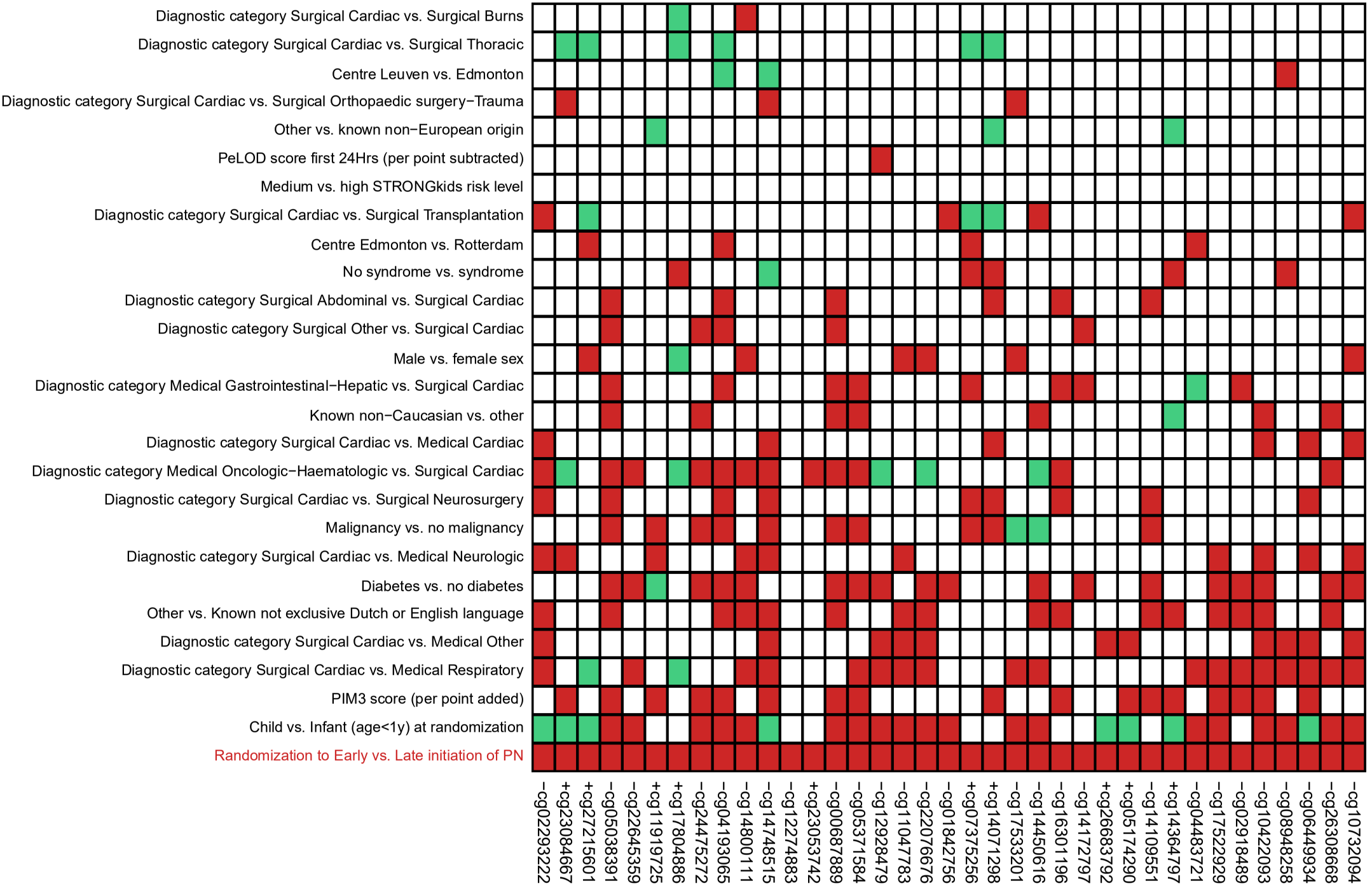
cg09021674
Control
Patient


Diagnostic category Surgical Cardiac vs. Surgical Burns
Diagnostic category Surgical Cardiac vs. Surgical Thoracic
Centre Leuven vs. Edmonton
Diagnostic category Surgical Cardiac vs. Surgical Orthopaedic surgery−Trauma
Other vs. known non−European origin
PeLOD score first 24Hrs (per point subtracted)
Medium vs. high STRONGkids risk level
Diagnostic category Surgical Cardiac vs. Surgical Transplantation
Centre Edmonton vs. Rotterdam
No syndrome vs. syndrome
Diagnostic category Surgical Abdominal vs. Surgical Cardiac
Diagnostic category Surgical Other vs. Surgical Cardiac
Male vs. female sex
Diagnostic category Medical Gastrointestinal−Hepatic vs. Surgical Cardiac
Known non−Caucasian vs. other
Diagnostic category Surgical Cardiac vs. Medical Cardiac
Diagnostic category Medical Oncologic−Haematologic vs. Surgical Cardiac
Diagnostic category Surgical Cardiac vs. Surgical Neurosurgery
Malignancy vs. no malignancy
Diagnostic category Surgical Cardiac vs. Medical Neurologic
Diabetes vs. no diabetes
Other vs. Known not exclusive Dutch or English language
Diagnostic category Surgical Cardiac vs. Medical Other
Diagnostic category Surgical Cardiac vs. Medical Respiratory
PIM3 score (per point added)
Child vs. Infant (age<1y) at randomization
Randomization to Early vs. Late initiation of PN
−cg02293222
+cg23084667
+cg27215601
−cg05038391
−cg22645359
+cg11919725
+cg17804886
−cg24475272
−cg04193065
−cg14800111
−cg14748515
−cg12274883
+cg23053742
−cg00687889
−cg05371584
−cg12928479
−cg11047783
−cg22076676
−cg01842756
+cg07375256
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−cg17533201
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+cg26683792
+cg05174290
−cg14109551
+cg14364797
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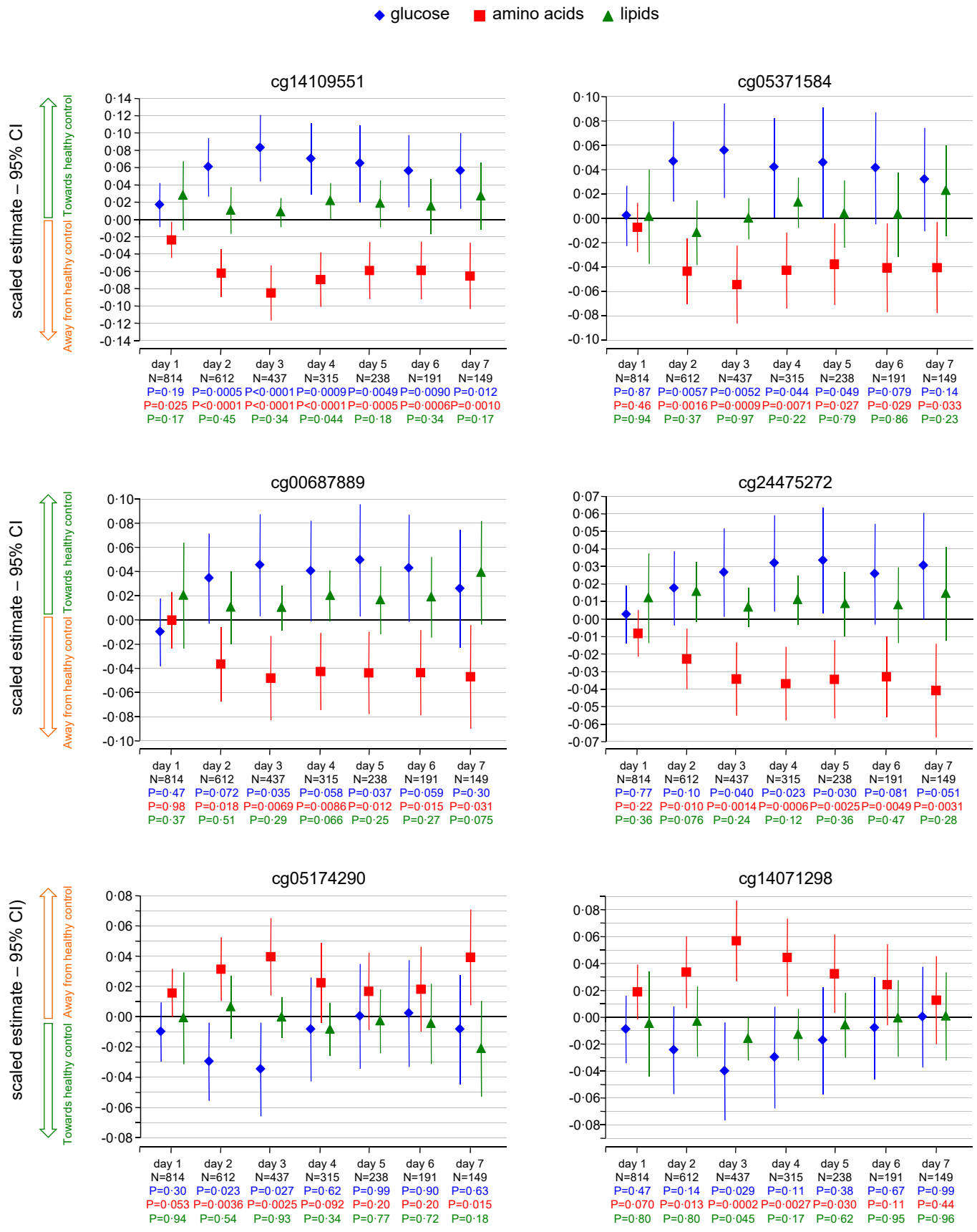
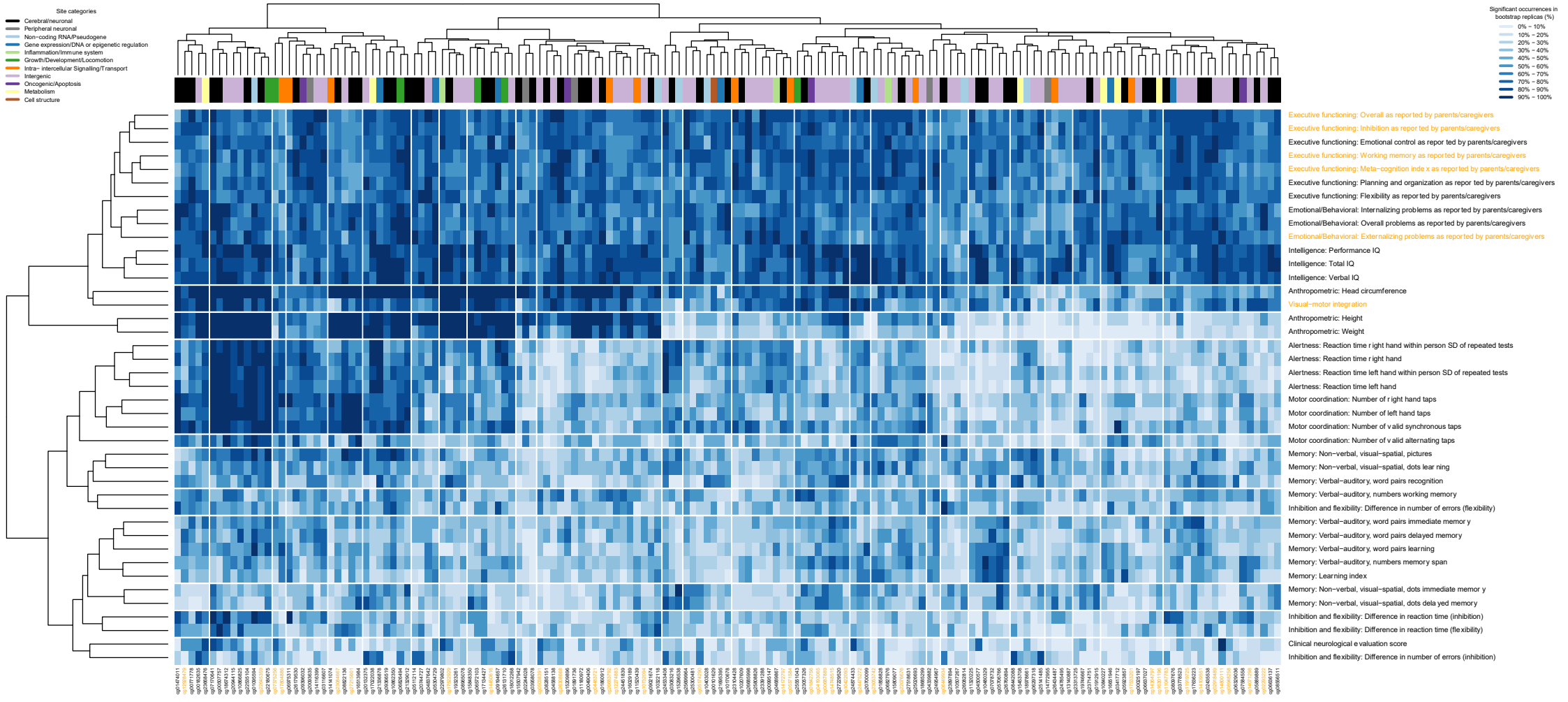
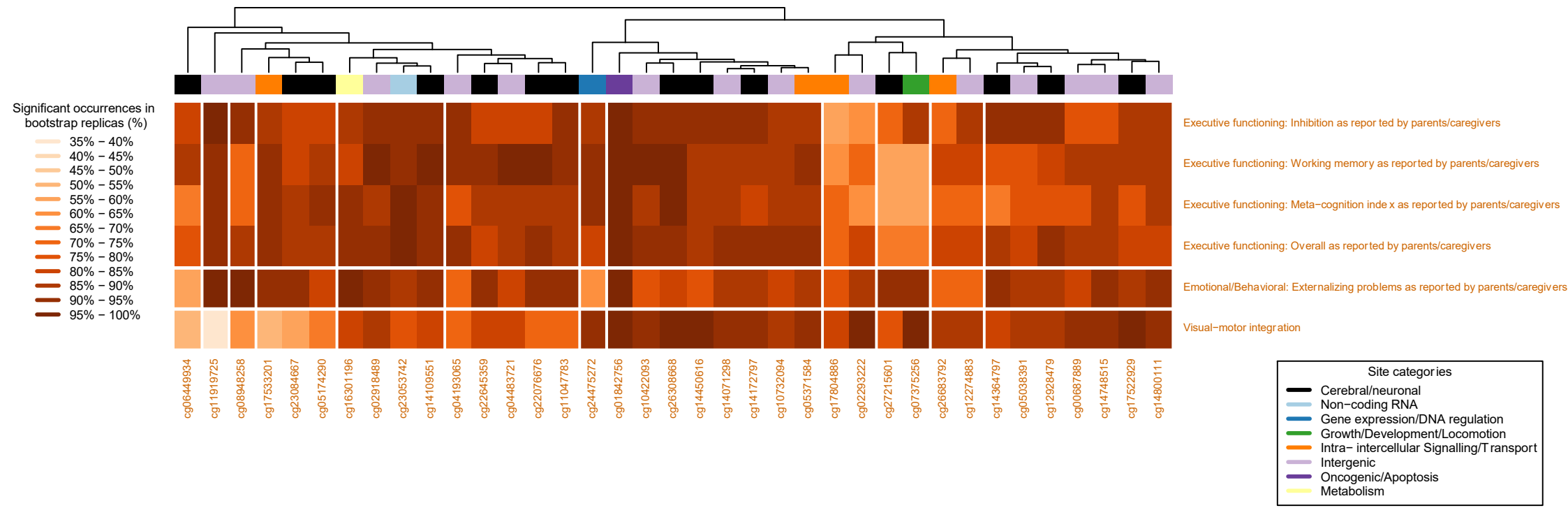

