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Cross-sectional Study

DNA methylation profiling of CD04$^+$/CD08$^+$ T cells reveals pathogenic mechanisms in increasing hyperglycemia: PIRAMIDE pilot study

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ABSTRACT

Background: DNA methylation can play a pathogenic role in the early stages of hyperglycemia linking homeostasis imbalance and vascular damage.
Material and methods: We investigated DNA methylome by RRBS in CD04$^+$ and CD08$^+$ T cells from healthy subjects (HS) to pre-diabetics (Pre-Diab) and type 2 diabetic (T2D) patients to identify early biomarkers of glucose impairment and vascular damage. Our cross-sectional study enrolled 14 individuals from HS state to increasing hyperglycemia (pilot study, PIRAMIDE trial, NCT03792607).
Results: Globally, differentially methylated regions (DMRs) were mostly annotated to promoter regions. Hypermethylated DMRs were greater than hypomethylated in CD04$^+$ T cells whereas CD08$^+$ T showed an opposite trend. Moreover, DMRs overlapping between Pre-Diab and T2D patients were mostly hypermethylated in both T cells. Interestingly, *SPARC* was the most hypomethylated gene in Pre-Diab and its methylation level gradually decreased in T2D patients. Besides, *SPARC* showed a significant positive correlation with DBP (+0.76), HDL (+0.54), Creatinine (+0.83), LVDd (+0.98), LVSD (+0.98), LAD (+0.98), LVPWd (+0.84), AODd (+0.81), HR (+0.72), Triglycerides (+0.83), LAD (+0.69) and AODd (+0.52) whereas a negative correlation with Cholesterol (−0.52) and LDL (−0.71) in T2D.
Conclusion: SPARC hypomethylation in CD08$^+$ T cells may be a useful biomarker of vascular complications in Pre-Diab with a possible role for primary prevention warranting further multicenter clinical trials to validate our findings.

1. Introduction

According to the World Health Organization (WHO), the prevalence of prediabetes (Pre-Diab) has increased in the past 10 years and its incidence will continue to increase in the coming years [1,2]. This intermediate metabolic state between normoglycemia and diabetes is associated with an increased risk of type 2 diabetes (T2D), and cardiovascular diseases (CVDs) [1,2]. Currently, it is estimated that Pre-Diab affects about 40% of the adult individuals of which about 5–10% can develop T2D while the remaining part could present macrovascular complications, mostly coronary heart disease (CHD) and microvascular damage [3–5]. Pre-Diab and T2D are highly heterogeneous at the molecular and clinical levels in which environmental risk factors, such as poor diet and sedentary life, play a relevant role in affecting the individual genetic background associated with the risk of CVD onset. Thus, the identification of novel candidate genes affecting individual sensitiveness to CVDs in increasing hyperglycemia offers a relevant route to

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Abbreviations

ACEi	acetylcholinesterase inhibitors
ACS	acute coronary syndrome
AODd	air-operated double-diaphragm pumps
ARBs	angiotensin II receptor blockers
BMI	body mass index
CD36	cluster of differentiation 36
CCL18	C–C motif chemokine ligand 18
CHD	coronary heart disease
CpG	cytosine-phosphate-guanine;
CVDs	cardiovascular diseases
DBP	diastolic blood pressure
DMRs	differentially methylated regions
ERBB	Erb-B receptor tyrosine kinase
FASLG	Fas ligand;
FDR	false discovery rate
FC	fold change
GEO	gene expression omnibus
GO	gene ontology
GIGYF2	GRB10 interacting GYF protein 2
HbA1c	haemoglobin A1c
HDL:	high-density lipoprotein
HMM	multivariate hidden markov model
HS	healthy subjects
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
IL-1	interleukin 1
IL6R	inerleukin-6 receptor
KLK10	kallikrein related peptidase 10
LAD	left anterior descending artery
LDL:	low-density lipoprotein
LVDd	left ventricular diastolic dysfunction
LVPWd	left ventricle posterior wall thickness
MAPK	mitogen-activated protein kinase
MT1X	metallothionein 1X
NLRP7	NLR family pyrin domain containing 7
OGTT	oral glucose tolerance test
Pre-Diab	prediabetics
PBMCs	peripheral blood mononuclear cells
PPAR	peroxisome proliferator-activated receptors
PPIs	protein protein interactions
PPP2R5C	serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit gamma isoform
PSMB 10	proteasome 20S subunit beta 10
PYCR1	pyrroline-5-carboxylate reductase 1
RRBS	reduced representation bisulfite sequencing
SBP	systolic blood pressure
SMAD3	small mother against decapentaplegic
SOS1	SOS ras/rac guanine nucleotide exchange factor 1
SPARC	secreted protein acidic and cysteine rich
T2D	type 2 diabetes
TAB2	TGF-beta activated kinase 1 (MAP3K7) binding protein 2
TSS	transcription start sites
WHO	world health organization
ZNF564	Zinc Finger Protein 564

