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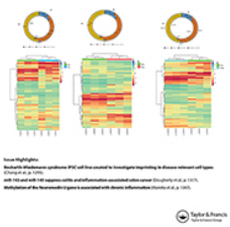

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RESEARCH PAPER

NMU DNA methylation in blood is associated with metabolic and inflammatory indices: results from the Moli-sani study

Annalisa Marotta [a], Fabrizia Noro [a], Roberta Parisi [a], Alessandro Gialluisi [a], Alfonsina Tirozzi [a], Amalia De Curtis [a], Simona Costanzo [a], Augusto Di Castelnuovo [b], Chiara Cerletti [a], Maria Benedetta Donati [a], Giovanni de Gaetano [a], Licia Iacoviello [a,c], Benedetta Izzi [a,*], and Francesco Gianfagna [b,c,*] on behalf of the Moli-sani study Investigators.[b,c]

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ABSTRACT

Neuromedin U (NMU) is a neuropeptide involved in gut-brain axis, energy balance and immune response. We aimed at analysing the association between *NMU* epigenetic variability and metabolic indices and the potential mediating role of low-grade inflammation in a general population of Italian adults.

NMU Blood DNA methylation levels at two CpG islands (*NMU76* and *NMU32*) were analysed using pyrosequencing in a randomly selected sub-cohort of 1,160 subjects from the Moli-sani study (≥35years; 49.20% men). Multivariable regressions adjusted for age, sex, smoking, alcohol and vegetable consumption were performed to estimate the associations between methylation and metabolic phenotypes (BMI, waist-to-hip ratio, blood pressure, glucose, HOMA-IR, lipids, lipoprotein(a) and apolipoproteins). Mediation analysis was performed to identify the influence of low-grade inflammation in the association using a composite index based on C reactive protein, granulocyte-to-lymphocyte ratio (GLR), platelet and white blood cell counts (INFLA-score).

Using principal component analysis four methylation factors were identified: *NMU76-F1, NMU76-F2, NMU32-F1* and *NMU32-F2*. *NMU76-F1* was FDR significantly associated with total cholesterol (for 1 SD increase: β = 4.5 ± 1.4 mg/dL of, R^2 = 10.8%, p = 0.001), ApoB (0.03 ± 0.01 g/L, 12.2%, p = 0.0004), with INFLA-score (1.05 ± 0.22, *p* = 2.7E-6) and GLR (-0.27 ± 0.03, 30.4%, *p* = 1.3E-20). GLR and lymphocyte numbers mediate the association of *NMU76-F1* with cholesterol (24.0% of total effect, Sobel p = 0.013) and ApoB (42.6%, p = 9E-7), respectively.

These findings suggest that *NMU* promoter methylation patterns could mark a pathway linking lipids with haematopoiesis and systemic inflammation.

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DNA methylation; low-grade inflammation; metabolic indices; neuromedin U; cardiovascular risk; white blood cell count; apolipoprotein B; granulocyte-to-lymphocyte ratio

Introduction

Metabolic disturbances include a set of interconnected characteristics such as dyslipidaemia, elevation of arterial blood pressure (BP), unbalanced glucose homeostasis, insulin resistance and abdominal obesity that directly increase the risk of cardiovascular diseases (CVD). More recently, other abnormalities such as chronic low-grade inflammation and a prothrombotic state have also been associated with metabolic alterations [1]. All these conditions often occur together in the so-called metabolic syndrome and interact to increase cardiovascular risk. However, the underlying mechanisms are not yet known. Chronic low-grade inflammation, in particular, has been proposed as one of the main factors involved both in the onset of metabolic features and in their pathophysiological consequences [2].

Neuromedin U (NMU) is a multifunctional hypothalamic neuropeptide widely expressed in several body districts [3] which exerts its function through two G-protein-coupled receptors: Neuromedin U Receptor 1 (NMUR1), mainly present in peripheral organs, and Neuromedin U Receptor 2 (NMUR2), preferentially expressed in the central nervous system [4]. Besides playing a role in various metabolic functions, numerous studies performed using murine models points to the importance of this neuropeptide in both inflammation and

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Supplemental data for this article can be accessed here.

metabolic factors. NMU has been found to be involved in inflammatory and immune responses [5], as it is expressed in human mast cells, monocytes and dendritic cells [6]. The NMU–NMUR1 neuronal signalling circuit provides a selective mechanism through which the enteric nervous system and the innate immune system are integrated to promote rapid type 2 cytokine responses that can induce antimicrobial, inflammatory and tissue-protective type 2 responses at mucosal sites [7]. Our group reported that NMU can potentiate platelet activation induced by weak agonists through interaction with NMUR1 [8]. These data also suggest a previously unrecognized role for NMU as a regulator of (platelet assisted) inflammatory processes. A significant association of *NMU* genetic variants with overweight and obesity in adults and children has been found [9] and we have described *NMU* haplotype and polymorphism variability to be associated with obesity [10], food preferences [11], and bone health in a population of European children [12].

Epigenetic modifications have been recognized as underlying phenomena to the onset of metabolic diseases and inflammation [13]. Exposure to a prenatal famine environment has been associated with a persistent decrease in DNA methylation of genes closely linked to growth, nutrient metabolism, cardiovascular function and inflammation [14]. Furthermore, an association between peripheral white blood cell DNA methylation and waist circumference was found in specific CpG sites of several genes mainly related to inflammation, obesity, diabetes and atherosclerosis [15].

The identification of specific pathways, as well as of their markers, underlying the development of inflammatory-mediated metabolic disturbances could be crucial to suggest new targets for preventive interventions and new biomarkers for risk assessment. We aimed at investigating the possible association of *NMU* DNA methylation levels with metabolic indices and the potential mediating role of low-grade inflammation.

Materials and methods

Study population

The study population was composed of subjects of the Moli-sani study (N = 24,325; 49.20% men; ≥ 35 years) randomly recruited from the general population of Molise Region, in 2005–2010 [16]. For this study we used data from a randomly selected subcohort of 1,160 subjects, with the exclusion of subjects with unreliable questionnaires or with missing values in core variables (N = 1,015). The analysed sub-cohort and the whole Moli-sani population showed similar values in all variables considered for this study (Table S1). The Moli-sani study complies with the Declaration of Helsinki and was approved by the Ethical Committee of the Catholic University in Rome, Italy. All participants provided written informed consent.