glucose
amino acids
lipids
cg14109551
cg05371584
cg00687889
cg24475272
cg05174290
cg14071298
scaled estimate – 95% CI
scaled estimate – 95% CI)
Towards healthy control
Away from healthy control
day 1 N=814 P=0·19 P=0·025 P=0·17
day 2 N=612 P=0·0005 P<0·0001 P=0·45
day 3 N=437 P<0·0001 P<0·0001 P=0·34
day 4 N=315 P=0·0009 P<0·0001 P=0·044
day 5 N=238 P=0·0049 P=0·0005 P=0·18
day 6 N=191 P=0·0090 P=0·0006 P=0·34
day 7 N=149 P=0·012 P=0·0010 P=0·17
day 1 N=814 P=0·87 P=0·46 P=0·94
day 2 N=612 P=0·0057 P=0·0016 P=0·37
day 3 N=437 P=0·0052 P=0·0009 P=0·97
day 4 N=315 P=0·044 P=0·0071 P=0·22
day 5 N=238 P=0·049 P=0·027 P=0·79
day 6 N=191 P=0·079 P=0·029 P=0·86
day 7 N=149 P=0·14 P=0·033 P=0·23
day 1 N=814 P=0·47 P=0·98 P=0·37
day 2 N=612 P=0·072 P=0·018 P=0·51
day 3 N=437 P=0·035 P=0·0069 P=0·29
day 4 N=315 P=0·058 P=0·0086 P=0·066
day 5 N=238 P=0·037 P=0·012 P=0·25
day 6 N=191 P=0·059 P=0·015 P=0·27
day 7 N=149 P=0·30 P=0·031 P=0·075
day 1 N=814 P=0·77 P=0·22 P=0·36
day 2 N=612 P=0·10 P=0·010 P=0·076
day 3 N=437 P=0·040 P=0·0014 P=0·24
day 4 N=315 P=0·023 P=0·0006 P=0·12
day 5 N=238 P=0·030 P=0·0025 P=0·36
day 6 N=191 P=0·081 P=0·0049 P=0·47
day 7 N=149 P=0·051 P=0·0031 P=0·28
day 1 N=814 P=0·30 P=0·053 P=0·94
day 2 N=612 P=0·023 P=0·0036 P=0·54
day 3 N=437 P=0·027 P=0·0025 P=0·93
day 4 N=315 P=0·62 P=0·092 P=0·34
day 5 N=238 P=0·99 P=0·20 P=0·77
day 6 N=191 P=0·90 P=0·20 P=0·72
day 7 N=149 P=0·63 P=0·015 P=0·18
day 1 N=814 P=0·47 P=0·070 P=0·80
day 2 N=612 P=0·14 P=0·013 P=0·80
day 3 N=437 P=0·029 P=0·0002 P=0·045
day 4 N=315 P=0·11 P=0·0027 P=0·17
day 5 N=238 P=0·38 P=0·030 P=0·62
day 6 N=191 P=0·67 P=0·11 P=0·95
day 7 N=149 P=0·99 P=0·44 P=0·96


Site categories
Cerebral/neuronal
Peripheral neuronal
Non−coding RNA/Pseudogene
Gene expression/DNA or epigenetic regulation
Inflammation/Immune system
Growth/Development/Locomotion
Intra− intercellular Signalling/Transport
Intergenic
Oncogenic/Apoptosis
Metabolism
Cell structure
Significant occurrences in bootstrap replicas (%)
0% − 10%
10% − 20%
20% − 30%
30% − 40%
40% − 50%
50% − 60%
60% − 70%
70% − 80%
80% − 90%
90% − 100%
Executive functioning: Overall as reported by parents/caregivers
Executive functioning: Inhibition as reported by parents/caregivers
Executive functioning: Emotional control as reported by parents/caregivers
Executive functioning: Working memory as reported by parents/caregivers
Executive functioning: Meta−cognition index as reported by parents/caregivers
Executive functioning: Planning and organization as reported by parents/caregivers
Executive functioning: Flexibility as reported by parents/caregivers
Emotional/Behavioral: Internalizing problems as reported by parents/caregivers
Emotional/Behavioral: Overall problems as reported by parents/caregivers
Emotional/Behavioral: Externalizing problems as reported by parents/caregivers
Intelligence: Performance IQ
Intelligence: Total IQ
Intelligence: Verbal IQ
Anthropometric: Head circumference
Visual−motor integration
Anthropometric: Height
Anthropometric: Weight
Alertness: Reaction time right hand within person SD of repeated tests
Alertness: Reaction time right hand
Alertness: Reaction time left hand within person SD of repeated tests
Alertness: Reaction time left hand
Motor coordination: Number of right hand taps
Motor coordination: Number of left hand taps
Motor coordination: Number of valid synchronous taps
Motor coordination: Number of valid alternating taps
Memory: Non−verbal, visual−spatial, pictures
Memory: Non−verbal, visual−spatial, dots learning
Memory: Verbal−auditory, word pairs recognition
Memory: Verbal−auditory, numbers working memory
Inhibition and flexibility: Difference in number of errors (flexibility)
Memory: Verbal−auditory, word pairs immediate memory
Memory: Verbal−auditory, word pairs delayed memory
Memory: Verbal−auditory, word pairs learning
Memory: Verbal−auditory, numbers memory span
Memory: Learning index
Memory: Non−verbal, visual−spatial, dots immediate memory
Memory: Non−verbal, visual−spatial, dots delayed memory
Inhibition and flexibility: Difference in reaction time (inhibition)
Inhibition and flexibility: Difference in reaction time (flexibility)
Clinical neurological evaluation score
Inhibition and flexibility: Difference in number of errors (inhibition)


Significant occurrences in bootstrap replicas (%)
35% − 40%
40% − 45%
45% − 50%
50% − 55%
55% − 60%
60% − 65%
65% − 70%
70% − 75%
75% − 80%
80% − 85%
85% − 90%
90% − 95%
95% − 100%
Executive functioning: Inhibition as reported by parents/caregivers
Executive functioning: Working memory as reported by parents/caregivers
Executive functioning: Meta−cognition index as reported by parents/caregivers
Executive functioning: Overall as reported by parents/caregivers
Emotional/Behavioral: Externalizing problems as reported by parents/caregivers
Visual−motor integration
cg06449934
cg11919725
cg08948258
cg17533201
cg23084667
cg05174290
cg16301196
cg02918489
cg23053742
cg14109551
cg04193065
cg22645359
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cg22076676
cg11047783
cg24475272
cg01842756
cg10422093
cg26308668
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cg07375256
cg26683792
cg12274883
cg14364797
cg05038391
cg12928479
cg00687889
cg14748515
cg17522929
cg14800111
Site categories
Cerebral/neuronal
Non−coding RNA
Gene expression/DNA regulation
Growth/Development/Locomotion
Intra− intercellular Signalling/Transport
Intergenic
Oncogenic/Apoptosis
Metabolism

SUPPLEMENTARY MATERIAL

Effect of Early Parenteral Nutrition during Paediatric Critical Illness on DNA Methylation as a Potential Mediator of Impaired Neurocognitive Development: A pre-planned secondary analysis of the PEPaNIC international, randomised, controlled trial

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Supplementary Appendix Methods:

Methods S1. Outcomes measured at 2 years' follow-up
Methods S2. DNA extraction and bisulfite conversion
Methods S3. Quality assessments of the DNA methylation data
Methods S4. Selection of patients and healthy control cohorts for identification of differential DNA methylation upon PICU admission
Methods S5. Definition of "Syndrome"
Methods S6. Association of the average macronutrient doses with the DNA methylation status of the CpG-sites differentially methylated by early-PN
Methods S7. Multivariable forward and backward feature selection on multiple imputed databases for linear and logistic regression models
Methods S8. Cross-tissue comparisons

Supplementary Appendix Figures:

Figure S1. Quality control of the epigenome-wide DNA methylation data of patient-control cohort 1
Figure S2. Quality control of the epigenome-wide DNA methylation data of patient-control cohort 2
Figure S3. Quality control of the epigenome-wide DNA methylation data of patients upon PICU discharge and healthy control children
Figure S4. Daily caloric intake in the first week of intensive care
Figure S5. Impact of early-PN, versus late-PN, on DNA methylation, adjusted for baseline risk factors and for incidence of new infection, duration of PICU-stay, duration of mechanical ventilatory support and hypoglycaemia
Figure S6. Summary of the associations of the average macronutrient doses with the DNA methylation status of the CpG-sites differentially methylated by early-PN
Figure S7. Role of the 159 differentially methylated CpG-sites evoked by critical illness in explaining impaired long-term outcome in linear models
Figure S8. Role of the 37 differentially methylated CpG-sites evoked by early-PN in explaining its adverse impact on neurocognitive development in linear models

Supplementary Appendix Tables:

Table S1. CpG-sites differentially methylated between patients upon PICU discharge and healthy control children
Table S2. Impact of early-PN, versus late-PN, on DNA methylation, adjusted for baseline risk factors
Table S3. Impact of early-PN, versus late-PN, on DNA methylation, adjusted for baseline risk factors and for incidence of new infection, duration of PICU-stay, duration of mechanical ventilatory support and hypoglycaemia
Table S4. Added explanatory power provided by the differentially methylated CpG-sites in predicting the long-term outcomes
Table S5. Classification of CpG-sites according to function
Table S6. Added explanatory power provided by the CpG-sites differentially methylated by early-PN in predicting the neurocognitive outcomes adversely affected by early-PN

References

Supplementary Methods

Methods S1. Outcomes measured at 2 years' follow-up [1]

Medical assessment

Anthropometric data
At the beginning of the follow-up visit, height (in cm), body weight (in kg) and head circumference (in cm) were measured.

Health status
In an interview with the parents, the need for medical support of all kind during the past two years for healthy control children and during the 2 years following the index PICU admission for patients, was recorded. The hospital admissions because of surgery or a medical reason, and the occurrence of a psychiatric diagnosis were documented.

Clinical neurological examination
In order to assess whether there were gross neurological abnormalities, during a structured clinical neurological examination, signs of major neurologic dysfunction were detected in the following domains: interaction/language skills, gross motor function, involuntary movements, reflexes, coordination and balance, fine motor function, cranial nerves, and special senses (sensory, visual, and auditory function). These were all scored normal or abnormal. An abnormal result for each of these domains was given 1 point and the sum was made of all the abnormal results, with a range of 0-8.

Neurocognitive testing

A broad range of neurocognitive functions, including general intellectual functioning, visual-motor integration, attention, motor coordination, inhibitory control and cognitive flexibility, verbal and visual-spatial learning, and memory were evaluated, as previously reported.[2]

Executive functioning
Executive functioning was measured with the Behaviour Rating Inventory of Executive Function (BRIEF-P 2·5-5 years, BRIEF 6-18 years), filled out by the parents/caregivers of the child. Overlapping scales and indices of both questionnaires (Inhibition, Flexibility, Emotional Control, Working Memory, Planning and Organisation, Meta-cognition) and a Total Score were analyzed (T-scores, with mean 50 and SD 10).[3,4]

Behaviour
Emotional and behavioural problems were assessed by the parent/caregiver with the Child behaviour Checklist (CBCL 1·5-5 years or CBCL 6-18 years).[5,6] Internalising, externalising, and total problems were analyzed (T-scores, with mean 50 and SD 10).[5,6]

Intelligence
General intellectual ability was assessed with use of age-appropriate versions of the Wechsler Intelligence Quotient (IQ) tests. The Wechsler Preschool and Primary Scale of Intelligence (WPPSI-III-NL)[7] was used for children aged 2·5 years – 5 years 11 months (one version for age range 2 years 6 months – 3 years 11 months, and another version for age range 4 years – 5 years 11 months), the Wechsler Intelligence Scale for Children (WISC-III-NL)[8] was used for children aged 6 years – 16 years 11 months, and the Wechsler Adult Intelligence Scale (WAIS-IV-NL)[9] for adolescents who were 17 years or older. For all these tests Total IQ, Verbal IQ, and Performance IQ scores (Test-mean 100, SD 15) were computed.

Visual-motor integration
We used the Beery Developmental Test of Visual-Motor Integration, 6th Edition (VMI) to assess the ability to integrate their visual and motor functions (total Scaled Score, Test-mean 10, SD 3). This involves eye-hand coordination.[10]

Alertness, motor-coordination, and executive functions
To measure alertness, motor-coordination and executive function, the validated Amsterdam Neuropsychological Tasks (ANT) program was used.[11] The ANT is a computer-aided assessment battery of reaction time (RT) tasks that allows for the systematic evaluation of information processing capacities.
Children 4 years and older performed ANT-Baseline Speed (BS), ANT-Tapping (TP), and Response Organisation Objects (ROO). The ANT-BS evaluated alertness by measuring simple RT to visual stimuli (mean RT and SD of

RT were obtained for the right and left hand separately). The ANT-TP assessed motor coordination for the right hand, left hand, bimanual alternating, and bimanual synchronous. The ANT-ROO measured inhibitory control and cognitive flexibility by calculating the differences in RT and the differences in number of errors between tests of increasing demand.

Memory

Auditory/verbal memory and Visual-spatial/non-verbal memory were assessed with use of four tests from the Children's Memory Scale (CMS) for children between 5 and 16 years 11 months.[12]

As to verbal memory, CMS-Numbers assessed short-term verbal memory span (forward digit recall) and verbal working memory load (backward digit recall). The CMS-Word Pairs (recall a list of word pairs) assessed short-term and long-term verbal memory, and recognition.

As to non-verbal memory, CMS-Picture Locations (remembering and recall of pictures in various locations) assessed immediate visual memory. CMS-Dot Locations (remembering and recall of the location of dots) assessed immediate and delayed visual memory.

For CMS-Numbers, raw scores for verbal memory span, CMS-numbers forward, and verbal working memory load, CMS-numbers backward were reported. For CMS-Word Pairs, CMS-Picture Locations, and CMS-Dot Locations, proportional scores were analyzed (proportion of correct responses ranging from 0 to 1, with higher scores reflecting better performance).

The CMS-Learning index is a standardised score of the sum of the three learning trials of the CMS-Word Pairs and the learning trial of the CMS-Dot Locations subtests. The range of the score is 50-150, with a higher score representing a better learning ability.

Methods S2. DNA extraction and bisulfite conversion

DNA extraction was performed with the Maxwell®RSC instrument and the corresponding blood DNA purification kit (Promega Benelux b.v., Leiden, The Netherlands). All DNA concentrations were accurately quantified with the Qubit® 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA).
Bisulfite conversion of the DNA (400 ng) was performed with the EZ and EZ-96 DNA Methylation-Direct® Kits (Zymo Research, Irvine, CA).

Methods S3. Quality assessments of the DNA methylation data

Several quality assessments were performed with use of Partek Genomics Suite® 7.0 (Partek, St. Louis, MO).

Methylation β-values, ranging from 0 (no methylation) to 1 (full methylation), and M-values, which are the log2 ratios of the intensities of methylated probes versus unmethylated probes,[13] were obtained after functional normalisation of the raw intensities.[14]

First, sample histogram formation assessed the bi-peak curve of the M-value distribution among the samples in the low- and high-end range, and principal component analysis (PCA) assessed the variance in the dataset. Next, probes with a mean detection P-value >0·01 were excluded, to ensure that signals were expressed above the background defined by negative control probes. Also probes on X and Y chromosomes and probes spanning single nucleotide polymorphisms (SNPs) were discarded. Indeed, in previous research among autosomal CpG-sites that displayed significant methylation differences between the two sexes, significant enrichment of cross-reactive probes co-hybridising to the sex chromosomes was observed.[15] Moreover, some probes target CpGs that are known to overlap with SNPs, and thus methylation levels detected by these probes could be the reflection of underlying genetic polymorphisms.[15]

M-values were compared between groups with ANOVA. A false discovery rate (FDR) <0·05 was used to account for multiple testing inherent to epigenome-wide analyses.[16] Based on the RefSeq annotations, CpG-sites were assigned to corresponding genes and relative location to gene transcripts. Promoter sequences were defined as 1500 base pairs upstream of the transcription start site.[17] Subsequently, pathway analyses were performed based on information from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database with use of Partek Pathway® (Partek, St. Louis, MO) and gene ontology analyses.[18]

Methods S4. Selection of patients and healthy control cohorts for identification of differential methylation upon PICU admission

In order to investigate whether DNA methylation was altered between PICU-admission and discharge it was first necessary -to avoid bias- to exclude from all further analyses all CpG-sites that were differentially methylated in patients upon PICU-admission as compared with healthy children, as these differences reflect pre-morbid conditions and illness-induced alterations in leukocyte composition.[19-23] As minor elective surgery is not often performed under the age of 1 year, infants were underrepresented in the healthy control group. However, age is an important determinant of DNA methylation.[24] Therefore, two "patient versus healthy control" cohorts were selected to identify the differentially methylated CpG-sites between patients upon PICU-admission and healthy children to maximally avoid bias (Figure_1).