clarify the molecular mechanisms underlying the early pathogenesis of vascular damage, a necessary prerequisite for the rational development of novel preventive biomarkers and drug targets.

DNA methylation can directly impact gene expression at transcriptional level [6], and several clinical studies demonstrated that peripheral insulin resistance, hyperglycemia, and inflammation can significantly alter DNA methylome of circulating cells in early stages by providing a putative mechanistic link between glucose homeostasis imbalance and vascular damage [7–13]. DNA methylation mainly occurs at cytosine bases of cytosine-phosphate-guanine (CpG) dinucleotides which are enriched in gene promoters and "CpG islands"representing large regions with about 50% of CpG [6]. Generally, hypermethylation of CpG sites in gene promoters, or associated CpG islands, can inversely modulate gene expression in a spatio-temporal manner [6]. We hypothesized that DNA methylation changes can appear already at the Pre-Diab state or during the transition to T2D providing putative novel candidate genes underlying vascular damage which might be useful as early biomarkers for primary prevention.

Our group has a longstanding experience in epigenetics and CVDs [14–23]. This pilot study is part of the clinical trial PIRAMIDE (NCT03792607) aimed at investigating early epigenetic interactions in diabetes and its progression by combining big data and network-oriented analysis [24]. We performed a very complex DNA methylome analysis on both circulating $CD04^+$ and $CD08^+$ T cells isolated from healthy subjects (HS), Pre-Diab, and T2D patients (Fig. 1A). We aim to identify differentially methylated regions (DMRs) and annotated genes to clarify if these circulating cells may carry out detrimental signals underlying early vascular damage. Indeed, as previously reported in patients with acute coronary syndrome (ACS) [25], which are strongly associated with hyperglycemia [26], alterations of methylation signatures in both $CD04^+$ and $CD08^+$ T cells provided potential clinical biomarkers and therapeutic targets [26–28]. Since DNA methylation into regulatory regions is highly correlated with cell-specific patterns of repressive chromatin marks [6], we chose the reduced representation bisulfite sequencing (RRBS) platform which provides an enrichment of both promoters and CpG islands [29]. By using liquid-based assays, the identification of novel early and non-invasive molecular biomarkers may help physicians to select high-risk hyperglycemic patients, and stratify the risk of developing CHD and vascular complications [30].

2. Materials and methods

2.1. Patient enrollment

In this pilot study, we enrolled a subgroup of patients from our ongoing PIRAMIDE clinical trial [24] (NCT03792607) by including a total of 14 individuals classified in HS, Pre-Diab, and T2D. Following, the main clinical characteristics distributed among the three groups: body mass index was higher in Pre-diab and T2D patients, even if not statistically significant. Otherwise, glycemia, Hb1Ac, insulin, total Cholesterol, and LDL-C showed significant increased levels from normo- to hyperglycemia. Our study population was recruited at the Department of Advanced Medical and Surgical Sciences (DAMMS), University of Campania "Luigi Vanvitelli". Patients were clinically diagnosed as Pre-Diab and T2D based on clinical history, symptoms, and laboratory tests, according to the current guidelines [31]. Specifically, Pre-Diab was diagnosed by evidence of fasting plasma glucose $\geq$5.6 mmol/L but <7.0 mmol/L (100–125 mg/dL; impaired fasting glucose, IFG), a 2-h glucose $\geq$7.8 mmol/L but <11.1 mmol/L during a 75 g oral glucose tolerance test (OGTT) (140–199 mg/dL; impaired glucose tolerance, IGT), or a plasma hemoglobin (Hb) A1c $\geq$5.7% but <6.4%. T2D was diagnosed by evidence of IFG>7.0 mmol/L (>125 mg/dL), post-prandial glycemia >11.1 mmol/L (>200 mg/dL), and evidence of HbA1c>6.6%. Patients with a known history of malignancy disorders, active infections, and chronic or immune-mediated diseases were excluded from the study to avoid confounder effects. As controls, we