Data collection

Detailed, structured questionnaires on medical history and lifestyle were administered to all subjects. A full description of collected data is reported elsewhere [17]. Physical activity during leisure time was assessed by a structured questionnaire and expressed as daily energy expenditure in metabolic equivalent task-hours (MET-h/day). Food intake during the year before enrolment was assessed by the validated Italian EPIC food frequency questionnaire [18]. Adherence to the Mediterranean diet was defined according to the Mediterranean Diet Score [19]. Hypertension was defined as systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg or treatment for hypertension. Hypercholesterolemia was defined if total cholesterol ≥240 mg/dL or by use of specific medication. Diabetes was defined as fasting blood glucose ≥126 mg/dL, or on the basis of specific pharmacological treatment. Metabolic syndrome (MetS) was defined according to the Adult Treatment Panel III criteria [20]. Body weight and height were measured on a standard beam balance scale with an attached ruler with subjects wearing no shoes and only light indoor clothing. BMI was calculated as weight(kg)/height(m)^2. Waist and hip circumferences were measured according to the National Institutes of Health, Heart, Lung, and Blood Guidelines [21].

Biochemical analyses in the Moli-sani cohort

Blood samples were obtained between 07:00 and 09:00 from participants who had fasted overnight

and had refrained from smoking for at least 6 h. Hemochromocytometric analyses were performed on freshly collected blood by a cell counter (Coulter HMX, Beckman Coulter, IL, Milan, Italy) within 3 hours from venipuncture. High sensitivity C-reactive protein (hs-CRP) was measured in freshly prepared serum samples by a particle-enhanced immunoturbidimetric assay (IL 350 Instrumentation Laboratory, Milan, Italy). Quality control for hs-CRP was maintained using in-house serum pool and internal laboratory standard; inter- and intra-day CV for hs-CRP were 5.5% and 4.2%, respectively. Serum lipids and glucose were assayed by enzymatic reaction methods using an automatic analyser (ILab 350, Instrumentation Laboratory, Milan, Italy). The concentration of low-density lipoprotein (LDL) cholesterol was calculated using Friedewald's formula. These analyses were performed in the centralized Moli-sani laboratory. Insulin, apolipoprotein A and B and Lp(a) measurements were performed using automated immunoassay on samples frozen under liquid nitrogen and subsequently transferred to the BiomarCaRE project centralized laboratory [22]. HOMA-IR (Homoeostatic Model Assessment of Insulin Resistance) were calculated as insulin (mU/L)*glucose(mg/dL)/405 [23].

To assess overall low-grade inflammation condition, we used the INFLA-score index [24–26], a composite score built up by combining deciles of hs-CRP, white blood cells (WBC), platelet count (PLT) and granulocyte-to-lymphocyte ratio (GLR). For all four components, the condition of being in the lowest deciles (1 to 4) was negatively scored from −4 to −1, being in the mid-deciles (5 or 6) was assigned a score of zero (0), being in the highest deciles (7 to 10) was scored from +1 to +4. The INFLA-score ranged between −16 and 16. An increase in this score indicates an increase in the global low-grade inflammation condition.

DNA methylation analysis

NMU methylation analysis was performed on white blood cell DNA using the Pyrosequencer Q48 Autoprep (QIAGEN) platform. Buffy coats of peripheral blood cells were freshly isolated from whole blood samples collected in sodium citrate EDTA by centrifugation at 3000 rpm for 20 min at RT and frozen under liquid nitrogen. DNA was subsequently extracted using a silica matrix-based method as previously described [27]. We selected two regions in *NMU* (chr4:55,595,229–55,636,698, GRCh38/hg38, Figure 1) for the methylation study: the CpG island 76 (CGI76, chr4:55,635,746–55,636,498), in the promoter and the CpG island 32 (CGI32, chr4:55,619,-358–55,619,556), in intron 2. For both CpG islands studied (*NMU76*: chr4:55,635,769–55,636,032 and *NMU32*: chr4:55,619,257–55,619,498, GRCh38/hg38 Assembly), amplicons covering 11 CpG sites for each island were designed using Pyromark Assay Design software (*NMU76*: Forward primer, 5`-TGGAAATTTAGAGGATGAGGGAATTTTA-3`, Reverse primer, 5`- biotinACCCTCTTTCTCT CACCTTTAC-3`; Sequencing primer, 5`-AGGGAATTTTATTAGTTTTTATTTA-3`; *NMU32*: Forward primer, 5`-GGTTTA TTTTATTAGGGAGTGTTAGATAG-3`, Reverse primer, 5`-biotinAACCCTCTAAACCAAATA TAAAATATAATC-3`, Sequencing primer, 5`-GTAGGGAGAGGTATTGTTT-3`). After discarding CpG values that did not pass our quality control check as described in the method section, 10 and 7 CpG sites, belonging to *NMU76* and *NMU32*, respectively, were included in further analysis. To exclude possible intra-plate differences, a sample of HUVECs was carried on in each plate as an internal control. Bisulphite treatment was conducted on 1 μg of genomic DNA using the EZ-96 DNA methylation kit (ZYMO RESEARCH) according to the manufacturer's instructions, except for the incubation protocol during the conversion, performed for 16 hours as described [28]. All PCR amplifications were performed in duplicate. The 2 assays were tested using fully methylated and unmethylated controls (EpiTect PCR Control DNA Set, QIAGEN).

For the CpG-specific analysis, data were discarded when the duplicate measurements had a standard deviation (SD) ≥ 5% for the *NMU76* (sample range of methylation levels 4–12%) and a SD ≥ 10% for the *NMU32* (sample range 15–45%).