First, from the healthy children a subgroup was selected that had a similar age and gender distribution than the total patient group with upon PICU admission DNA available ("patient-control cohort 1"). For this procedure, we first calculated the proportion of infants (younger than one year) in the patient group, as well as the proportions of children for each year added (i.e. percentage of children between 1 and 2 years old, between 2 and 3 years old, and so on). For the selection of the healthy control group, we retained all healthy infants (in view of the underrepresentation of this group). Starting from this number of healthy infants, we then applied similar proportions as identified in the different age categories of the patients to calculate how many healthy children needed to be included in each of the age categories. We then randomly selected the required number of healthy children in each age range, ensuring to retain a similar distribution of boys and girls.

Second, demographically comparable cohorts of patients and healthy controls were selected via propensity score matching with SPSS R-menu R3.1 (Foundation for Statistical Computing) in IBM SPSS Statistics 24.0.0.0 (SPSS, Chicago, IL) ("patient-control cohort 2"). This procedure used logistic regression to estimate propensity scores with age and gender as covariates, and one-to-one nearest neighbour matching without replacement, with the caliper set at 0·05.

All CpG-sites that were differentially methylated between patients and controls in one or both of these two comparisons, with a false discovery rate below 0·05 to adjust for multiple testing, were discarded from further analyses.

Methods S5. Definition of "Syndrome"

A prerandomisation syndrome or illness *a priori* defined as affecting or possibly affecting neurocognitive development, and which is subdivided in the following categories:

- Genetically confirmed syndrome or pathogenic chromosomal abnormality
- Clearly defined syndrome, association or malformation without (identified) genetic aberration
- Polymalformative syndrome of unknown etiology
- Clear auditory or visual impairment without specified syndrome
- Congenital hypothyroidism due to thyroid agenesis
- Brain tumor or tumor with intracranial metastatic disease
- Paedopsychiatric disorder (e.g. autism spectrum disorder, (treatment for) attention deficit hyperactivity disorder)
- Severe medical disorder, not primarily neurologic, but suspected to alter psychomotor and/or mental performance
- Severe neonatal problem (e.g. severe asphyxia)
- Severe craniocerebral trauma or near-drowning
- Severe infectious encephalitis or drug-induced encephalopathy
- Infectious meningitis, encephalitis or Guillain-Barré
- Resuscitation and/or need for extracorporeal membrane oxygenation prior to randomisation
- Severe convulsions or stroke prior to randomisation

Methods S6. Association of the average macronutrient doses with the DNA methylation status of the CpG-sites differentially methylated by early-PN

Doses of glucose, lipids, and amino acids administered to the patients were transformed from crude grams per day into percentage of reference doses for age and weight published in nutritional guidelines, as described previously.[25] These reference doses were not necessarily those that were used as nutritional targets in the participating centres. They were only used because this type of analyses required expression of the administered doses of the three macronutrients in an interchangeable and comparable unity for children of different ages and weights (for whom recommended macronutrient doses per kg body weight differ), without which the analyses would be flawed.

As the PEPaNIC trial investigated the effect on outcome of early-PN versus late-PN over the first 7 days after admission to the PICU, with doses titrated differently for each macronutrient, we analysed independent associations between the methylation status of the CpG-sites affected by early-PN and average daily doses of each macronutrient, expressed as percentage of reference doses, up to each of the 7 days for all patients who were still in the PICU on these respective days. Multivariable linear regression analyses were performed, with the three macronutrients entered together into the model as continuous variables in place of the randomisation group, adjusted for predefined baseline risk factors. These risk factors were treatment centre, age, race,[26] gender, geographic origin,[26] language, history of malignancy, diabetes, predefined "syndrome" (Methods_S5), type (diagnostic group) and severity of illness (Paediatric Index of Mortality-3 [PIM3][27] score estimating mortality risk and Paediatric Logistic Organ Dysfunction [PELOD][28] score of the first 24 h estimating severity of organ dysfunction), and severity of malnutrition risk (STRONGkids score dichotomised for medium risk [score 2–3] and high risk [score 4–5]).[29]

Methods S7. Multivariable forward and backward feature selection on multiple imputed databases for linear and logistic regression models

To investigate any potential role of altered DNA methylation evoked by the critical illness in explaining impaired long-term outcomes, multivariable linear and logistic regression analyses were performed adjusting for baseline risk factors, with stepwise variable selection according to Rubin's method,[30,31] described as follows:

1. Given M_0, the model that only includes the intercept but no covariates, and *n* candidate covariates.
2. Fit a new model M_{k+1}consisting of the *k* covariates in the currently selected model (M_k) plus one of the remaining *n-k* unselected covariates. Repeat for the same covariate in each of the *m* imputed datasets. Evaluate the pooled P-value in the multivariable model for this covariate *k+1*. If the P-value is below the threshold of 0·2 add this covariate to the currently selected model M_k.
3. Re-check the pooled multivariable P-values for all other covariates already in the currently selected model M_{k+1}. Remove from the currently selected model M_{k+1} all covariates with a multivariable pooled P-value above the threshold of 0·2. Add the removed covariates to the unselected covariates set.
4. Repeat steps 2 and 3 until all covariates have been evaluated and until all multivariable pooled P-values in the model are below the 0·2 threshold.

Methods S8. Cross-tissue comparisons

To investigate correspondence between blood and brain in the differentially methylated CpG-sites on the last PICU-day, the BECon tool for interpreting DNA methylation findings from blood in the context of brain [32,33] and the Brain Epigenomics blood brain DNA methylation comparison tool[34] were used. Both tools were developed based on probes present on the Illumina 450K Beadchip array.

Based on paired blood and post-mortem brain tissue data from 16 individuals, BECon allows interrogation of the correlation level between methylation in blood and three brain regions: BA7 (parietal), BA10 (frontal), and BA20 (temporal). Informative CpG-sites are those for which correlation between DNA methylation in blood and DNA methylation in brain $\geq$ 0.36, 0.40 and 0.33 for the three respective regions, and for which DNA methylation in blood is sufficiently variable.[32,33]

Brain Epigenomics allows to interrogate the correlation between DNA methylation in blood and the DNA methylation of four brain regions: prefrontal cortex, entorhinal cortex, superior temporal gyrus and cerebellum, based on 71-75 matched samples. Correlations are reported together with p-values.[34]

Supplementary Figures

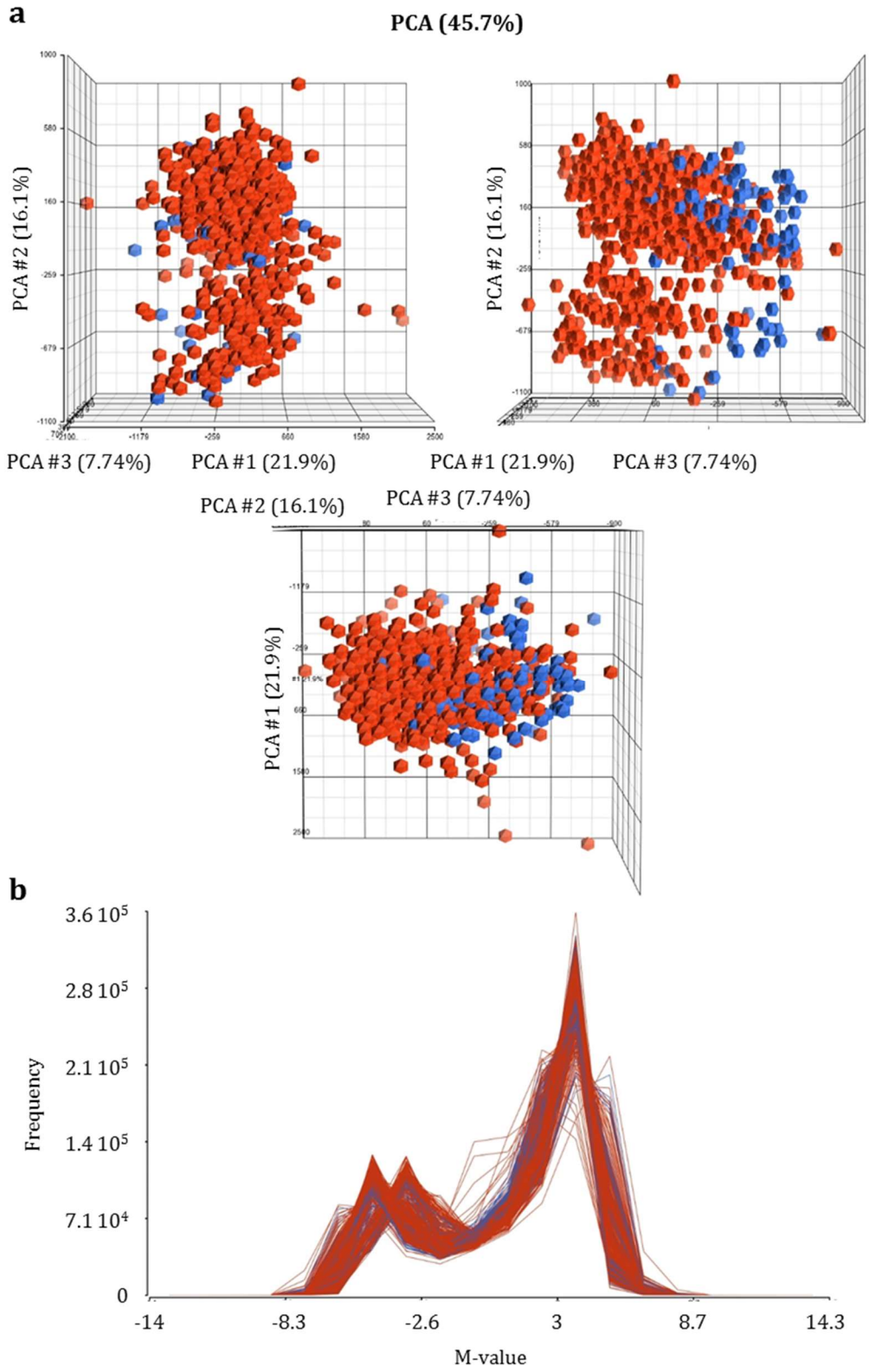


Figure S1. Quality control of the epigenome-wide DNA methylation data of patient-control cohort 1
From the healthy children, a subgroup was selected (n=119, blue dots and lines) that had a similar age and gender distribution than the total patient group with upon PICU admission DNA available (n=694, red dots and lines) (Methods_S4). Panel A shows the first three components of the Principal Component Analysis (PCA), which is a technique used to bring out strong patterns in a dataset by reducing the dimensionality via creating new uncorrelated variables that successively maximise variance. These three components explained 45·7% of the variance in the data. Panel B shows the sample histogram of the frequency of the M-values of the probes.

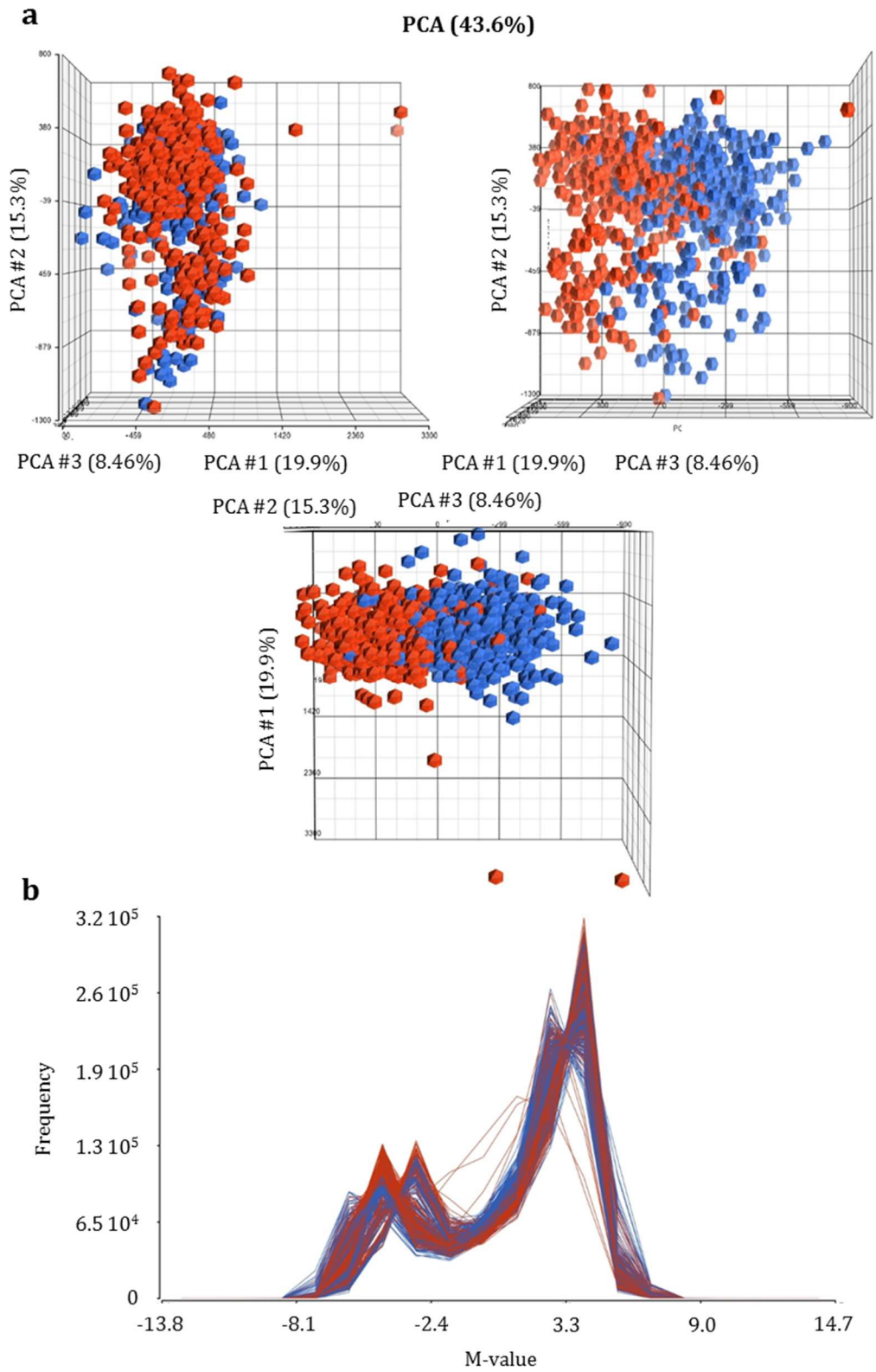


Figure S2. Quality control of the epigenome-wide DNA methylation data of patient-control cohort 2
Demographically comparable cohorts of patients with upon PICU admission DNA available (n=344, red dots and lines) and healthy controls (n=344, blue dots and lines) were selected via propensity score matching (Methods_S4). Panel A shows the first three components of the Principal Component Analysis (PCA), which is a technique used to bring out strong patterns in a dataset by reducing the dimensionality via creating new uncorrelated variables that successively maximise variance. These three components explained 43·6% of the variance in the data. Panel B shows the sample histogram of the frequency of the M-values of the probes.