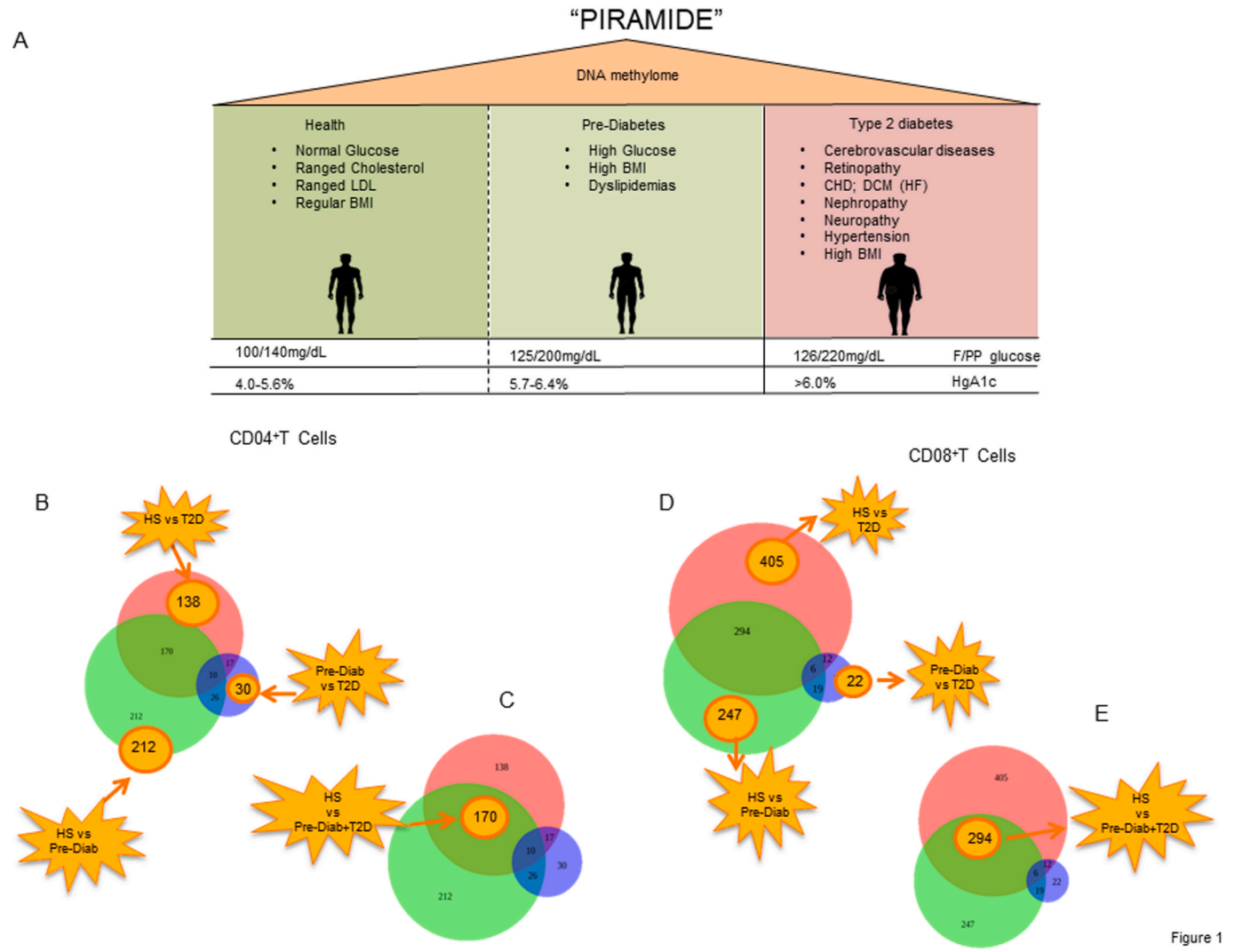

Fig. 1. A) Dynamics of DNA methylation in different stages of impaired glucose homeostasis. PIRAMIDE clinical trial aimed at investigating early epigenetic-sensitive regulatory networks in different stages ranging from normoglycemia to Pre-Diab and conclamate T2D. **B–C-D-E) Distribution of overlapping and unique DMRs.** Venn diagrams show the distribution of unique and overlapping DMR-related genes which have been identified in our three groups. The number of unique (B and D) and overlapping (C and E) DMRs in both $CD04^{+}$ and $CD08^{+}$ T cells is reported.