Statistical analysis

All analyses were performed using SAS/STAT software (Version 9.4 for Windows©2009. SAS

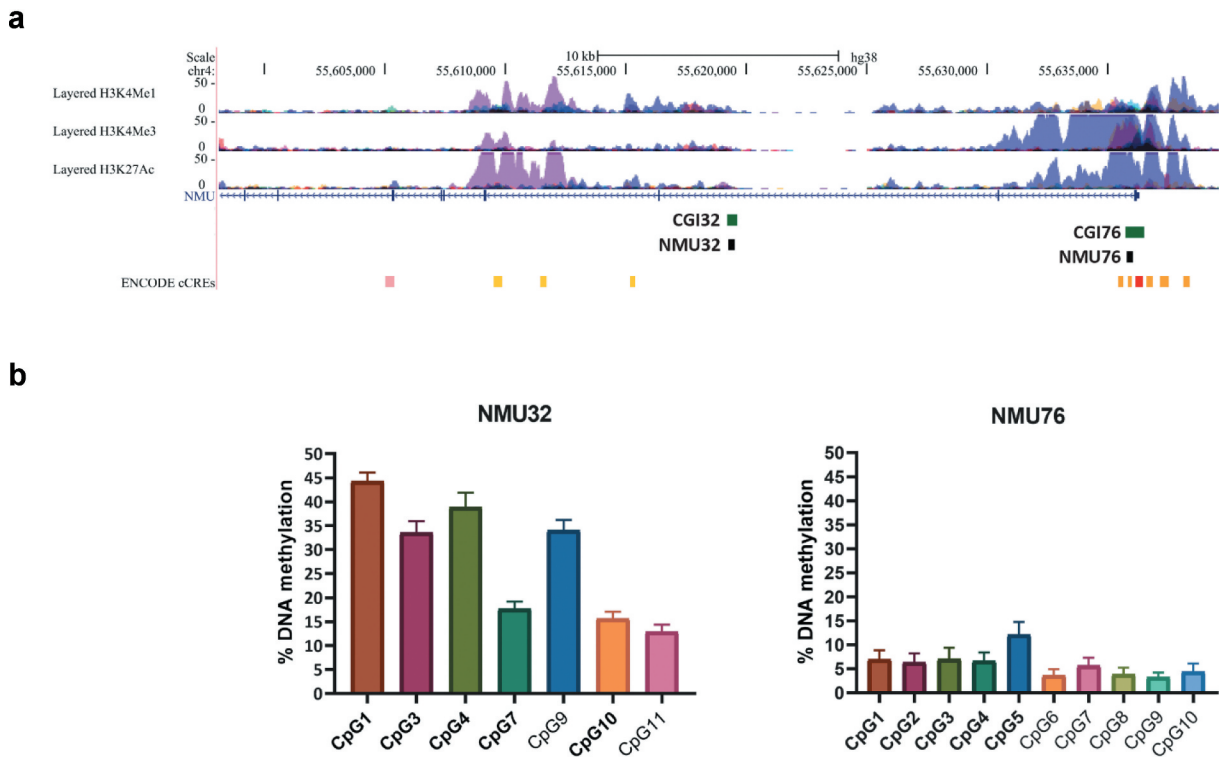

Figure 1. Genomic location of the NMU regions used for the methylation study.
(a) *NMU* genomic structure (chr4:55,595,231–55,636,298, GRCh38/hg38 Assembly). *NMU* is depicted as blue line (intronic regions) and blue boxes (exons). Two CpG islands (CGIs) are located in the gene (*CGI32* chr4:55,619,358–55,619,556, and *CGI76* chr4:55,635,746–55,636,498) and are depicted as dark green boxes (CGIs are defined based on the formula described by Gardiner-Garden et al. J Mol Biol. 1987;196(2):261–282). Pyrosequenced regions within the two CGIs (*NMU76*: chr4:55,635,769–55,636,032 and *NMU32*: chr4:55,619,257–55,619,498) are depicted as black boxes. *NMU76* co-localizes with a region marked by H3K4Me3 and H3K27Ac (displayed as coloured overlayed histograms (data produced by the Bernstein Laboratory at the Broad Institute and the University of California, Santa Cruz and part of the ENCODE database) and with a proximal enhancer-like cis-regulatory element (orange box, data based on the ENCODE Registry of candidate cis-Regulatory Elements (cCREs) and produced by the Zlab at UMass Medical Centre, ENCODE Data Analysis Centre). **(b) *NMU* DNA methylation distribution in the Moli-sani cohort**. Mean and SD histogram bar representation of *NMU32* and *NMU76* CpG site DNA methylation. CpG sites belonging to *NMU32-F1* and *NMU76-F1* are depicted in bold.

Institute Inc. and SAS are registered trademarks of SAS Institute Inc., Cary, NC, USA). Mean and SD were computed for continuous variables and frequencies for categorical variables. All continuous variables, including methylation data, were also transformed to z-scores (mean = 0; SD = 1). Because *NMU* CpG sites methylation showed significant inter-correlations (Table S2) within each region, we used principal component analysis (PCA) on the correlation matrix of the *NMU* CpG sites to identify common underlying factors (patterns) that explain the largest variation in *NMU* methylation [29,30]. Only the subjects having all the CpG sites successfully measured for each CpG island were included in the analysis: 1022 for *NMU*76 and 1016 subjects for *NMU*32, respectively. Criteria for factor selection were eigenvalue > 0.7 as revealed by the scree test and the interpretability of the final solution. The finally selected factors were transformed by the orthogonal varimax rotation to keep independent latent variables for subsequent analysis, and then standardized. Each subject received a factor score, calculated by summing the observed methylation site values, each weighted by factor loadings [31].