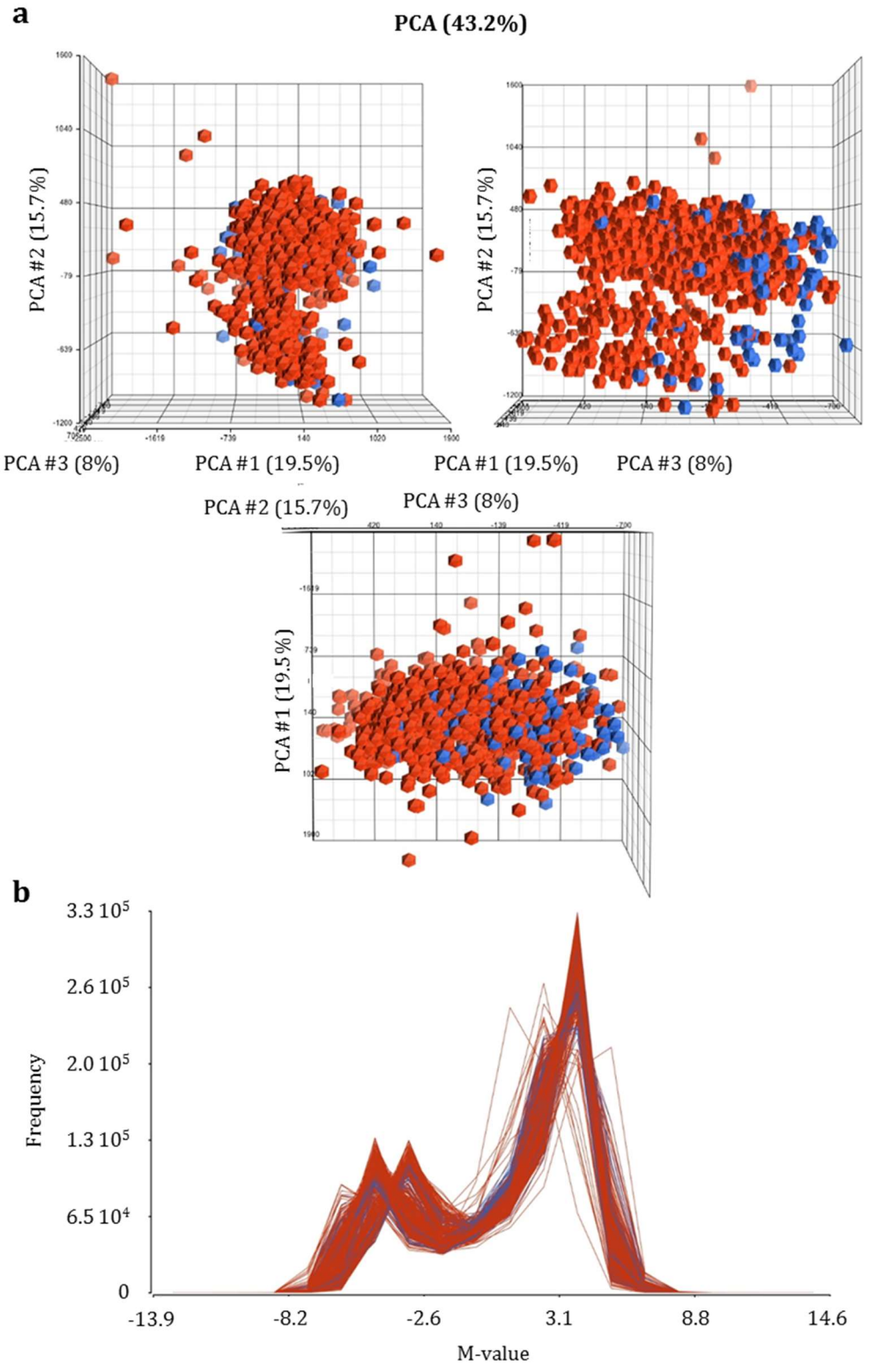


Figure S3. Quality control of the epigenome-wide DNA methylation data of patients upon PICU-discharge and healthy control children

Upon PICU discharge, DNA was available from 814 patients (red dots and lines) and was compared with 119 healthy control children with comparable age and gender distribution (blue dots and lines). Panel A shows the first three components of the Principal Component Analysis (PCA), which is a technique used to bring out strong patterns in a dataset by reducing the dimensionality via creating new uncorrelated variables that successively maximise variance. These first three components explained 43·2% of the variance in the data. Panel B shows the sample histogram of the frequency of the M-values of the probes.

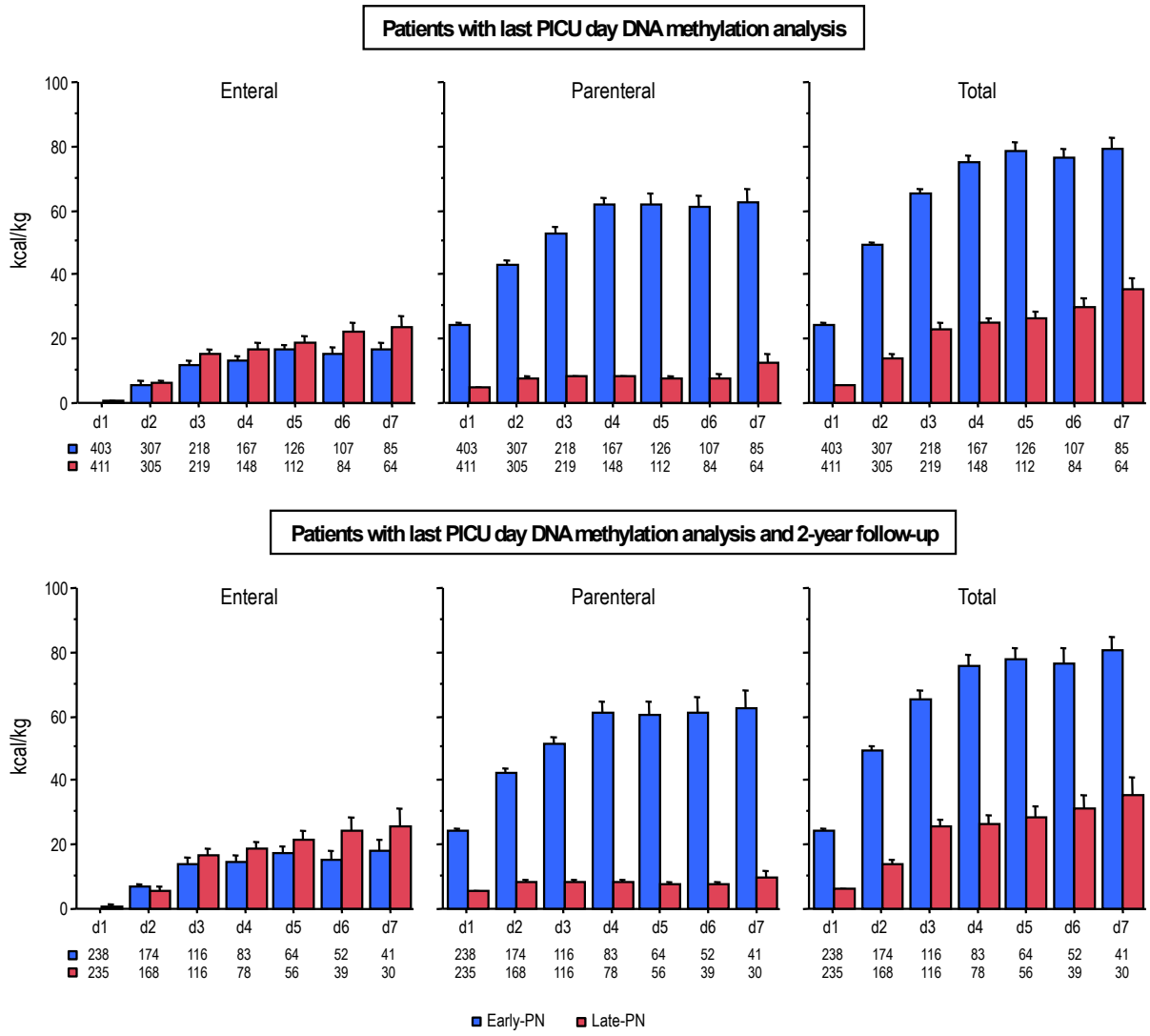
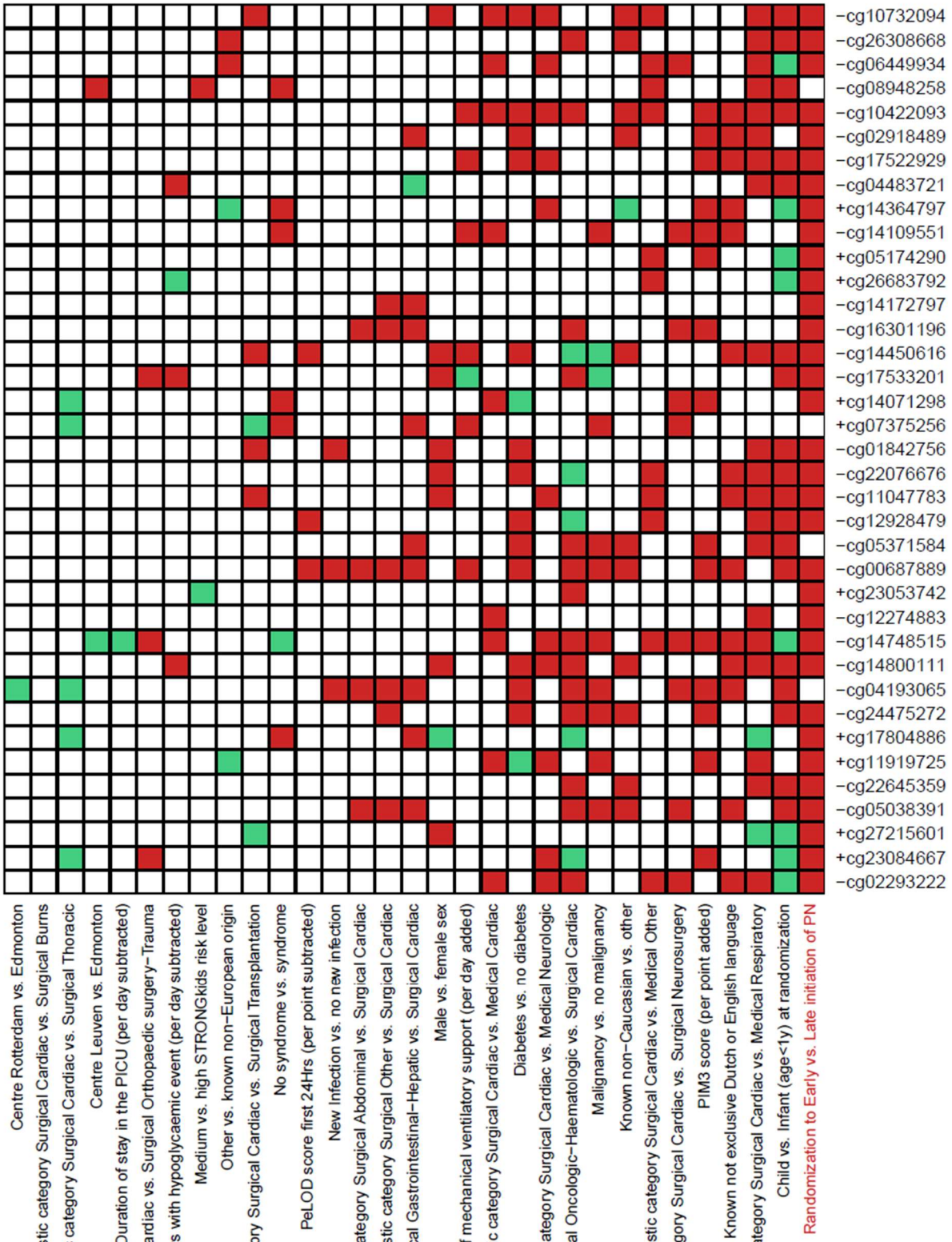


Figure S4. Daily caloric intake in the first week of intensive care
Daily amounts of energy (kilocalories per kg per day) provided by the enteral route, the parenteral route, or both (total energy) are shown for the first 7 days in the PICU for the patients included in the DNA methylation analyses and for the patients with data available on DNA methylation status and outcome 2 years after PICU admission. Energy targets differed according to age and weight of the children. Bars depict means and whiskers indicate standard errors. Blue and red bars represent patients of the early-PN group and of the late-PN group, respectively. n indicates the number of patients still in the PICU on the respective days. d: day; PICU: paediatric intensive care unit; PN: parenteral nutrition.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+cg27215601
+cg23084667
−cg02293222
Centre Rotterdam vs. Edmonton
Diagnostic category Surgical Cardiac vs. Surgical Burns
Diagnostic category Surgical Cardiac vs. Surgical Thoracic
Centre Leuven vs. Edmonton
Duration of stay in the PICU (per day subtracted)
Diagnostic category Surgical Cardiac vs. Surgical Orthopaedic surgery−Trauma
Days with hypoglycaemic event (per day subtracted)
Medium vs. high STRONGkids risk level
Other vs. known non−European origin
Diagnostic category Surgical Cardiac vs. Surgical Transplantation
No syndrome vs. syndrome
PeLOD score first 24Hrs (per point subtracted)
New Infection vs. no new infection
Diagnostic category Surgical Abdominal vs. Surgical Cardiac
Diagnostic category Surgical Other vs. Surgical Cardiac
Diagnostic category Medical Gastrointestinal−Hepatic vs. Surgical Cardiac
Male vs. female sex
Duration of mechanical ventilatory support (per day added)
Diagnostic category Surgical Cardiac vs. Medical Cardiac
Diabetes vs. no diabetes
Diagnostic category Surgical Cardiac vs. Medical Neurologic
Diagnostic category Medical Oncologic−Haematologic vs. Surgical Cardiac
Malignancy vs. no malignancy
Known non−Caucasian vs. other
Diagnostic category Surgical Cardiac vs. Medical Other
Diagnostic category Surgical Cardiac vs. Surgical Neurosurgery
PIM3 score (per point added)
Other vs. Known not exclusive Dutch or English language
Diagnostic category Surgical Cardiac vs. Medical Respiratory
Child vs. Infant (age<1y) at randomization
Randomization to Early vs. Late initiation of PN

Figure S5. Impact of early-PN, versus late-PN, on DNA methylation, adjusted for baseline risk factors and for incidence of new infection, duration of PICU-stay, duration of mechanical ventilatory support and hypoglycaemia

Summary of the 100 bootstrapped multivariable linear regression analyses for identification of the robustly significant (with a P<0·05 in at least 50 replicated analyses), independent associations of the nutritional management strategy (early-PN versus late-PN), baseline risk factors, incidence of new infection, duration of PICU-stay, duration of mechanical ventilatory support and hypoglycaemia, with the methylation beta-values of the differentially methylated CpG-sites identified upon PICU discharge. Each row corresponds to 100 bootstrap replicates of linear regression models for the given CpG-site and columns correspond to the model covariates. A "+" in front of a CpG-site indicates more methylation in patients than in controls. A "-" in front of a CpG-site indicates less methylation in patients than in controls. Colored boxes indicate robust independent associations with CpG-sites. Green colored boxes indicate where the covariate alters DNA methylation towards healthy control values, suggesting a protective effect. Red colored boxes indicate where the covariate alters DNA methylation away from healthy control values, suggesting a harmful effect. PIM3: Paediatric Index of Mortality 3; PN: parenteral nutrition; PeLOD: Paediatric logistic organ dysfunction; STRONGkids: Screening Tool for Risk on Nutritional Status and Growth.