selected subjects free of hyperglycemia and with no signs and symptoms of vascular damages or medication use. This study was approved by the local Ethical Committee (Protocol N. 114) and all patients signed a written informed consent. The study was conducted following the principles of the Declaration of Helsinki.

2.2. Clinical data analysis

Statistical analysis was performed to compare clinical characteristics for Pre-Diab and T2D groups by using R software (version 3.03). Continuous variables were expressed as mean ± standard deviation or standard error. Unpaired Student's t-test was used for comparison between two groups. Categorical variables were expressed as percentages and were compared using the Chi-Square test or Fisher's exact test. A p-value of <0.05 was considered significant.

2.3. Cell isolation and DNA extraction

Peripheral venous blood samples (about 25 mL) were collected from HS and patients and processed immediately after the blood draw. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by Ficoll gradient using Histopaque®-1077 (Sigma-Aldrich), according to manufacturer's instructions. Then, T cells were separated and purified, and genomic DNA (gDNA) samples were immediately extracted according to manufacturer's instructions (Supplementary S1) (Supplementary Fig. 1).

2.4. Library preparation

Sequencing was performed at the Genomix4Life S.r.l. (Salerno, Italy). Briefly, 2 μg of gDNA were used for each library preparation. DNA samples were digested by *Msp*I restriction enzyme and purified with the GeneJet PCR Purification Kit (Thermo Fisher Scientific). All libraries were prepared by TruSeq Library Prep Kit (Illumina) and bisulfite conversion was obtained using the EZ DNA Methylation-Gold Kit (Zymo Research). DNA amplification reaction was performed using Pfu-TurboCxHotstart DNA Polymerase (Agilent Technologies, USA) and the amplified fragments were purified by AMPure XP Beads. Finally, they were quantified by the Agilent 4200 TapeStation (Agilent Technologies). Each DNA library was analyzed by paired-end sequencing read (2 × 75 cycles) on Illumina Nextseq 500.

2.5. DNA sequence processing and alignment

Raw reads were assessed for quality by using FastQC (v011.8, Babraham Bioinformatics, UK) and trimmed to remove Illumina adaptors and low-quality reads using TrimGalore (v0.6.3, Babraham Bioinformatics, UK) with the default settings (Supplementary S2).

2.6. Downstream bioinformatic analysis

2.6.1. Identification of differentially methylated regions (DMRs)

We identified DMRs by using the R package (v1.10.0) [32]. For both T cell types, we explored and compared three DNA methylation profiles: 1) HS *vs* Pre-Diab patients, 2) HS *vs* T2D patients, and 3) Pre-Diab *vs* T2D patients. To prevent PCR bias and increase the power of the statistical test, CpG sites covering less than 10 reads or more than the 99.9th percentile of coverage distribution in each sample were filtered out. Coverage values between samples were normalized as by default. Read coverage per base and correlation plots were calculated and displayed in Supplementary Fig. 2. To define DMRs, we used a tiling window of 1 Kb. DMRs among the three groups were defined as regions with more than 25% methylation differences ($|\Delta M|$) and *q-value* <0.01, after applying logistic regression by using the SLIM method to correct *p-value* for multiple hypothesis testing. Positive and negative values indicate hyper- and hypomethylation in patients, respectively. DNA methylation profiles covering >300,000 CpG dinucleotides in both T cell types isolated from each study participant were generated by using Illumina Nextseq 500 platform. Then, multiple t-tests were performed. Overall, circulating T cells revealed no statistically significant changes in global DNA methylation trend in HS vs increasing hyperglycemia (Supplementary Table 1).

2.6.2. Gene annotation and functional analysis

By using the R package ChIPseeker (v1.20.0) [33], we represented the % of DMRs located into promoters, coding sequences, introns, distal intergenic regions, 5′ UTR and 3′ UTR in all three datasets, for both T cells (Supplementary S3).