We first analysed the associations between the identified patterns and lifestyles, to identify potential confounders for the subsequent analyses. Then, we studied the association between *NMU* methylation factors and metabolic features

in linear and logistic regression models. We included anthropometric variables (BMI, waist, waist-to-hip ratio), hypertension (systolic and diastolic blood pressure), glucose (blood glucose, insulin levels and HOMA-IR) and lipid indices (total-, LDL- and HDL-cholesterol, lipoprotein-(a), apolipoprotein A and B), and the related categorical variables (overweight or obesity, hypertension, diabetes and hypercholesterolemia). Similarly, linear regression models were used to assess the association between *NMU* DNA methylation patterns and inflammatory parameters, represented by INFLA-score and its components hs-CRP, WBC, PLT and GLR, as well as the WBC subpopulations. Age, sex, smoking (current- vs previous- vs ever-smokers), and lifestyles associated with any methylation factor and with any outcome at $p < 0.05$ were used as covariates. A Benjamini-Hochberg false discovery rate (FDR) method was used to adjust p values for multiple testing considering all analyses performed for each methylation region. A FDR p value (pFDR) <0.05 was considered statistically significant. Using the same models, a mediation analysis was conducted to analyse the proportion of the associations between factors (predictor) and metabolic parameters (dependent variable) explained by the effect of inflammatory variables (mediator). The direct (independent of mediator) and indirect (mediated) effects of predictor on dependent variable were evaluated assessing changes in the regression coefficient with and without including the mediator in the model. The Sobel test was used to assess whether the indirect effect through the mediator on the relationship was significantly different from zero [32]. The analysis was conducted considering only predictor, mediator and dependent variables showing statistically significant associations among them in previous analyses.

Prediction of NMU DNA binding factors

To detect potential regulatory effects of methylation at the CpG sites investigated, we searched for putative binding sites by using the PROMO software [33]. The full CpG island regions analysed in the methylation study were used as DNA sequence bait in the search. The prediction was made by focusing only on the human species and transcription factors, setting the minimum sequence similarity threshold for transcription factor binding detection to 85%.

Results

NMU DNA methylation factor identification

Characteristics of the Moli-sani sub-cohort included in this analysis are reported in Table 1. *NMU76* had a mean and SD methylation range across its CpG sites of 6.03 ± 2.58 while *NMU32* showed on average higher methylation levels compared to *NMU76* of 28.16 ± 12.48. Population mean and SD for each of the *NMU* CpG sites studied (10 in the *NMU76* and 7 in the *NMU32*) are reported in Figure 1b and Table S3. PCA identified two main methylation factors for both *NMU76* and *NMU32: NMU76*-F1, *NMU76*-F2, *NMU32*-F1 and *NMU32*-F2. Factor loadings of individual CpG sites obtained after varimax rotation are reported in Table S4. *NMU76*-F1, that explains 83.0% of *NMU76* variance, is characterized by high positive loadings of CpGs 1–5 (Figure 1b and Table S4) while *NMU32*-F1, explaining 81.2% of methylation variance at *NMU32*, is mainly characterized by positive loadings of CpGs 1,3,4,7 and 10 (Figure 1b and Table S4).

NMU DNA methylation and metabolic indices

We first analysed the potential confounders testing the associations between lifestyle variables and methylation factors (Table 1). Beyond age and sex, we found smoking habit, vegetables and alcohol consumption to be associated with methylation factors. These variables were included in the subsequent models as covariates.

We analysed the associations between metabolic indices and methylation patterns (N = 1022, Table 2) or single sites (N = 1160, Table S3). We found that *NMU76*-F1 was significantly associated with total cholesterol (β = 4.5 ± 1.4 mg/dl for increase of 1 SD of Factor1, pFDR = 0.024) and ApoB (0.03 ± 0.01 g/L, pFDR = 0.021). Nominally significant (positive) associations were also observed for *NMU76*-F1 with LDL-cholesterol and triglycerides, which, however, did not survive FDR

Table 1. Association between CpG factors and potential confounders.

					F1				*F2*				*F1*				*F2*			
Variable	N	Mean or n	SD or %	N	Est	±	SE	*p*	Est	±	SE	*p*	*N*	Est	±	SE	*p*	Est	±	SE	*p*
Age (years, mean±SD)	1160	55.4	11.7	1022	**0.018**	±	**0.002**	3E-14	**0.012**	±	**0.002**	**2E-07**	1016	−0.001	±	0.002	0.66	−0.002	±	0.002	0.28
Males (n, %)	1160	571	49.2%	1022	0.058	±	0.055	0.29	0.009	±	0.054	0.87	1016	**0.196**	±	**0.058**	**0.0007**	**−0.135**	±	**0.052**	**0.009**
Eversmokers (n, %)	1158	601	51.9%	1020	**−0.159**	±	**0.058**	**0.0064**	−0.101	±	0.058	0.08	1015	−0.093	±	0.062	0.13	−0.002	±	0.055	0.97
Leisure-time physical activity (MET-h/day)*	1160	3.55	3.98	1022	0.013	±	0.028	0.63	0.029	±	0.028	0.29	1016	0.023	±	0.030	0.45	−0.006	±	0.027	0.83
Mediterranean Diet adherence score	1160	4.75	1.61	1022	0.009	±	0.017	0.60	0.008	±	0.017	0.65	1016	−0.011	±	0.018	0.54	0.011	±	0.016	0.48
Food intake (g/day, mean±SD)*	1160	2199	679	1022	−0.024	±	0.03	0.43	0.004	±	0.03	0.90	1016	−0.017	±	0.031	0.58	−0.028	±	0.028	0.31
Fruit intake (g/day, mean±SD)*	1160	389.3	226.9	1022	0.034	±	0.027	0.20	0.049	±	0.027	0.07	1016	0.037	±	0.028	0.19	0.014	±	0.025	0.58
Vegetables intake (g/day, mean±SD)*	1160	170.3	84.0	1022	**0.061**	±	**0.027**	**0.025**	0.051	±	0.027	0.06	1016	−0.010	±	0.030	0.75	0.007	±	0.027	0.80
Alcohol intake (g/day, mean±SD)*	1160	17.30	23.28	1022	−0.038	±	0.032	0.24	0.040	±	0.032	0.22	1016	0.008	±	0.032	0.79	**−0.058**	±	**0.029**	**0.042**

Linear regression analysis using age and sex as covariates. * Est and SE from standardized values; statistically significant ($p < 0.05$) results are shown in bold;

correction (Table 2). Considering single CpG sites, *NMU76* CpG 4 resulted associated with ApoB levels (0.03 ± 0.01 g/L, p = 2E-5; pFDR = 0.004) after appropriate FDR adjustment (Table S3); a stepwise multivariate analysis including all *NMU76* sites (backward elimination for sites with $p > 0.05$) confirmed the association between ApoB and CpG 4 (0.04 ± 0.01 g/L, p = 0.0002) and showed a nominally significant association for CpG 9 (−0.02 ± 0.01 g/L, p = 0.026). As for total cholesterol, N*MU76* CpG 3, 4 and 5 resulted associated, although with only nominal statistical significance (Table S3). As for *NMU32*, we found nominally significant, positive associations of *NMU32-F1* with ApoB levels and MetS, and of *NMU32-F2* with diabetes mellitus and hypercholesterolemia; however, they were no more significant after FDR correction (Table 2). Similarly, we did not find FDR-significant associations considering single *NMU32* CpG sites (Table S3).