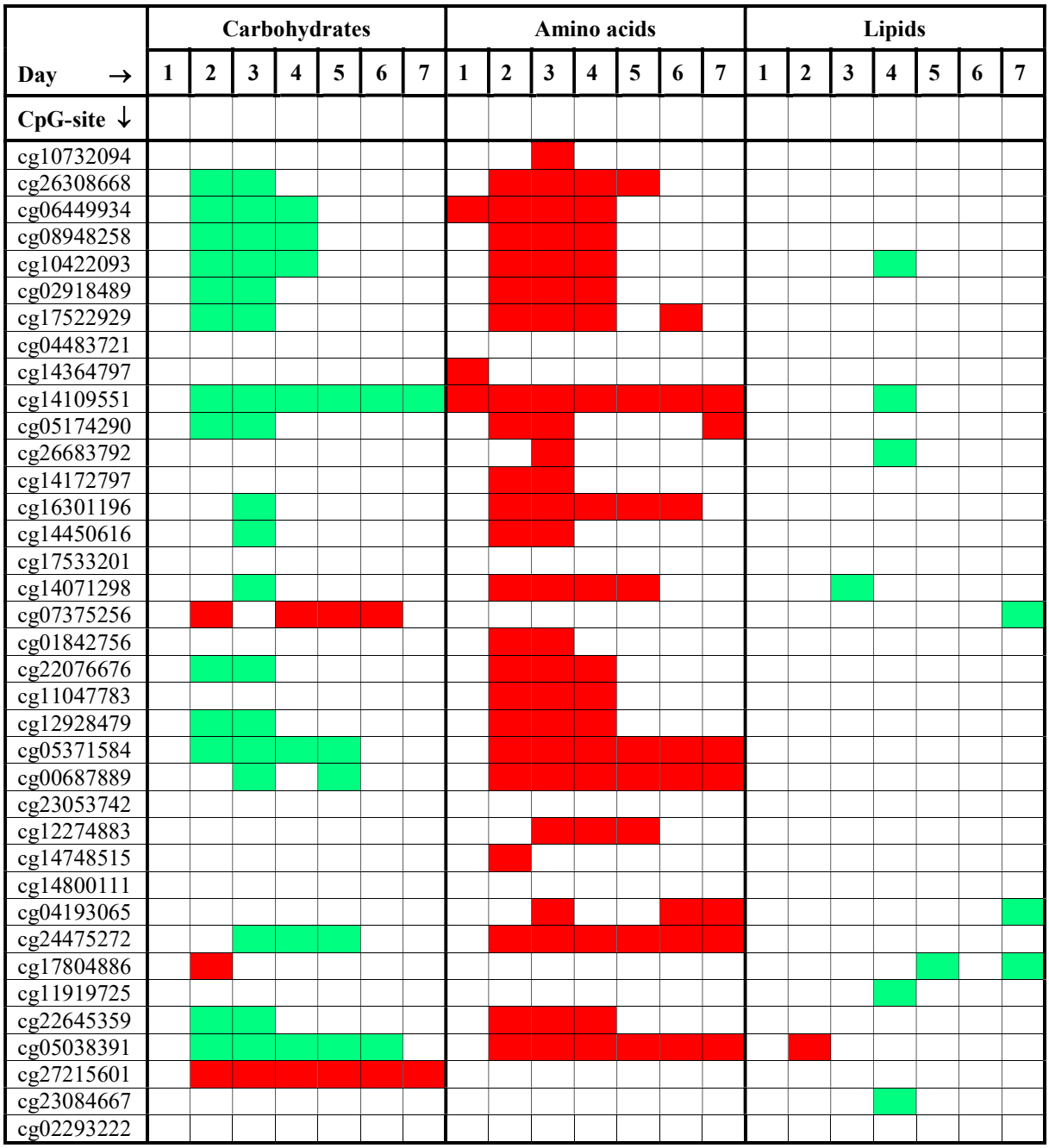

Figure S6. Summary of the associations of the average macronutrient doses with the DNA methylation status of the CpG-sites differentially methylated by early-PN

For each of the first 7 days in the PICU (the time window of the randomised intervention), associations of the average daily doses of each of the individual macronutrients up to that day with methylation status of the CpG-sites affected by early-PN are summarised. The three macronutrients were entered simultaneously as continuous variables. The analyses were adjusted for treatment centre, age, race,[26] gender, geographic origin,[26] language, history of malignancy, diabetes, predefined "syndrome" (Methods_S5), type and severity of illness,[27,28] and severity of malnutrition risk.[29] Red colored boxes indicate where the macronutrient alters DNA methylation away from healthy control values, suggesting a harmful effect. Green colored boxes indicate where the macronutrient alters DNA methylation towards healthy control values, suggesting a protective effect.

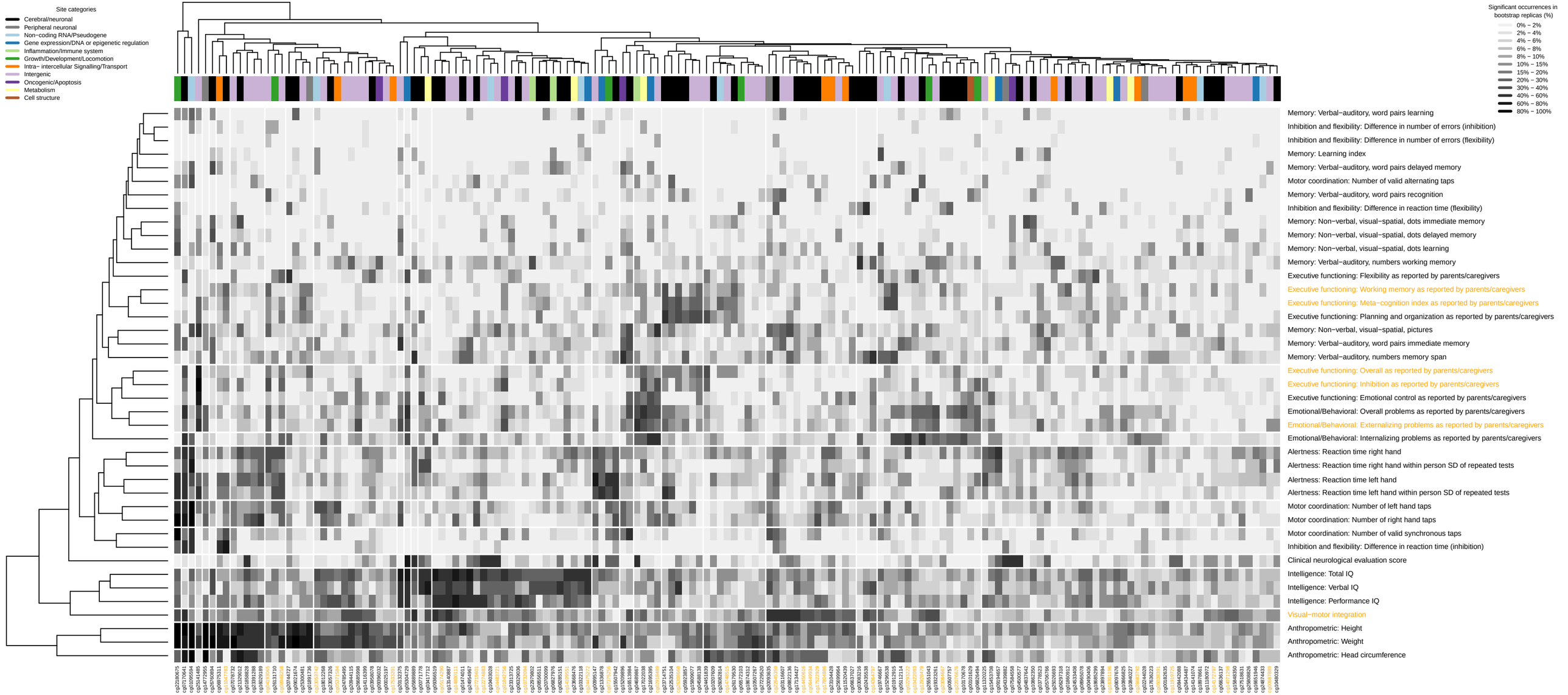
Site categories
Cerebral/neuronal
Peripheral neuronal
Non−coding RNA/Pseudogene
Gene expression/DNA or epigenetic regulation
Inflammation/Immune system
Growth/Development/Locomotion
Intra− intercellular Signalling/Transport
Intergenic
Oncogenic/Apoptosis
Metabolism
Cell structure
Significant occurrences in bootstrap replicas (%)
0% − 2%
2% − 4%
4% − 6%
6% − 8%
8% − 10%
10% − 15%
15% − 20%
20% − 30%
30% − 40%
40% − 60%
60% − 80%
80% − 100%
Memory: Verbal−auditory, word pairs learning
Inhibition and flexibility: Difference in number of errors (inhibition)
Inhibition and flexibility: Difference in number of errors (flexibility)
Memory: Learning index
Memory: Verbal−auditory, word pairs delayed memory
Motor coordination: Number of valid alternating taps
Memory: Verbal−auditory, word pairs recognition
Inhibition and flexibility: Difference in reaction time (flexibility)
Memory: Non−verbal, visual−spatial, dots immediate memory
Memory: Non−verbal, visual−spatial, dots delayed memory
Memory: Non−verbal, visual−spatial, dots learning
Memory: Verbal−auditory, numbers working memory
Executive functioning: Flexibility as reported by parents/caregivers
Executive functioning: Working memory as reported by parents/caregivers
Executive functioning: Meta−cognition index as reported by parents/caregivers
Executive functioning: Planning and organization as reported by parents/caregivers
Memory: Non−verbal, visual−spatial, pictures
Memory: Verbal−auditory, word pairs immediate memory
Memory: Verbal−auditory, numbers memory span
Executive functioning: Overall as reported by parents/caregivers
Executive functioning: Inhibition as reported by parents/caregivers
Executive functioning: Emotional control as reported by parents/caregivers
Emotional/Behavioral: Overall problems as reported by parents/caregivers
Emotional/Behavioral: Externalizing problems as reported by parents/caregivers
Emotional/Behavioral: Internalizing problems as reported by parents/caregivers
Alertness: Reaction time right hand
Alertness: Reaction time right hand within person SD of repeated tests
Alertness: Reaction time left hand
Alertness: Reaction time left hand within person SD of repeated tests
Motor coordination: Number of left hand taps
Motor coordination: Number of right hand taps
Motor coordination: Number of valid synchronous taps
Inhibition and flexibility: Difference in reaction time (inhibition)
Clinical neurological evaluation score
Intelligence: Total IQ
Intelligence: Verbal IQ
Intelligence: Performance IQ
Visual−motor integration
Anthropometric: Height
Anthropometric: Weight
Anthropometric: Head circumference

Figure S7. Role of the 159 differentially methylated CpG-sites evoked by critical illness in explaining impaired long-term outcome in linear models

Each row corresponds to 100 bootstrap replicates of a multivariable linear regression model for the given outcome assessed at 2-years follow-up and columns correspond to the 159 differentially methylated CpG-sites. CpG-sites and outcomes in orange font correspond to those affected by the nutritional management strategy (early-PN versus late-PN). All models resulted from stepwise backward-forward variable selection according to Rubin's method (Methods_S7)[30,31] including the baseline risk factors age, centre, race,[26] gender, geographic origin,[26] language, history of malignancy, a predefined "syndrome" (Methods_S5), diagnosis and severity of illness (PIM3 and PeLOD),[27,28] risk of malnutrition (STRONGkids)[29] and the 159 CpG-sites becoming differentially methylated in patients during PICU-stay as compared with matched healthy children. The robustness of these results was evaluated in 100 bootstrapped replicates.[35] Color intensity of the boxes reflects the frequency with which a CpG-site was found to be independently and significantly ($P<0{\cdot}05$) associated with the outcomes in the 100 bootstrapped replicated analyses, with darker grey colors corresponding to a higher frequency. Outcomes and CpG-sites were clustered based on these frequencies. Dendrograms show clustering hierarchy. The base of the column dendrogram is color-coded according to CpG-site functional classes "Cerebral/neuronal", "Peripheral neuronal," "Growth/Development/Locomotion", "Metabolism", "Gene expression/DNA regulation/Epigenetic regulation", "Inflammation/Immune system", "Cell structure", "Intra/intercellular Signalling/Transport", "Non-coding RNA/pseudogene", "Oncogenic/Apoptosis", or "Intergenic" (Table_S3).

For anthropometrics, particularly strong associations were observed for groups of CpG-sites in genes involved in growth, bone formation, myogenesis, brain development, axon guidance, intercellular adhesion, apoptosis, Ras signalling, neuronal functioning, signal transduction in brain, and neurodegeneration. For measures of intelligence, the strongest associations were observed for CpG-sites in genes involved in neurocognitive development/functioning with neuronal network formation and information processing, verbal learning and recall, concentration, memory, mental retardation/neurodegeneration, neuropsychiatric and attention deficit disorders, neuromuscular development, apoptosis and transcriptional regulation. For motor coordination the strongest associations were observed for CpG-sites involved in skeletal muscle and brain development, connectivity and signal transduction in brain, visual motor/fine motor function, neuronal network formation and information processing, (working) memory, behaviour, muscle disorders/nerve degeneration, neuropsychiatric, neurodegenerative and attention deficit disorders, and apoptosis. For visual-motor integration the strongest associations were observed for CpG-sites in genes involved in peripheral nerve maturation, sensory nerve conduction, neuromuscular development, neuronal development, visual motor/fine motor function, concentration and attention deficit, memory, apoptosis, and signal transduction in brain. For the executive functions, the strongest associations were observed for CpG-sites in genes involved in brain development, cognitive functioning, signal transduction in brain, learning and memory; concentration, visual-motor/fine-motor function, emotional functioning, behaviour, locomotor activity, mental retardation, neurodegenerative, neuropsychiatric and attention deficit disorders, apoptosis, metabolism, and transcriptional regulation. For behavioural problems the strongest associations were observed for CpG-sites in genes involved in brain development, signal transduction in the brain, social function and behaviour, attention deficit hyperactivity, neuropsychiatric and neurodegenerative disorders, verbal learning and recall, concentration, memory, psychomotor speed, cellular energy metabolism, apoptosis, and transcriptional regulation. For alertness, the strongest associations were observed for CpG-sites in genes involved in neurodevelopment, neuromuscular development, visual motor/fine motor function, concentration, locomotor activity, psychomotor speed, memory, speech, social and emotional function, behaviour, neurodegenerative, neuropsychiatric and attention deficit disorders, transcriptional regulation, and metabolism. Also several strong associations were observed between various outcomes and intergenic CpG-sites (Table_S1).

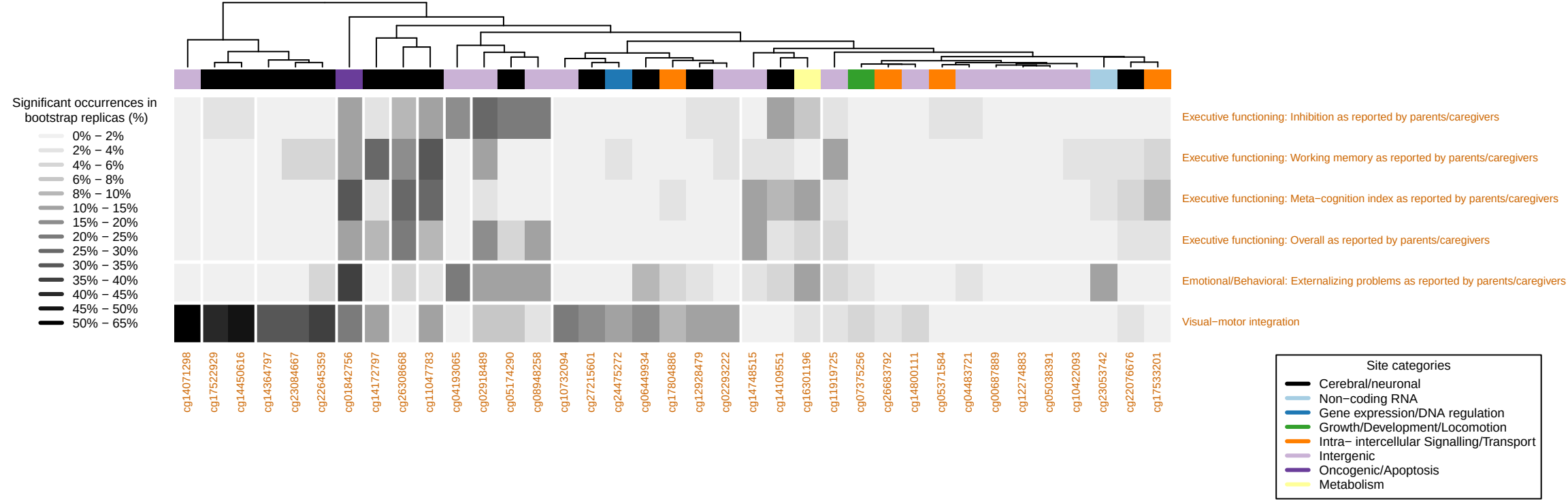
Significant occurrences in bootstrap replicas (%)
0% − 2%
2% − 4%
4% − 6%
6% − 8%
8% − 10%
10% − 15%
15% − 20%
20% − 25%
25% − 30%
30% − 35%
35% − 40%
40% − 45%
45% − 50%
50% − 65%
Executive functioning: Inhibition as reported by parents/caregivers
Executive functioning: Working memory as reported by parents/caregivers
Executive functioning: Meta−cognition index as reported by parents/caregivers
Executive functioning: Overall as reported by parents/caregivers
Emotional/Behavioral: Externalizing problems as reported by parents/caregivers
Visual−motor integration
cg14071298
cg17522929
cg14450616
cg14364797
cg23084667
cg22645359
cg01842756
cg14172797
cg26308668
cg11047783
cg04193065
cg02918489
cg05174290
cg08948258
cg10732094
cg27215601
cg24475272
cg06449934
cg17804886
cg12928479
cg02293222
cg14748515
cg14109551
cg16301196
cg11919725
cg07375256
cg26683792
cg14800111
cg05371584
cg04483721
cg00687889
cg12274883
cg05038391
cg10422093
cg23053742
cg22076676
cg17533201
Site categories
Cerebral/neuronal
Non−coding RNA
Gene expression/DNA regulation
Growth/Development/Locomotion
Intra− intercellular Signalling/Transport
Intergenic
Oncogenic/Apoptosis
Metabolism