2.6.3. Correlation analysis

After DMRs annotation, we considered methylation levels of relevant overlapping DMR-related genes from both T cell types for correlation analysis. A significant association between their methylation status (hyper- and hypo-) and quantitative clinical parameters/laboratory tests for both T cell types and from each disease group was calculated by Spearman's Correlation, using as threshold *corr*> 0.5.

2.6.4. Chromatin state discovery and characterization

We used the ChromHMM (v1.19) software [34] to characterize epigenomic regions according to 18 chromatin states, defined from both $CD04^+$ and $CD08^+$ T cells, and grouped in HS, Pre-Diab, and T2D. ChromHMM acquires chromatin state signatures by using a multivariate Hidden Markov Model (HMM) that fits the combinatorial and spatial presence or absence of each chromatin state. From these signatures, ChromHMM generates a genome-wide annotation for each cell type and condition by calculating the most probable state in each genomic segment. Then, we annotated two methylated groups by applying the R package ChIPseeker and we found 564 genes annotated to hypo-states and 8389 annotated to hyper-states. Finally, we performed functional analysis for only promoters regions (≤1 Kb), by using g: Profiler web server.

Raw data have been deposited in the NCBI Gene Expression Omnibus (GEO) database under the accession number PRJNA600866 (https://www.ncbi.nlm.nih.gov/sra).

2.6.5. Gene prediction analysis

GeneMANIA tool was performed to perform gene function predictions based on GO annotations patterns for genes of interest [35]. Only physical interactions were selected.

3. Results

3.1. Differentially methylated regions (DMRs)

To characterize the most significant DMRs in distinct Pre-Diab and T2D groups, we firstly showed the distribution values for each sample. Annotated DMRs were mapped according to their distance from established CpG islands. Globally, DNA methylation changes were rather concentrated among CpG islands located in the promoter regions (about 30–35% of total DMRs), for both T cell types and each comparison (Supplementary Fig. 3). To individuate the overlapping changes during increasing hyperglycemia, we calculated the number of annotated DMR-related genes by using the Venn Diagram (Fig. 2A–D). Moreover, we performed heatmaps to show the DMR methylation changes in both T cells (Supplementary Fig. 4A-B) (Supplementary Tables 2 and 3). The summary of significant DMRs is shown in Supplementary Table 4. Moreover, we identified the genomic locations which were most impacted by changes in DNA methylation between HS and increasing hyperglycemia. Then, we discerned hypo- and hypermethylated DMRs-related genes (Supplementary Fig. 4C-D). In general, we observed that the number of hypermethylated DMRs was greater than those hypomethylated in $CD04^+$ T cells; on the contrary, $CD08^+$ T cells showed a higher number of hypomethylated DMRs than hypermethylated ones.

3.1.1. Analysis of $CD04^+$ T cells

We identified 437 DMRs (FDR<0.05) and 418 annotated genes, of which 35% (n = 154) were hypo- and 65% (n = 283) were hypermethylated in HS vs Pre-Diab (Supplementary Table 5). Moreover, from the comparison between HS and T2D, we identified 351 DMRs (FDR <0.05) associated with 335 annotated genes, of which 32% (n = 112) were hypo- and 68% (n = 239) were hypermethylated (Supplementary Table 6). Finally, from the comparison between Pre-Diab and T2D groups, we identified 84 DMRs (FDR <0.05) associated with 83 annotated genes of which 51% (n = 43) were hypo- and 49% (n = 41) were hypermethylated (Supplementary Fig. 4C).

3.1.2. Analysis of $CD08^+$ T cells

We identified 594 DMRs (FDR < 0.05) of which 74% (n = 438) were hypo- and 26% (n = 156) were hypermethylated associated with 566 annotated genes by comparing HS *vs* Pre-Diab group (Supplementary Table 7). From the comparison between HS and T2D, we identified 786 DMRs (FDR <0.05), of which 68% (n = 535) were hypo- and 32% (n = 251) were hypermethylated and associated with 717 annotated genes (Supplementary Table 8). Finally, from the comparison between Pre-Diab and T2D groups, we identified 62 DMRs (FDR <0.05) of which 37% (n = 23) were hypo- and 63% (n = 39) were hypermethylated and associated with 59 annotated genes (Supplementary Fig. 4C).