NMU DNA methylation and inflammation

Since inflammation is tightly linked to the development of metabolic features, we investigated the associations between *NMU* methylation and low-grade inflammation markers (Table 3). *NMU*76-F1 methylation was significantly associated with INFLAscore (−1.05 ± 0.22, p = 2.7E-6) and, among the four components of the INFLA-score, with GLR (−0.27 ± 0.03, p = 1.3E-20). Moreover, we identified an inverse relationship between *NMU76*-F1 and granulocyte numbers (−0.19 ± 0.04 10^9cells/L) and a positive one with lymphocyte numbers (0.18 ± 0.02 10^9cells/L), which remained significant after FDR correction (pFDR<0.0001; Table 3). Considering single CpG site analysis, several sites – especially those loading on *NMU76*-F1 – were FDR-significantly associated with INFLA-score, GLR and lymphocyte number (Table S5); a multivariate analysis including all *NMU76* sites confirmed this result, while did not reveal strong leading effects from specific CpG sites (data not shown). As for *NMU32* region, no associations were found between its methylation patterns and inflammation related parameters. As for NMU32 region, nosignificant association was found between its methylation patterns and inflammationrelated parameters.

Table 2. Association between metabolic phenotypes and CpG factors.

				NMU76								*NMU32*							
				F1				*F2*					*F1*				*F2*			
Continuous variables	N	Mean	SD	N	Est	±	SE	*p*	Est	±	SE	*p*	N	Est	±	SE	*p*	Est	±	SE	*p*
BMI (kg/m^2)	1158	28.05	4.51	1018	0.135	±	0.16	0.4	0.234	±	0.161	0.15	1013	0.201	±	0.154	0.19	0.029	±	0.172	0.86
Weight (kg)	1158	74.75	14.08	1018	0.251	±	0.445	0.57	0.477	±	0.448	0.29	1013	0.525	±	0.419	0.21	−0.323	±	0.471	0.49
Waist circumference (cm)	1158	95.60	12.10	1018	0.213	±	0.415	0.61	0.643	±	0.418	0.12	1013	0.709	±	0.395	0.07	0.014	±	0.444	0.98
Waist-to-hip ratio	1158	0.92	0.07	1018	0.00	±	0.002	0.92	0.002	±	0.002	0.47	1013	0.003	±	0.002	0.26	0.001	±	0.003	0.83
Blood glucose* (mg/dL)	1155	100.77	26.14	1016	−0.014	±	0.887	0.84	0.999	±	0.89	0.14	1011	−0.668	±	0.875	0.86	1.744	±	0.980	0.042
Insulin (pmol/l)	1125	50.32	29.28	989	−0.978	±	1.051	0.35	1.058	±	1.058	0.32	980	0.646	±	1.038	0.53	0.394	±	1.152	0.73
HOMA-IR	1120	1.89	1.76	985	−0.077	±	0.063	0.22	0.073	±	0.063	0.25	976	−0.016	±	0.064	0.81	0.058	±	0.071	0.42
C-peptide (ng/ml)	1119	1.41	0.64	984	−0.014	±	0.023	0.54	0.008	±	0.023	0.74	974	−0.001	±	0.023	0.98	0.001	±	0.025	0.96
Systolic blood pression (mmHg)	1160	143.43	20.69	1020	0.764	±	0.637	0.23	0.611	±	0.64	0.34	1015	0.147	±	0.599	0.81	−0.137	±	0.671	0.84
Diastolic blood pression (mmHg)	1160	84.15	9.16	1020	0.578	±	0.323	0.07	0.15	±	0.325	0.64	1015	0.226	±	0.306	0.46	0.009	±	0.344	0.98
Total cholesterol (mg/dl)	1155	211.50	37.88	1016	4.447	±	1.358	0.0011°	−1.54	±	1.37	0.26	1011	1.125	±	1.280	0.38	1.581	±	1.436	0.27
LDL-cholesterol (mg/dl)	1135	129.95	32.34	999	3.394	±	1.181	0.0042	−2.529	±	1.204	0.036	993	1.339	±	1.109	0.23	1.636	±	1.242	0.19
Apolipoprotein B (g/l)	1141	1.02	0.24	1001	0.031	±	0.009	0.00047°	−0.01	±	0.009	0.27	996	0.018	±	0.008	0.033	0.002	±	0.009	0.82
HDL-cholesterol (mg/dl)	1155	55.77	13.41	1016	0.112	±	0.448	0.80	0.563	±	0.45	0.21	1011	−0.616	±	0.427	0.15	0.272	±	0.479	0.57
Apolipoprotein AI (g/l)	1136	1.66	0.33	996	0.018	±	0.011	0.11	0.008	±	0.011	0.46	992	−0.005	±	0.011	0.67	0.006	±	0.012	0.63
Lipoprotein(a) (mg/dl)	1103	17.60	18.49	968	0.800	±	0.679	0.24	0.524	±	0.681	0.44	961	−0.517	±	0.640	0.42	0.930	±	0.718	0.20
Triglycerides (mg/dl)	1155	132.60	82.75	1016	5.832	±	2.857	0.041	2.093	±	2.874	0.47	1011	−0.398	±	2.755	0.89	−1.056	±	3.091	0.73
Categorical variables	N	n	%	N	OR (95%CI)				OR (95%CI)				N	OR (95%CI)				OR (95%CI)			
Overweight or obesity	1158	840	72.5%	1018	1.01	(0.86–1.19)			1.08	(0.91–1.27)			1013	1.10	(0.94–1.29)			1.07	(0.90–1.28)		
Diabetes mellitus	1148	106	9.2%	1011	1.05	(0.82–1.33)			1.08	(0.84–1.39)			1006	0.95	(0.74–1.23)			1.09	(0.83–1.43)		
Hypertension	1153	713	61.8%	1013	1.04	(0.88–1.23)			1.05	(0.89–1.23)			1009	1.06	(0.91–1.24)			1.04	(0.87–1.24)		
Hypercholesterolemia	1134	313	27.6%	995	1.23	(1.05–1.44)			0.95	(0.81–1.12)			993	1.05	(0.90–1.22)			1.19	(1.00–1.42)		
Metabolic syndrome	1153	322	27.9%	1014	0.97	(0.83–1.15)			1.04	(0.88–1.22)			1009	1.17	(1.00–1.35)			0.98	(0.83–1.16)		