Figure S8. Role of the 37 differentially methylated CpG-sites evoked by early-PN in explaining its adverse impact on neurocognitive development in linear models

Each row corresponds to 100 bootstrap replicates of multivariable linear regression models for the 6 neurocognitive outcomes negatively affected by early-PN, versus late-PN. Columns correspond to the 37 CpG-sites that were differentially methylated by early-PN. All models resulted from stepwise backward-forward variable selection according to Rubin's method (Methods_S7)[30,31] from the baseline risk factors age, centre, race,[26] gender, geographic origin,[26] language, history of malignancy, a predefined "syndrome" (Methods_S5), diagnosis and severity of illness (PIM3 and PeLOD),[27,28] risk of malnutrition (STRONGkids)[29] and the 37 differentially methylated CpG-sites evoked by early-PN. The robustness of these results was evaluated in 100 bootstrapped replicates.[35] Color intensity of the boxes reflects the frequency with which a CpG-site was found to be independently and significantly ($P<0{\cdot}05$) associated with the outcomes in the 100 bootstrapped replicated analyses, with darker grey colors corresponding to a higher frequency. Outcomes and CpG-sites were clustered based on these frequencies. Dendrograms show clustering hierarchy. The base of the column dendrogram is color-coded according to CpG-site functional classes "Cerebral/neuronal", "Peripheral neuronal," "Growth/Development/Locomotion", "Metabolism", "Gene expression/DNA regulation/Epigenetic regulation", "Inflammation/Immune system", "Cell structure", "Intra/intercellular Signalling/Transport", "Non-coding RNA/pseudogene", "Oncogenic/Apoptosis", or "Intergenic" (Table_S3).

The CpG-sites that were most often retained in the bootstrapped linear models as independently associated with either the 4 executive functions, the externalising behaviour or the visual-motor integration were cg26308668 in srGAP1 (involved in neuronal development and migration, linked to mental retardation), cg14364797 in FNBP1 (involved in neuronal network formation and information processing), cg11047783 in KAT6B (involved in cerebral cortex development, attention deficit hyperactivity disorder (ADHD) and intellectual disability), cg14109551 in CEP85L (involved in brain tumors, ADHD and bipolar disorder), cg22645359 in TCF7L2 (involved in Wnt signalling and related to neurodevelopment and plasticity of mature neurons), cg14172797 in PRKCA (involved in memory, mood regulation, behaviour, and apoptosis), cg06449934 in GPER1 (involved in memory and cognition, social and spatial recognition learning, working memory, synaptic transmission, and anxiety), cg05174290 in ATAD2B (involved in neuronal differentiation), cg27215601 in NSMCE2 (involved in bipolar disorder, genome maintenance, and transcription), cg23084667 in GRAP2 (involved in protein-tyrosine kinase signalling and linked to RET signalling which is involved in brain development and maturation of dopaminergic neurons), cg17522929 in PKM (involved in brain development and neuronal differentiation), cg14450616 in PLD3 (involved in neuronal development, survival and neurotransmission, visual learning, memory and flexibility), cg12928479 in NLRC5 (involved in neuroimmune and neuroinflammatory processes), cg01842756 in RNF217 (involved in apoptosis), cg16301196 in PLA2G15 (involved in lipid metabolism), cg24475272 in SETD7 (involved in histone methylation), and several intergenic CpG-sites (Figure_4; Table_S1).

Supplementary Tables

Table S1. CpG-sites differentially methylated between patients upon PICU discharge and healthy control children, with CpG-sites of which methylation was altered by early-PN marked in grey

CpG-site	Gene symbol	Gene-related protein function	P-value	Gene section	More methylated in
cg01474011	CUL3	polyubiquitination and degradation of proteins; controlling brain size and connectivity; muscle disorders and nerve degeneration; neuropsychiatric disorders	1·04923e-32	Intron/ Promoter	Control
cg10732094			1·67761e-18	Intergenic	Control
cg26308668	SRGAP1	regulating neuronal development and migration; mental retardation; autism; schizophrenia	2·13492e-17	Intron	Control
cg18674312			7·16483e-13	Intergenic	Control
cg06449934	GPER1	numerous intracellular signalling pathways among the cardiovascular, endocrine, reproductive, immune and central nervous systems; regulation of hippocampal memory and cognition; social and spatial recognition learning; working memory; synaptic transmission; anxiety	7·34083e-13	5' UTR	Control
cg15453708	PRKAG2	regulating cellular energy metabolism including inhibition of protein, carbohydrate and lipid biosynthesis, and cell growth and proliferation	8·68961e-13	5' UTR/ Intron	Control
cg08948258			1·80219e-12	Intergenic	Control
cg00025197			7·21064e-12	Intergenic	Control
cg15848350			1·49199e-11	Intergenic	Control
cg10422093			2·36286e-11	Intergenic	Control
cg09366519	HSD11B1	cortisol metabolism; verbal learning and recall, concentration, and memory, depression, dementia, and delirium	2·64893e-11	5' UTR/ Promoter	Control
cg02918489			3·0712e-11	Intergenic	Control
cg07170641	MS4A6A	signal transduction in hematopoietic cells and in the brain	3·33165e-11	5' UTR/ Intron	Control
cg08442088	DBNL	reorganisation of the actin cytoskeleton; formation of cell projections, such as neurites; morphogenesis of neurons and synapse formation	6·62839e-11	5' UTR/ Intron	Control
cg17522929	PKM	glycolysis; transcriptional activation; caspase-independent cell death of tumor cells; may mediate metabolic effects of thyroid hormone; involved in bacterial pathogenesis (adherence of bacteria to human cells); brain development; neuronal differentiation	1·04581e-10	5' UTR/ Intron	Control
cg01860912	IL19	transcriptional activation by activating STAT3; inflammatory responses; microglia activation; Alzheimer's disease	1·13534e-10	Intron	Control
cg04483721			1·88177e-10	Intergenic	Control
cg03958078	IGFBP7	regulating IGF availability; cell growth regulation; senescence; apoptosis; cancer; memory consolidation; neurogenesis	4·18313e-10	Intron	Patient
cg14364797	FNBP1	regulation of the actin cytoskeleton; spine formation/neurite branching; neuronal network formation; information processing	4·85693e-10	3' UTR	Patient
cg14109551	CEP85L	brain tumors; attention deficit hyperactivity disorder (ADHD); bipolar disorders	5·2671e-10	Intron	Control

cg02244028	SCN11A	action potential initiation and propagation in excitable cells, including nerve, muscle, and neuroendocrine cells; mediating brain-derived neurotrophic factor-evoked membrane depolarisation; major effector of peripheral inflammatory pain hypersensitivity	6·98287e-10	5' UTR	Control
cg04837642			8·5543e-10	Intergenic	Control
cg00101629	KAZN	desmosome assembly, cell adhesion, cytoskeletal organisation, and epidermal differentiation	9·46095e-10	Intron	Control
cg23668476	TPST1	catalyzing the O-sulfation of tyrosine residues of polypeptides	9·79535e-10	Intron	Control
cg05174290	ATAD2B	chromatin-related function; neuronal differentiation; tumor progression	1·09857e-09	Intron/ Non-coding	Patient
cg26683792	SLC35E1	unknown (putative transporter)	1·54675e-09	Intron	Patient
cg08097676	METTL16	regulating S-adenosyl-methionine homeostasis	2·18203e-09	Intron	Control
cg14116399			2·69626e-09	Intergenic	Control
cg00771778	LYST	sorting endosomal resident proteins into late multivesicular endosomes; cognitive decline, parkinsonism, cerebellar deficits, spasticity and peripheral neuropathy in Chediak-Higashi syndrome	3·11466e-09	5' UTR/ Non-coding	Control
cg11180972	CHST11	sulfating chondroitin; skeletal development; brain development; locomotion; ADHD	3·32475e-09	Intron	Control
cg24954967			3·60567e-09	Intergenic	Control
cg00995147			3·85827e-09	Intergenic	Control
cg14172797	PRKCA	many different cellular processes, such as cell proliferation, differentiation and apoptosis, cell cycle checkpoint, and cell volume control; cancer development; episodic remembering (memory); mood regulation; behaviour; choroid gliomas; highly expressed in brain	6·01096e-09	Intron	Control
cg16301196	PLA2G15	hydrolyzing lysophosphatidylcholine to glycerophosphorylcholine and a free fatty acid; phospholipid degradation	6·01122e-09	3' UTR	Control
cg16651946	UBP1	transcriptional activation	8·97361e-09	Intron	Control
cg10480329	CENPA	propagating centromere identity through replication and cell division; concentration, psychomotor speed; verbal memory	9·59681e-09	Intron	Control
cg14450616	PLD3	hydrolysis of membrane phospholipids and processing of amyloid-beta precursor protein; neuronal development and survival, neurotransmission; visual learning, memory, speed and flexibility; Alzheimer's disease; highly expressed in the brain	9·60705e-09	5' UTR	Control
cg10170678	RIN3	Ras signalling pathway; neuronal signalling; Alzheimer's disease	1·05881e-08	Intron	Patient
cg14772955	KIAA0319L	axon guidance	1·71378e-08	Promoter	Control
cg17533201	RAB11FIP4	regulating endocytic traffic; cytokinesis	1·91728e-08	Intron	Control
cg26179530	AAK1	regulating clathrin-mediated endocytosis; transcriptional activation; neurotrophic factor signalling and dendrite growth regulation; cognitive functioning/learning and memory; neurodegenerative disorders; schizophrenia	2·11396e-08	Intron	Control
cg26434487	RABGAP1L	endocytosis; intracellular protein transport; cell migration	2·28886e-08	5' UTR/ Intron	Patient

cg23714751	MKL1	regulating smooth muscle cell differentiation; brain development; neuronal survival; Alzheimer's disease; schizophrenia; skeletal myogenic differentiation; growth	2·37827e-08	Intron	Control
cg14071298			2·54664e-08	Intergenic	Patient
cg07375256	ZSCAN25	transcriptional regulation (DNA binding and protein-protein interactions); genetic variation in ZSCAN25 has been associated with body weight, hip and brachial circumference.	3·0847e-08	Intron	Patient
cg00490406	AIM2	innate immune response; neuronal morphology regulation; behaviour, auditory fear memory and locomotor activity; neurological/psychiatric diseases	3·72256e-08	Promoter	Control
cg20944115			4·91057e-08	Intergenic	Control
cg21695395	ETS1	transcriptional regulation of numerous genes; stem cell development; cell senescence and death; tumorigenesis; angiogenesis	4·95399e-08	Intron	Patient
cg05304729	MNDA	transcriptional regulation in the myeloid lineage	5·5916e-08	Promoter	Patient
cg25808826			5·92518e-08	Intergenic	Control
cg21329012	MAP3K2	MAP kinase and NF-kappaB signalling pathways; BDNF functioning in central nervous system neurons; bone formation; myogenesis	5·99492e-08	Intron	Control
cg01842756	RNF217	apoptosis signalling	6·05342e-08	Intron/ Non-coding	Control
cg24451839			8·55229e-08	Intergenic	Control
cg15296538	AACSP1	pseudogene	8·99578e-08	Non-coding	Control
cg10866825			1·00012e-07	Intergenic	Control
cg03116607			1·06136e-07	Intergenic	Control
cg00182635			1·09129e-07	Intergenic	Control
cg22076676	THADA	apoptosis; adaptive thermogenesis; homeostasis; neuroinflammation and multiple sclerosis	1·15757e-07	Intron/ Non-coding	Control
cg11047783	KAT6B	transcriptional regulation; cerebral cortex development; cognition; ADHD; intellectual disability; microcephaly and growth regulation	1·22556e-07	Intron	Control
cg10322118	B4GALT1-AS1	non-coding RNA	1·29633e-07	Non-coding	Patient
cg01095594	LOC101927043	non-coding RNA	1·37489e-07	Non-coding	Control
cg03778523			1·41884e-07	Intergenic	Control
cg18978661	LINC01599	non-coding RNA	1·42119e-07	Non-coding	Control
cg18012268			1·44128e-07	Intergenic	Control
cg12928479	NLRC5	regulating the NF-kappa-B and type I interferon signalling pathways; immunity; neuroimmune and neuroinflammatory processes	1·46443e-07	Exon	Control
cg03083216	XRCC4	DNA repair; physical development; central nervous system development	1·53098e-07	Intron	Control
cg05706766			1·82628e-07	Intergenic	Control
cg01912915			2·18924e-07	Intergenic	Control
cg10207609	CD36	regulating many signalling processes; fatty acid transport; brain lipid sensing and nervous control of energy balance;	2·51116e-07	5' UTR/ Promoter	Control

		behaviour, spatial learning and memory; neuropsychiatric and neurodegenerative disorders			
cg17022038	MAST3	unknown; microtubule-associated serine/threonine-protein kinase gene-3	2·60968e-07	Exon	Patient
cg01891736	LITAF	regulating TNF-alpha expression; endosomal protein trafficking, targeting proteins for lysosomal degradation; Charcot Marie Tooth disease type 1C peripheral neuropathy	2·74131e-07	5' UTR/ Non-coding	Patient
cg23104428	TTC7A	regulation of phosphatidylinositol 4-phosphate synthesis; targeting PI4kinase to plasmamembrane; intestinal development	2·84889e-07	Intron	Control
cg05371584	TBC1D8	unknown (putative role in GTPase-activation of Rab family proteins)	2·90514e-07	Intron/ Non-coding	Control
cg09396032	GSE1	oncogene	2·90638e-07	5' UTR/ Intron	Patient
cg26131710	GRHL1	transcriptional regulation during epithelial development; fetal growth restriction	3·05169e-07	Intron	Control
cg05923857	TCF7L2	transcriptional regulation in the Wnt signalling pathway; blood glucose homeostasis; neurodevelopment and plasticity of mature neurons; memory, visual-motor/fine-motor function; spatial learning, emotional functioning; behaviour; speech; schizophrenia; ADHD; anxiety and bipolar disorders	3·2048e-07	Exon	Control
cg05112114	INO80	transcriptional regulation; DNA replication and repair; microcephaly; developmental delay; weight regulation	3·39423e-07	Intron/ Non-coding	Control
cg04500577	INPP4B	regulating phosphatidylinositol signalling pathways; callosal axon formation; cortical development; regulating nerve conduction velocity; cognition	3·60272e-07	5' UTR	Patient
cg06827976	FGR	regulating immune responses; cell migration; cytoskeleton reorganisation	3·71808e-07	5' UTR	Patient
cg15209896	STPG1	apoptosis	4·33224e-07	5' UTR	Control
cg00687889			4·61645e-07	Intergenic	Control
cg06636137	PHF10	proliferation of neural progenitors; dendrite growth	5·40824e-07	Intron	Control
cg13140887			6·00644e-07	Intergenic	Control
cg07078732			6·59023e-07	Intergenic	Control
cg08965078			7·55456e-07	Intergenic	Control
cg23313725			8·06001e-07	Intergenic	Control
cg24785495			9·40639e-07	Intergenic	Control
cg25026693	UBASH3B	promoting accumulation of activated target receptors, such as T-cell receptors and EGFR, on the cell surface	9·45502e-07	Intron	Control
cg19323261			9·5725e-07	Intergenic	Control
cg21336878	LYAR	ribosomal RNA processing; transcription regulation	9·96407e-07	5' UTR	Control
cg25300481			1·01322e-06	Intergenic	Control
cg10507267			1·14643e-06	Intergenic	Control
cg15809077			1·25402e-06	Intergenic	Control
cg23897894			1·2621e-06	Intergenic	Control
cg23391288			1·28174e-06	Intergenic	Control