3.1.3. Overlapping DMRs in $CD04^+$ T of HS vs Pre-diab and T2D

By analyzing the heatmap, we noticed that there were some clusters of DMR-related genes which retained the same hyper- or hypomethylation level in increasing hyperglycemia, as depicted in red boxes (Supplementary Fig. 5A). We focused on the top 18 highly significant DMR-related genes, which were shared in HS *vs* increasing hyperglycemia, of which 13 were hyper- and 5 were hypomethylated (Fig. 2A and B) (Supplementary Table 9). Moreover, we reported two opposite trends for DNA methylation in HS vs increasing hyperglycemia. The first trend demonstrated an increasing grade of DNA methylation from normo- to increasing hyperglycemia. *GIGYF2* gene resulted in the highest hypermethylated gene ($q = 2.85E\text{-}19$ and $q = 1.78E\text{-}21$ in Pre-Diab and T2D, respectively). However, the *ZNF564* gene showed the highest fold change (FC = 15) of differential methylation between Pre-Diab and T2D (Fig. 2A). The second trend demonstrated an increased level of hypomethylation from normo- to increasing hyperglycemia. In particular, the

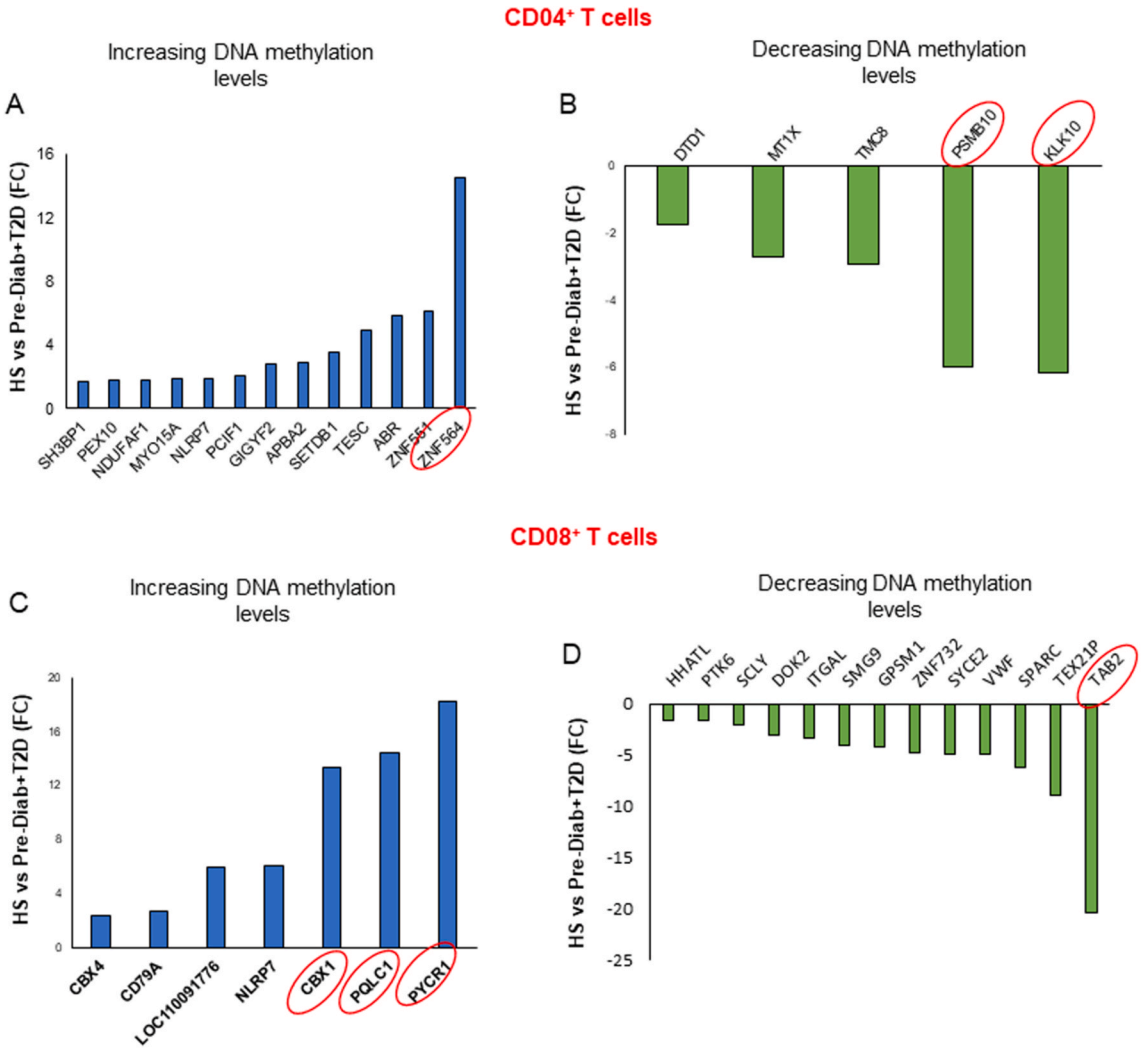

Fig. 2. Trend of methylation in overlapping DMR-related genes. The bar plots show the fold change (FC) of methylation level s associated to the top overlapping DMR-related genes in CD04$^+$ (A and B) and CD08$^+$ (C and D) T cells. Red circles indicate DMR-related genes with the higher FC of methylation in HS vs Pre-Diab and T2D (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

MT1X gene resulted in the highest hypomethylated gene ($q = 3.01E-09$ and $q = 6.21E-10$ in Pre-Diab and T2D, respectively) (Fig. 2B). However, *KLK10* and *PSMB10* genes showed the highest FC of differential methylation (FC=6) both in Pre-Diab and T2D. Interestingly, *MT1X* gene presented an inverse correlation with SBP (−0.70), DBP (−0.82) and AODd (−0.64) in Pre-Diab group and with SBP (−0.55), Glucose (−0.54), Cholesterol (−0.64), LDL (−0.71), LVSD (−0.65) in T2D patients, as well as a positive correlation with Triglycerides (+0.66).

3.1.4. *Overlapping DMRs in CD08$^+$ T of HS vs Pre-diab and T2D*