Linear (continuous variables) and logistic (categorical, yes vs no) regression analyses using age, sex, eversmokers and vegetable and alcohol consumption as covariates. * *p* values from analyses using log-transformed variable; statistically significant ($p < 0.05$) results are shown in bold; ° results statistically significant after FDR correction considering all analyses for each region

Table 3. Association between CpG factors and inflammatory indices.

				NMU76							*NMU32*						
				F1				*F2*			*F1*				*F2*		
Variable	N	Mean or n	SD or %	N	Est	±	SE	p	Est	±	SE	p	N	Est	±	SE	P
INFLAscore	1114	−0.38	6.13	976	−1.048	±	0.223	3E-6°	0.104	±	0.224	0.64	976	0.076	±	0.213	0.72	0.213	±	0.240	0.37
C-reactive protein (mg/L)	1160	2.71	3.33	1020	−0.104	±	0.118	0.38	0.187	±	0.118	0.11	1015	−0.108	±	0.118	0.36	−0.206	±	0.132	0.12
Platelets (10^9/l)	1160	243.04	63.84	1020	−0.386	±	2.209	0.86	−1.157	±	2.221	0.60	1015	3.645	±	2.094	0.08	4.109	±	2.348	0.08
WBC (10^9/l)	1160	6.21	2.18	1020	−0.018	±	0.057	0.76	0.037	±	0.057	0.52	1015	−0.041	±	0.077	0.60	0.044	±	0.086	0.61
Granulocyte-to-lymphocyte	1113	1.98	0.80	975	−0.267	±	0.028	7E-21°	−0.016	±	0.029	0.58	975	−0.047	±	0.028	0.09	0.007	±	0.031	0.82
Lymphocytes (10^9/l)	1113	2.06	1.47	975	0.179	±	0.022	1E-15°	0.02	±	0.023	0.39	975	0.033	±	0.054	0.54	0.033	±	0.060	0.58
Monocytes (10^9/l)	1113	0.37	0.19	975	−0.002	±	0.005	0.75	0.002	±	0.005	0.70	975	0.000	±	0.007	0.99	−0.001	±	0.008	0.85
Granulocytes (10^9/l)	1113	3.78	1.27	975	−0.189	±	0.044	2E-5°	0.011	±	0.045	0.80	975	−0.076	±	0.043	0.08	0.020	±	0.049	0.68

Linear regression analysis using age, sex, eversmokers and vegetable and alcohol consumption as covariates. statistically significant ($p < 0.05$) results are shown in bold; ° results statistically significant (all $p < 0.0001$) after FDR correction considering all analyses for each region

Transcription factor analysis using PROMO/ TRANSFAC

We studied the prediction of transcription factor binding sites of the genomic regions encompassing the most significantly associated (surviving FDR correction) CpG sites with either the metabolic or the inflammation-related markers namely: CpG 2, CpG 3, CpG 4, CpG7 and CpG8, all belonging to *NMU76*. *NMU76* CpG 4 co-localizes with a putative binding site for EBF (Figure 2), while

CpG 2, CpG 3, CpG 7 and CpG 8 all contain binding sites for p53 and EIIaE-A (adenovirus E2a-inducible early promoter – element A) transcription factors (Figure 2).

Mediation analysis

Since *NMU76*-F1 was associated with both lipid-related variables and inflammation markers, we investigated a potential link among all these variables. GLR was inversely associated with both total cholesterol (−5.4 ± 1.4 mg/dl, p = 0.00014) and ApoB (−0.04 ± 0.01 g/L, p = 2.3E-6) levels, and lymphocyte numbers with higher ApoB levels (0.02 ± 0.01 g/L, p = 5.0E-5; Table S5). We thus tested the associations of both metabolic and inflammatory variables with *NMU76* methylation in the same model, to estimate the extent of the overlaps between the effects of the above mentioned variables. As shown in Supplementary Figure 1, *NMU76*-F1 explained 10.9% of cholesterol and 12.3% of ApoB variance, independently on inflammation. When inflammatory variables were added to the model, these effects were reduced up to 8.3% (for cholesterol, adjusted for GLR) and 7.1% (for ApoB, adjusting for lymphocyte count), respectively. These reductions were statistically significant (for GLR on cholesterol, 24.0% of total effect, Sobel p = 0.013; for lymphocyte count on ApoB, 42.6%, p = 9E-7). Conversely, considering the association with inflammatory variables, *NMU76*-F1 explained 30.4% of GLR and 25.6% of lymphocyte count, independently on metabolic parameters. In this case, the effect of metabolic parameters was limited (cholesterol on GLR, 0.9% of total effect, p = 0.04; ApoB on lymphocyte number, 2.4%, p = 0.001).