cg11320225			1·5591e-06	Intergenic	Control
cg22535104	HIP1	clathrin-mediated endocytosis and trafficking; regulating AMPA receptor trafficking in the central nervous system; regulating presynaptic nerve terminal activity	1·5757e-06	Intron	Control
cg08285151	HDAC9	histone deacetylation; inhibiting skeletal myogenesis; involved in heart development; protecting neurons from apoptosis	1·66871e-06	Intron/ 5'UTR/ Promoter	Control
cg00507757	CS	catalyzing the synthesis of citrate from oxaloacetate and acetyl coenzyme A; neurological/ neurodegenerative diseases; autism spectrum and bipolar disorders; social function and behaviour	1·7824e-06	Intron	Patient
cg04339882	CROCC	ciliary rootlet formation; sensory neuron function	1·91119e-06	Intron	Control
cg17134427	ZFR	RNA binding protein involved in nucleocytoplasmic shuttling in neurons; post-implantation and gastrulation stages of development; pancreatic cancer; highly expressed in brain	2·03974e-06	Exon/Non-coding	Control
cg27518631			2·05884e-06	Intergenic	Control
cg06297318			2·06403e-06	Intergenic	Control
cg26760894	TRAF3IP2-AS1	long non-coding RNA; cocaine abuse; bipolar disease	2·26946e-06	Non-coding	Patient
cg26082814	LOC101928841	unknown	2·30918e-06	5' UTR	Control
cg04689867	ADGRE1	cell adhesion; cell-cell interactions in the immune system	2·36198e-06	Promoter	Control
cg25699964			2·47585e-06	Intergenic	Control
cg20093635	COL15A1	stabilising microvessels and muscle cells; peripheral nerve maturation; sensory nerve conduction; neuromuscular development	2·48466e-06	Intron	Control
cg10403028	TEX41	non-coding RNA	2·50114e-06	Non-coding	Control
cg06826494	SVIL	myogenesis (myogenic membrane structure and differentiation); cell migration	2·59468e-06	Exon	Control
cg23053742	LACTB2-AS1	non-coding RNA	2·74109e-06	Non-coding	Patient
cg12274883			2·78855e-06	Intergenic	Control
cg05889889	RTTN	axial rotation, left-right organ specification; differentiation of the neural tube, and looping of the heart tube during development; maintenance of normal ciliary structure	2·8705e-06	Intron	Control
cg20132375			3·01235e-06	Intergenic	Control
cg14141074	TBC1D1	cell cycle and differentiation of various tissues; insulin-stimulated glucose uptake into cells (trafficking of GLUT-4)	3·17527e-06	Promoter/ Intron	Patient
cg14748515			3·23217e-06	Intergenic	Control
cg14800111			3·29157e-06	Intergenic	Control
cg04193065			3·37213e-06	Intergenic	Control
cg07584558	DEDD2	receptor-induced apoptosis	3·60917e-06	3' UTR/ Non-coding	Patient
cg10862350	PDE4B	signal transduction processes a.o. in central nervous system; schizophrenia; bipolar disorder	3·87418e-06	Intron	Control
cg01858828			3·9731e-06	Intergenic	Control

cg24475272	SETD7	histone methylation, with impact on transcriptional activation of genes such as collagenase or insulin	4·14626e-06	3' UTR/ Intron	Control
cg17804886	RASA3	negatively regulating the Ras signalling pathway	4·18977e-06	5' UTR/ Intron	Patient
cg06329036	SORCS2	expression of BDNF (brain-derived neurotrophic factor, regulator of structural, synaptic and morphological plasticity of the brain); high expression in central neurvous system; bipolar disorder, schizophrenia; Alzheimer's disease	4·43609e-06	Intron	Control
cg11919725			4·78016e-06	Intergenic	Patient
cg20744727	CD93	intercellular adhesion and clearance of apoptotic cells; phagocytic capacity of activated microglia, associated with neurodegeneration	4·82546e-06	Promoter	Control
cg22645359	TCF7L2	transcriptional regulation in the Wnt signalling pathway; blood glucose homeostasis; related to neurodevelopment and plasticity of mature neurons; memory, visual motor/fine motor function, emotional functioning, behaviour and speech; schizophrenia; ADHD	5·0259e-06	3' UTR/ Intron	Control
cg05038391			5·205e-06	Intergenic	Control
cg27215601	NSMCE2	nuclear transport, transcription, chromosome segregation and DNA repair; key role in genome maintenance, suppression of mitotic recombination; dwarfism; bipolar disorder	5·28548e-06	Intron/ Non-coding	Patient
cg06975311	SIPA1	regulating the Ras signalling pathway; cell cycle progression	5·45604e-06	Promoter	Patient
cg02435538	RHBDD2	unknown (putative involvement in intramembrane proteolysis); highly expressed in brain	5·46515e-06	Promoter	Control
cg23084667	GRAP2	leukocyte-specific protein-tyrosine kinase signaling; RET signalling which is involved in brain development and maturation of dopaminergic neurons	5·49153e-06	5' UTR	Patient
cg16029189			5·51594e-06	Intergenic	Control
cg27229520			5·54735e-06	Intergenic	Control
cg03417712	SARDH	sarcosine degradation	6·03672e-06	Intron	Control
cg09194657	TUBGCP2	microtubule nucleation at the centrosome	6·69048e-06	Intron/ Non-coding	Control
cg02293222			7·31748e-06	Intergenic	Control
cg18685299			8·78038e-06	Intergenic	Control
cg24533408			9·66289e-06	Intergenic	Control
cg19746667			1·00928e-05	Intergenic	Control
cg06637027	CD86	T-lymphocyte proliferation and interleukin-2 production; microglia activation; cognitive/ neurodegenerative disturbances	1·02439e-05	Promoter	Control
cg17636223			1·16598e-05	Intergenic	Control
cg26685998			1·20457e-05	Intergenic	Control
cg11520439	NIPSNAP3B	vesicular trafficking	1·2255e-05	3' UTR/ Non-coding	Control
cg06572103	TNFSF11	osteoclast differentiation and activation (and bone loss); T-cell proliferation and T-cell-dependent immune respons; apoptosis; skeletal pathologies: osteogenesis imperfecta, osteolysis in	1·2438e-05	5' UTR/ Intron	Patient

		diabetic neuropathy, bone cancer pain, Charchot Marie Tooth disease and other metabolic bone disorders			
cg22180675	MIR486-1	miRNA; skeletal muscle development	1·28525e-05	3' UTR/ Intron	Control
cg25141485			1·31228e-05	Intergenic	Patient
cg23057326	HIVEP3	transcriptional regulation; cell progression and differentiation; regulating bone formation; cognitive function; pain modulation	1·40672e-05	5' UTR	Control
cg16513984	TNFRSF1B	regulating anti-apoptotic pathways; protecting neurons from apoptosis by stimulating anti-oxidative pathways	1·4192e-05	Intron	Patient
cg22979802	CLNK	regulating immunoreceptor signalling	1·46218e-05	Intron	Control
cg15507942	CD36	regulating many signalling processes; fatty acid transport; brain lipid sensing and nervous control of energy balance; behaviour, spatial learning and memory; neuropsychiatric and neurodegenerative disorders	1·4825e-05	Promoter/5' UTR	Control
cg25551043	MYOF	plasmalemma repair mechanism of endothelial cells that permits rapid resealing of membranes disrupted by mechanical stress; endocytic recycling; Duchenne muscular dystrophy	1·53994e-05	Intron	Control
cg24874433	LINC00854	non-coding RNA	1·54394e-05	Non-coding	Patient
cg08822136			1·57673e-05	Intergenic	Patient
cg04588138			1·64447e-05	Intergenic	Control
cg10840227	TPP2	proteolysis; removing tripeptides from longer peptides	1·6673e-05	Intron	Patient
cg08556511	YAP1	regulation of the Hippo signalling pathway which is involved in development, growth, repair and homeostasis; progression of multiple cancers, including glioblastoma; brain development; Huntington disease	1·68105e-05	Intron	Control
cg20700099	DPP8	T-cell activation and immune function (possibly, based on similarities with DPP4); ischemia-triggered inflammation and neurodegeneration	1·76243e-05	Intron/ Non-coding	Control
cg09021674	EIF3E	initiation of protein synthesis; growth; intellectual ability; autism-spectrum and neurodegenerative disorders	1·9893e-05	Intron	Patient

All differentially methylated genes identified based on a false discovery rate <0·05 are shown and are ranked from the most to the least significantly affected CpG-sites. DNA methylation status of the CpG-sites in grey was significantly affected by Early-PN.

Source of gene-related protein function: Entrez Gene Summary at Pubmed.com and UniProt database (www.uniprot.org), complemented with updated literature searches for links with brain and development.

Abbreviations: AACSP1, Acetoacetyl-CoA Synthetase Pseudogene 1; AAK1, Adaptor-related protein complex 2 Associated Kinase 1; ADGRE1, Adhesion G Protein-Coupled Receptor E1; ADHD: attention deficit hyperactivity disorder; AIM2, Absent In Melanoma 2; ATAD2B, ATPase Family AAA Domain Containing 2B; B4GALT1-AS1, Beta-1,4-galactosyltransferase 1 Antisense ribonucleic acid 1; CD36, Cluster of Differentiation 36; CD86, Cluster of Differentiation 86; CD93, Cluster of Differentiation 93; CENPA, Centromere protein A; CEP85L, Centrosomal Protein 85 Like; CHST11, Carbohydrate Sulfotransferase 11; CLNK, Cytokine Dependent Hematopoietic Cell Linker; COL15A1, Collagen Type XV Alpha 1 Chain; CROCC, Ciliary Rootlet Coiled-Coil; CS, Citrate Synthase; CUL3, Cullin 3; DBNL, Drebrin Like; DEDD2, Death Effector Domain Containing 2; DPP8, Dipeptidyl Peptidase 8; EIF3E, Eukaryotic Translation Initiation Factor 3 Subunit E; ETS1, E26 Transformation-Specific Proto-Oncogene 1; FGR, Feline Gardner-Rasheed Sarcoma Proto-Oncogene; FNBP1, Formin Binding Protein 1; GPER1, G Protein-Coupled Oestrogen Receptor 1; GRAP2, Growth factor Receptor-bound Protein 2-Related Adaptor Protein 2; GRHL1, Grainyhead Like Transcription Factor 1; GSE1, Genetic Suppressor Element 1; HDAC9, Histone Deacetylase 9; HIP1, Huntingtin Interacting Protein 1; HIVEP3, Human Immunodeficiency

Virus Type I enhancer binding protein 3; HSD11B1, 11-Beta-Hydroxysteroid Dehydrogenase 1; IGFBP7, Insulin-like Growth Factor Binding Protein 7; IL19, Interleukin 19; INO80, inositol auxotroph 80; INPP4B, Inositol Polyphosphate-4-Phosphatase Type II B; KAT6B, Lysine Acetyltransferase 6B; KAZN, Kazrin; KIAA0319L, KIAA0319 Like; LACTB2-AS1, Lactamase Beta 2 Antisense ribonucleic acid 1; LINC00854, Long Intergenic Non-Protein Coding RNA 854; LINC01599, Long Intergenic Non-Protein Coding RNA 1599; LITAF, Lipopolysaccharide Induced TNF Factor; LOC101927043, Collagen Alpha-1(II) Chain-Like 101927043; LOC101928841, Collagen Alpha-1(II) Chain-Like 101928841; LYAR, Ly1 Antibody Reactive; LYST, Lysosomal Trafficking Regulator; MAP3K2, Mitogen-Activated Protein 3 Kinase 2; MAST3, Microtubule Associated Serine/Threonine Kinase 3; METTL16, Methyltransferase Like 16; MIR486-1, MicroRNA 486-1; MKL1, Megakaryoblastic Leukaemia (Translocation) 1; MNDA, Myeloid Cell Nuclear Differentiation Antigen; MS4A6A, Membrane Spanning 4-Domains A6A; MYOF, Myoferlin; NIPSNAP3B, Nipsnap Homolog 3B; NLRC5, NLR Family CARD Domain Containing 5; NSMCE2, Non-SMC Element 2 Methyl Methanesulfonate Sensitivity Gene 21 Homolog; PDE4B, Phosphodiesterase 4B; PHF10, PHD Finger Protein 10; PICU, paediatric intensive care unit; PKM, Pyruvate Kinase Muscle; PLA2G15, Phospholipase A2 Group XV; PLD3, Phospholipase D Family Member 3; PRKAG2, Protein Kinase AMP-Activated Non-Catalytic Subunit Gamma 2; PRKCA, Protein Kinase C Alpha; RAB11FIP4, RAB11 Family Interacting Protein 4; RABGAP1L, RAB GTPase Activating Protein 1 Like; RASA3, RAS P21 Protein Activator 3; RHBDD2, Rhomboid Domain Containing 2; RIN3, Ras And Rab Interactor 3; RNF217, Ring Finger Protein 217; RTTN, Rotatin; SARDH, Sarcosine Dehydrogenase; SCN11A, Sodium Voltage-Gated Channel Alpha Subunit 11; SETD7, SET Domain Containing Lysine Methyltransferase 7; SIPA1, Signal-Induced Proliferation-Associated 1; SLC35E1, Solute Carrier Family 35 Member E1; SORCS2, Sortilin Related VPS10 Domain Containing Receptor 2; SRGAP1, SLIT-ROBO Rho GTPase Activating Protein 1; STPG1, Sperm Tail PG-Rich Repeat Containing 1; SVIL, Supervillin; TBC1D1, TBC1 Domain Family Member 1; TBC1D8, TBC1 Domain Family Member 8; TCF7L2, Transcription Factor 7 Like 2; TEX41, Testis Expressed 41; THADA, Thyroid Adenoma-Associated Protein; TNFRSF1B, TNF Receptor Superfamily Member 1B; TNFSF11, TNF Superfamily Member 11; TPP2, Tripeptidyl Peptidase 2; TPST1, Tyrosylprotein Sulfotransferase 1; TRAF3IP2-AS1, TRAF3IP2 Antisense RNA 1; TTC7A, Tetratricopeptide Repeat Domain 7A; TUBGCP2, Tubulin Gamma Complex Associated Protein 2; UBASH3B, Ubiquitin Associated And SH3 Domain Containing B; UBP1, Upstream Binding Protein 1; XRCC4, X-Ray Repair Cross Complementing 4; YAP1, Yes Associated Protein 1; ZFR, Zinc Finger Ribonucleic acid binding protein; ZSCAN25, Zinc Finger And SCAN Domain Containing 25.