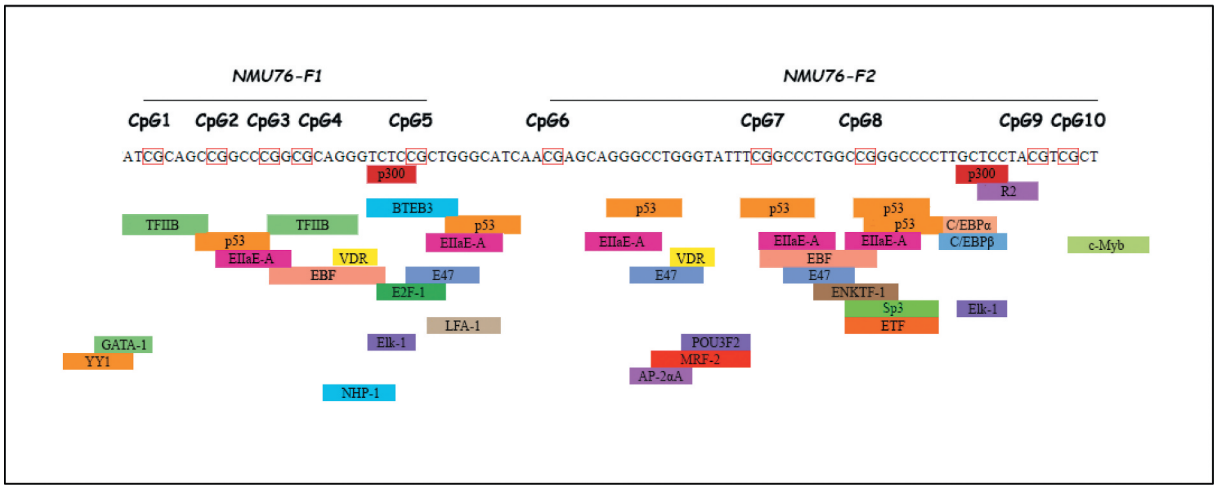

Figure 2. PROMO/TRANSFAC transcription factor prediction analysis of *NMU76* (chr4:55,635,809–55,635,902, GRCh38/hg38 assembly).

CpG sites belonging to the two *NMU76* Factors (*NMU76-F1* and *NMU76-F2*) identified in the analysis are indicated by empty red boxes. The length of each box indicating the transcription factor identifies its predicted binding sequence.

Discussion

We herein report the DNA methylation levels of two *NMU* CpG islands measured in blood samples belonging to a general population of Italian adults. Our study shows for the first time that *NMU* promoter DNA methylation is associated with increased lipid indices (levels of total cholesterol and of lipoprotein containing ApoB) and low-grade inflammation markers (mainly decreased GLR, with reduced granulocyte and increased lymphocyte number). Moreover, we found a significant overlap between these associations, suggesting potential common regulatory mechanisms for these two pathways influenced by NMU.

NMU is a neuropeptide with several functions involved in gut-brain axis, energy balance and immune response as reported in a large number of studies conducted in mouse models [3]. In humans, mainly in children, a possible link of *NMU* with overweight and obesity [9,10], food preferences [11] and bone health [12], all phenotypes related with metabolic parameters, has been reported.

We found a significant link between *NMU* DNA methylation variability and total cholesterol and ApoB levels, but not with HDL-cholesterol or ApoAI and Lp(a). Our results suggest an effect on pathways linked to the turn-over regulation of ApoB-containing lipoproteins, such as chylomicrons, VLDL and LDL, whose alteration has been consistently associated with cardiovascular risk [34]. These data support the rationale for NMU pathway to be a potential treatment target for dyslipidemia. A growing interest for NMU investigation in relation to human health has recently inspired research towards the development of NMU analogues for the treatment of metabolic disturbances [35–37].

We also found a robust negative association with INFLA-score, a marker of circulating low-grade inflammation, both for the main *NMU76* methylation factor and for several individual CpG sites of the same promoter region. The association was mainly and negatively driven by GLR and, in particular, by a decreasing effect on granulocyte number and an increasing effect on lymphocyte number, with no net effect on total WBC numbers. This could suggest a role for NMU in influencing haematopoiesis. The latter is strongly supported by previous studies on myeloid [38] and erythroid [39] lineages indicating NMU as an autocrine growth factor regulated by c-myb. A role in the activation and consumption as well as in the proliferation of selected WBC subpopulations, following an inflammatory stimulus, cannot be excluded. In numerous studies using animal models, NMU has been involved in inflammatory processes at different levels, especially through its binding with NMUR1 on different types of white

blood cells. Indeed, NMU was implicated in stimulation of cytokine production by T cells, mast cell-mediated inflammation [40], eosinophil infiltration into inflammatory sites by directly activating eosinophils [5], LPS-induced IL-6 expression in macrophages [41], IL-5 expression in lung lymphocytes co-stimulated with CGRP IL-33 [42], and anti-microbial and inflammatory type 2 responses at mucosal sites [7]. Data from the BLUEPRINT consortium (https://blueprint.haem.cam.ac.uk/bloodatlas/) showed that *NMU* and *NMUR1* are differentially expressed in several inflammatory human cells. In particular, *NMU* is highly expressed in HUVECs, whereas WBCs mostly express *NMUR1*.

We observed that part of the effect of the main *NMU* promoter methylation pattern on GLR and lipid indices was shared between these two pathways. The causal direction of these associations (*NMU* methylation affecting inflammation or lipids or the latter affecting *NMU* methylation) cannot be determined in our cross-sectional study. However, beside the known regulatory role of epigenetics in influencing both the onset of metabolic-related diseases [43] and of inflammatory states, in particular low-grade inflammation [44–46], other longitudinal studies are available on this topic. Some of them showed a longitudinal association between DNA methylation age measure [47] and biomarkers of inflammation or BMI and adoposity [48], suggesting that methylation could be the first exposure. Other studies, on the contrary, demonstrated that DNA methylation is the effect of certain metabolic conditions, including adiposity, over time [48–52]. From this perspective, *NMU* methylation could regulate (or simply mark an unknown pathway regulating) both blood cell numbers and lipids, or it could be the result of the cumulative effect of certain metabolic and inflammatory patterns over time. As for the first hypothesis, previous studies showed a link between leukocyte colony stimulating factors, able to change granulocyte-to-lymphocyte ratio, and both reduced expression of VLDL receptors and increased hepatic cholesterol biosynthesis [53]. As for the second hypothesis, lifestyles associated with metabolic disturbances could also change methylation levels [54]. In our analyses, however, we tested several lifestyle variables as potential covariates, finding significant associations with age, sex and smoking, while dietary variables as alcohol and vegetables intake were associated at lower statistical significance.