Table S2. Impact of early-PN, versus late-PN, on DNA methylation, adjusted for baseline risk factors

CpG-site	Percent of bootstrap replicas for which Early-PN versus Late-PN was independently associated with DNA methylation (p-value ≤ 0·05)	p-value median [95% CI][a]
cg27215601	99	0·0003 [0·0001 ; 0·0007]
cg11919725	98	0·0001 [0·00007 ; 0·0001]
cg17533201	94	0·00062 [0·0002 ; 0·001]
cg23084667	93	0·00108 [0·0006 ; 0·002]
cg06449934	82	0·00318 [0·0008 ; 0·005]
cg14071298	81	0·00596 [0·003 ; 0·01]
cg14364797	80	0·00488 [0·002 ; 0·008]
cg14450616	80	0·00309 [0·001 ; 0·007]
cg26308668	78	0·00615 [0·003 ; 0·01]
cg17804886	77	0·00695 [0·002 ; 0·02]
cg05038391	76	0·00550 [0·001 ; 0·01]
cg24475272	74	0·00861 [0·002 ; 0·01]
cg16301196	73	0·00488 [0·001 ; 0·01]
cg00687889	71	0·00755 [0·003 ; 0·01]
cg10422093	66	0·02 [0·01 ; 0·03]
cg10732094	65	0·01 [0·007 ; 0·03]
cg14109551	64	0·01 [0·01 ; 0·03]
cg05174290	64	0·01 [0·009 ; 0·04]
cg26683792	63	0·02 [0·01 ; 0·04]
cg23053742	63	0·02 [0·01 ; 0·04]
cg14800111	63	0·01 [0·002 ; 0·02]
cg22645359	60	0·03 [0·01 ; 0·04]
cg14172797	58	0·03 [0·01 ; 0·06]
cg11047783	57	0·02 [0·01 ; 0·05]
cg14748515	57	0·03 [0·01 ; 0·06]
cg17522929	56	0·03 [0·02 ; 0·06]
cg12928479	56	0·03 [0·01 ; 0·07]
cg08948258	55	0·03 [0·01 ; 0·06]
cg04483721	55	0·03 [0·02 ; 0·08]
cg01842756	54	0·03 [0·02 ; 0·06]
cg22076676	54	0·03 [0·02 ; 0·09]
cg04193065	54	0·03 [0·02 ; 0·08]
cg07375256	53	0·03 [0·01 ; 0·06]
cg05371584	52	0·04 [0·03 ; 0·08]
cg02918489	51	0·04 [0·02 ; 0·06]
cg12274883	51	0·04 [0·02 ; 0·08]
cg02293222	50	0·05 [0·02 ; 0·07]

[a] 95% CI for the median computed according to BCa method[36]

Table S3. Impact of early-PN, versus late-PN, on DNA methylation, adjusted for baseline risk factors and for incidence of new infection, duration of PICU-stay, duration of mechanical ventilatory support and hypoglycaemia

CpG-site	Percent of bootstrap replicas for which Early-PN versus Late-PN was independently associated with DNA methylation (p-value ≤ 0·05)	p-value median [95% CI][a]
cg11919725	97	0·0001 [0·00006 ; 0·0005]
cg17533201	95	0·0004 [0·0001 ; 0·001]
cg27215601	92	0·001 [0·0004 ; 0·002]
cg14450616	91	0·002 [0·001 ; 0·005]
cg23084667	91	0·0007 [0·0003 ; 0·001]
cg06449934	90	0·0005 [0·0002 ; 0·001]
cg24475272	88	0·002 [0·001 ; 0·004]
cg14109551	75	0·01 [0·006 ; 0·02]
cg16301196	75	0·009 [0·004 ; 0·01]
cg14071298	75	0·009 [0·003 ; 0·01]
cg26308668	74	0·009 [0·004 ; 0·01]
cg05174290	73	0·01 [0·007 ; 0·02]
cg00687889	72	0·01 [0·004 ; 0·02]
cg14364797	70	0·009 [0·004 ; 0·02]
cg14800111	68	0·01 [0·007 ; 0·02]
cg22645359	68	0·01 [0·009 ; 0·03]
cg05038391	67	0·008 [0·004 ; 0·01]
cg10732094	66	0·01 [0·004 ; 0·02]
cg26683792	66	0·01 [0·005 ; 0·02]
cg17804886	66	0·01 [0·006 ; 0·02]
cg11047783	65	0·02 [0·008 ; 0·03]
cg10422093	64	0·02 [0·01 ; 0·03]
cg14172797	64	0·03 [0·01 ; 0·03]
cg23053742	64	0·02 [0·01 ; 0·03]
cg22076676	63	0·02 [0·007 ; 0·04]
cg17522929	58	0·02 [0·01 ; 0·05]
cg12274883	57	0·03 [0·01 ; 0·05]
cg02293222	57	0·02 [0·01 ; 0·05]
cg12928479	56	0·03 [0·01 ; 0·06]
cg04483721	53	0·04 [0·02 ; 0·06]
cg01842756	53	0·03 [0·02 ; 0·06]
cg14748515	53	0·04 [0·02 ; 0·08]
cg02918489	50	0·04 [0·03 ; 0·07]
cg04193065	49	0·05 [0·02 ; 0·11]
cg05371584	47	0·05 [0·03 ; 0·10]
cg08948258	46	0·05 [0·02 ; 0·08]
cg07375256	43	0·08 [0·03 ; 0·15]

[a] 95% CI for the median computed according to BCa method[36]

Table S4. Added explanatory power provided by the differentially methylated CpG-sites in predicting the long-term outcomes

	Linear		Non-Linear	
Outcome assessed at 2-years follow-up	**R^2 [a]**	**R^2 fold-increase[b]**	**R^2 [c]**	**R^2 fold-increase[d]**
Anthropometric: Height	0·755	1·728	0·840	1·423
Anthropometric: Weight	0·657	2·105	0·777	1·551
Anthropometric: Head circumference	0·487	1·477	0·716	1·456
Clinical neurological evaluation score	0·303	1·298	0·584	1·238
Intelligence: Verbal IQ	0·230	1·326	0·647	1·904
Intelligence: Performance IQ	0·222	1·446	0·630	1·919
Intelligence: Total IQ	0·264	1·352	0·643	1·865
Visual-motor integration	0·309	1·273	0·637	1·559
Alertness: Reaction time right hand	0·286	7·815	0·652	2·040
Alertness: Reaction time left hand	0·308	6·667	0·629	2·034
Alertness: Reaction time right hand within person SD of repeated tests	0·230	12·938	0·632	2·409
Alertness: Reaction time left hand within person SD of repeated tests	0·291	6·094	0·631	2·011
Motor coordination: Number of left hand taps	0·339	5·656	0·671	1·804
Motor coordination: Number of right hand taps	0·344	5·789	0·663	1·876
Motor coordination: Number of valid alternating taps	0·114	4·523	0·599	1·880
Motor coordination: Number of valid synchronous taps	0·276	4·635	0·650	1·934
Inhibition and flexibility: Difference in reaction time (inhibition)	0·173	7·066	0·627	2·564
Inhibition and flexibility: Difference in number of errors (inhibition)	0·064	1·798	0·567	1·798
Inhibition and flexibility: Difference in reaction time (flexibility)	0·109	2·425	0·606	2·467
Inhibition and flexibility: Difference in number of errors (inhibition)	0·042	2·382	0·585	2·007
Memory: Verbal-auditory, numbers memory span	0·185	2·626	0·606	2·054
Memory: Verbal-auditory, numbers working memory	0·157	1·612	0·592	1·619
Memory: Verbal-auditory, word pairs learning	0·097	2·663	0·610	1·856
Memory: Verbal-auditory, word pairs immediate memory	0·158	1·672	0·604	1·814
Memory: Verbal-auditory, word pairs delayed memory	0·113	2·357	0·591	1·883
Memory: Verbal-auditory, word pairs recognition	0·123	3·454	0·568	1·906
Memory: Non-verbal, visual-spatial, pictures	0·182	3·228	0·596	1·822
Memory: Non-verbal, visual-spatial, dots learning	0·118	2·655	0·603	1·777
Memory: Non-verbal, visual-spatial, dots immediate memory	0·085	12·005	0·574	2·158
Memory: Non-verbal, visual-spatial, dots delayed memory	0·081	31·058	0·572	1·859
Memory: Learning index	0·085	2·646	0·591	1·685

Table S4. Added explanatory power provided by the differentially methylated CpG-sites in predicting the long-term outcomes (continued)

	Linear		Non-Linear	
Outcome assessed at 2-years follow-up	**R^2 [a]**	**R^2 fold-increase[b]**	**R^2 [c]**	**R^2 fold-increase[d]**
Emotional/Behavioural: Internalising problems as reported by parents/caregivers	0·143	1·989	0·623	1·858
Emotional/Behavioural: Externalising problems as reported by parents/caregivers	0·103	2·415	0·617	2·030
Emotional/Behavioural: Overall problems as reported by parents/caregivers	0·117	2·111	0·620	1·954
Executive functioning: Inhibition as reported by parents/caregivers	0·086	3·110	0·604	1·962
Executive functioning: Flexibility as reported by parents/caregivers	0·092	1·536	0·612	1·978
Executive functioning: Emotional control as reported by parents/caregivers	0·089	1·975	0·600	1·973
Executive functioning: Working memory as reported by parents/caregivers	0·095	1·786	0·602	2·040
Executive functioning: Planning and organisation as reported by parents/caregivers	0·082	3·000	0·607	2·174
Executive functioning: Meta-cognition index as reported by parents/caregivers	0·082	2·394	0·605	2·082
Executive functioning: Overall as reported by parents/caregivers	0·099	1·708	0·607	1·892

	Linear		Non-Linear	
	R^2 [a]	**R^2 fold-increase[b]**	**R^2 [c]**	**R^2 fold-increase[d]**
Aggregate outcomes [e]				
Anthropometric	0·633 ± 0·135	1·770 ± 0·315	0·778 ± 0·036	1·447 ± 0·038
Intelligence	0·239 ± 0·023	1·371 ± 0·057	0·640 ± 0·005	1·896 ± 0·016
Alertness	0·279 ± 0·034	8·379 ± 3·122	0·636 ± 0·006	2·123 ± 0·096
Motor coordination	0·268 ± 0·108	5·151 ± 0·664	0·646 ± 0·016	1·873 ± 0·026
Inhibition and flexibility	0·097 ± 0·058	3·418 ± 2·448	0·596 ± 0·014	2·207 ± 0·182
Memory	0·126 ± 0·040	5·998 ± 8·800	0·591 ± 0·003	1·858 ± 0·045
Emotional / Behavioural	0·121 ± 0·021	2·172 ± 0·220	0·621 ± 0·002	1·947 ± 0·050
Executive Functioning	0·089 ± 0·005	2·216 ± 0·632	0·605 ± 0·003	2·014 ± 0·034
Overall[e]	0·199 ± 0·154	4·092 ± 5·090	0·624 ± 0·006	1·905 ± 0·038

[a] Optimism-corrected R^2 of multivariable linear regression models for outcomes assessed at 2-years follow-up including 159 differentially methylated CpG-sites and baseline risk factors.
[b] Optimism-corrected R^2 fold-increase with respect to multivariable linear regression models including baseline risk factors only.
[c] Optimism-corrected R^2 of multivariable non-linear random forest regression models for outcomes assessed at 2-years follow-up including 159 differentially methylated CpG-sites and baseline risk factors.
[d] Optimism-corrected R^2 fold-increase with respect to multivariable non-linear random forest regression models including baseline risk factors only.
All linear models resulted from stepwise variable selection according to Rubin's method (Methods S7).[30,31] All models were evaluated in 100 bootstrapped replicates. Baseline risk factors included age, centre, race,[26] gender, geographic origin,[26] language, history of malignancy, diabetes, a predefined "syndrome" (Methods S5), diagnosis and severity of illness (PIM3 and PeLOD),[27,28] and risk of malnutrition (STRONGkids)].[29] R^2 for non-linear models computed according to Rubin's rules.[37]
[e] Aggregate outcomes reported as mean ± standard error of the mean (SD).
Abbreviations: PN, parenteral nutrition; IQ, intelligence quotient; SD, standard deviation.

Table S5. Classification of CpG-sites according to function

Cerebral/Neuronal	cg22645359	**Intra-intercellular Signalling/Transport**	cg05706766
cg00490406	cg23057326		cg06297318
cg00507757	cg23084667	cg05371584	cg07078732
cg00771778	cg23714751	cg06975311	cg08822136
cg01474011	cg26179530	cg11520439	cg08948258
cg01860912	cg26308668	cg14141074	cg08965078
cg02435538	cg26760894	cg17533201	cg10422093
cg03083216	cg27215601	cg17804886	cg10507267
cg03958078	**Peripheral neuronal**	cg23104428	cg10732094
cg04500577	cg01891736	cg25026693	cg10866825
cg05112114	cg02244028	cg26434487	cg11320225
cg05174290	cg04339882	cg26683792	cg11919725
cg05889889	cg14772955	**Non-coding RNA/ Pseudogene**	cg12274883
cg05923857	cg20093635		cg13140887
cg06329036	**Growth/Development/ Locomotion**	cg01095594	cg14071298
cg06449934		cg10322118	cg14116399
cg06636137	cg06572103	cg10403028	cg14748515
cg06637027	cg06826494	cg15296538	cg14800111
cg07170641	cg07375256	cg18978661	cg15809077
cg08285151	cg22180675	cg23053742	cg15848350
cg08442088	cg25551043	cg24874433	cg16029189
cg08556511	cg26131710	cg26082814	cg17636223
cg09021674	**Metabolism**	**Oncogenic/ Apoptosis**	cg18012268
cg09366519	cg03417712		cg18674312
cg10170678	cg10840227	cg01842756	cg18685299
cg10207609	cg15453708	cg07584558	cg19323261
cg10480329	cg16301196	cg09396032	cg19746667
cg10862350	cg17022038	cg15209896	cg20132375
cg11047783	cg23668476	**Intergenic**	cg20944115
cg11180972	**Gene expression/DNA regulation/ Epigenetic regulation**	cg00025197	cg23313725
cg12928479		cg00182635	cg23391288
cg14109551	cg05304729	cg00687889	cg23897894
cg14172797	cg08097676	cg00995147	cg24451839
cg14364797	cg09194657	cg01858828	cg24533408
cg14450616	cg16651946	cg01912915	cg24785495
cg15507942	cg21336878	cg02293222	cg24954967
cg16513984	cg21695395	cg02918489	cg25141485
cg17134427	cg24475272	cg03116607	cg25300481
cg17522929	**Inflammation/Immune system**	cg03778523	cg25699964
cg20700099	cg04689867	cg04193065	cg25808826
cg20744727	cg06827976	cg04483721	cg26685998
cg21329012	cg22979802	cg04588138	cg27229520
cg22076676	**Cell structure**	cg04837642	cg27518631
cg22535104	cg00101629	cg05038391	

Table S6. Added explanatory power provided by the CpG-sites differentially methylated by early-PN in predicting the neurocognitive outcomes adversely affected by early-PN

	Linear		Non-Linear	
Outcome assessed at 2-years follow-up	**R^2 [a]**	**Fold-increase in R^2 [b]**	**R^2 [c]**	**Fold-increase in R^2 [d]**
Executive functioning: Inhibition as reported by parents/caregivers	0·045	1·481	0·603	1·996
Visual-motor integration	0·267	1·082	0·637	1·527
Emotional/Behavioural: Externalising problems as reported by parents/caregivers	0·053	1·169	0·606	1·994
Executive functioning: Working memory as reported by parents/caregivers	0·064	1·185	0·606	2·053
Executive functioning: Meta-cognition index as reported by parents/caregivers	0·049	1·404	0·605	2·116
Executive functioning: Overall as reported by parents/caregivers	0·065	1·111	0·609	1·930
Aggregate outcomes [e]				
Overall	0·091 ± 0·086	1·239± 0·164	0·611 ± 0·005	1·936± 0·086

[a] Optimism-corrected R^2 of multivariable linear regression models for outcomes assessed at 2-years follow-up including 37 differentially methylated CpG-sites and baseline risk factors.
[b] Optimism-corrected R^2 fold-increase with respect to multivariable linear regression models including baseline risk factors only.
[c] Optimism-corrected R^2 of multivariable non-linear random forest regression models for outcomes assessed at 2-years follow-up including 37 differentially methylated CpG-sites and baseline risk factors.
[d] Optimism-corrected R^2 fold-increase with respect to multivariable non-linear random forest regression models including baseline risk factors only.
All linear models resulted from stepwise variable selection according to Rubin's method (Methods S7).[30,31] All models were evaluated in 100 bootstrapped replicates. Baseline risk factors included age, centre, race,[26] gender, geographic origin,[26] language, history of malignancy, diabetes, a predefined "syndrome" (Methods S5), diagnosis and severity of illness (PIM3 and PeLOD),[27,28] and risk of malnutrition (STRONGkids)].[29] R^2 for non-linear models computed according to Rubin's rules.[37]
[e] Aggregate outcomes reported as mean ± standard error of the mean (SD).
Abbreviations: PN, parenteral nutrition.

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