The most associated methylation factor with the analysed parameters tagged *NMU76* where 1. the putative *NMU* promoter is located and where 2. we identified a region rich in potential binding sites for transcription factors. DNA methylation dependent gene expression can be regulated by both these mechanisms [55]. Putative search for specific transcription factor binding sites in the *NMU76* identified binding sites for EBF (lipids associated CpG4, Figure 2), p53 and EIIaE-A (GLR and low-grade inflammation associated CpGs 2, 3, 7 and 8, Figure 2). Interestingly, EBF is a known transcription factor regulating adipogenesis [56]; p53 controls cell-cycle arrest, an important step for the finalization of myelopoiesis [57,58] and EIIaE-A has been described to be important in haematopoiesis [59,60].

Major strengths of this study are the relatively large sample size and the methodology applied to investigate *NMU* methylation associations with the metabolic and inflammatory phenotypes studied. This approach, recently validated for other two genes by our group in a smaller population [29,30], allowed us to reduce the number of tests to carry out and the statistical burden of correction for multiple testing, identifying few factors characterized by close CpG sites in specific *NMU* genomic regions. Moreover, the main factors identified in both regions explained a large part of gene methylation variance, suggesting that these factors properly mark the methylation variability of the regions they belong to.

This study also has some limitations. First, the cross-sectional design of the study has different implications. The mediation analysis lies on the hypothesis that DNA methylation is the causal factor, that occurred before metabolic and inflammatory changes and that inflammation mediates the relationship between methylation and lipids. Indeed, while inflammation explains a large portion of the association between methylation levels and lipid- related variables, these latters only marginally affect the association between methylation and inflammation. A further limitation is the availability of a single cohort, without

replication. However, the latter limitation is overcome by the availability of methylation measurements for multiple CpG sites in the same gene and in a large general population, by the very high level of statistical significance reached in several analyses and by the large portion of variance explained in clinically useful parameters (11% of cholesterol content and 26% of lymphocyte number variability). Caution is necessary in extending these results to larger population contexts, since data were collected in a single Italian region. However, the main characteristics of our population sample are comparable to those of the Italian Cardiovascular Epidemiology Observatory [61]. For this reason, our sample could be considered representative of at least the Italian population.

Our study opens up to future investigations to understand the epigenetic regulation of *NMU* in different tissues and cell types. This will help to clarify how methylation changes are responsible for altered *NMU* expression levels in health and disease. Overall, the findings reported here – linking *NMU* methylation patterns with both lipid- and inflammation-related markers - identify *NMU*, and more specifically the ApoB associated CpG 4, as a potential novel biomarker of circulating inflammation, lipid homoeostasis and cardiovascular risk assessment.

Data availability statement

The data that support the findings of this study are available from the corrisponding author, L.I., upon reasonable request. Mail to: licia.iacoviello@-moli-sani.org

Authors' contributions

AM, BI and FG designed the study and wrote the manuscript. AM performed the methylation experiments; FG performed statistical analyses; BI designed and supervised the methylation experiments; FN designed and performed the methylation experiments; RP and AT performed the sample DNA extraction; AG and AdC provided statistical support; SC managed the databases and provided statistical support; CC, MBD, GdG and LI were at the origin of the conception of the Moli-sani cohort and participated in the design of the study; all authors critically reviewed and approved the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors

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Appendix

Moli-sani Study Investigators

The enrolment phase of the Moli-sani Study was conducted at the Research Laboratories of the Catholic University in Campobasso (Italy), the follow up of the Moli-sani cohort is being conducted at the Department of Epidemiology and Prevention of the IRCCS Neuromed, Pozzilli, Italy.

Steering Committee: Licia Iacoviello*°(Chairperson), Giovanni de Gaetano* and Maria Benedetta Donati*.

Scientific secretariat: Marialaura Bonaccio*, Americo Bonanni*, Chiara Cerletti*, Simona Costanzo*, Amalia De Curtis*, Augusto Di Castelnuovo§, Francesco Gianfagna°§, Mariarosaria Persichillo*, Teresa Di Prospero* (Secretary).

Safety and Ethical Committee: Jos Vermylen (Catholic Univesity, Leuven, Belgio) (Chairperson), Ignacio De Paula Carrasco (Accademia Pontificia Pro Vita, Roma, Italy), Antonio Spagnuolo (Catholic University, Roma, Italy).

External Event adjudicating Committee: Deodato Assanelli (Brescia, Italy), Vincenzo Centritto (Campobasso, Italy).

Baseline and Follow-up data management: Simona Costanzo* (Coordinator), Marco Olivieri (Associazione Cuore Sano, Campobasso, Italy), Teresa Panzera*.

Data Analysis: Augusto Di Castelnuovo§ (Coordinator), Marialaura Bonaccio*, Simona Costanzo*, Simona Esposito*, Alessandro Gialluisi*, Francesco Gianfagna°§, Emilia Ruggiero*.

Biobank and biochemical laboratory: Amalia De Curtis* (Coordinator), Sara Magnacca§.

Genetic laboratory: Benedetta Izzi* (Coordinator), Annalisa Marotta*, Fabrizia Noro*, Roberta Parisi*, Alfonsina Tirozzi*.

Recruitment staff: Mariarosaria Persichillo* (Coordinator), Francesca Bracone*, Francesca De Lucia (Associazione Cuore Sano, Campobasso, Italy), Cristiana Mignogna*, Teresa Panzera*, Livia Rago*.

Communication and Press Office: Americo Bonanni*.

Regional Health Institutions: Direzione Generale per la Salute – Regione Molise; Azienda Sanitaria Regionale del Molise (ASReM, Italy); Molise Dati Spa (Campobasso, Italy); Offices of vital statistics of the Molise region.

Hospitals: Presidi Ospedalieri ASReM: Ospedale A. Cardarelli – Campobasso, Ospedale F. Veneziale – Isernia, Ospedale San Timoteo – Termoli (CB), Ospedale Ss. Rosario – Venafro (IS), Ospedale Vietri – Larino (CB), Ospedale San Francesco Caracciolo – Agnone (IS); Casa di Cura Villa Maria – Campobasso; Ospedale Gemelli Molise – Campobasso; IRCCS Neuromed – Pozzilli (IS).

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Baseline Recruitment staff is available at https://www.moli-sani.org/?page_id=173