We identified a total of 60 DMR-annotated genes, of which 40 were hypo- and 19 hypermethylated in HS *vs* hyperglycemia (Supplementary Fig. 5B). We focused on the top 20 highly significant DMR-related genes shared in HS *vs* increasing hyperglycemia, of which 7 were hyper- and 13 were hypomethylated (Supplementary Table 10). Also, we reported two opposite trends for DNA methylation in HS *vs* increasing hyperglycemia. The first trend demonstrated an increased level of DNA methylation from normo- to increasing hyperglycemia (Fig. 2C). *NLRP7* resulted the highest hypermethylated gene ($q = 4.50E-42$ and $q = 3.49E-60$ in Pre-Diab and T2D, respectively). On the other hand, the *PYCR1* gene showed the highest FCof differential methylation (FC=18) in HS *vs* increasing hyperglycemia. The second trend demonstrated a decreasing grade of methylation from normo- to increasing hyperglycemia (Fig. 2D). In particular, *SPARC* resulted the highest hypomethylated gene ($q = 3.77E-18$ and $q = 1.20E-14$ in Pre-Diab and T2D, respectively). *TAB2* gene showed the highest FC (FC= 6) ($q = 1.54E-15$ and $q = 1.51E-07$ in Pre-Diab and T2D, respectively). We noticed that *SPARC* characterized Pre-Diab condition, showing a positive correlation with DBP (+0.76), HDL (+0.54), Creatinine (+0.83), LVDd (+0.98), LVSD (+0.98), LAD (+0.98), LVPWd (+0.84), AODd (+0.81), HR (+0.72), Triglycerides (+0.83), LAD (+0.69) and AODd (+0.52) whereas a negative correlation with Cholesterol (−0.52) and LDL (−0.71) in T2D.

3.2. *Functional analysis*

DMR-related genes of CD04$^+$ T cells in Pre-Diab patients were mainly involved in the early damage to the micro-domains leading to abnormalities of eye morphology, physiology, and movement as well as neurogenesis (Supplementary Table 11). In CD08$^+$ T cells from Pre-Diab patients, DMRs were associated with abnormalities of the central nervous system mainly involving high mental function and brain morphology as well as eye abnormalities. Moreover, it raised during early damage of cardiac muscle tissue, newly developed blood vessels, as well as liver, limb, and muscle (Supplementary Table 12).

Besides, we evaluated the functional characteristics and signaling pathways associated with overlapping DMR-related genes in HS vs

increasing hyperglycemia (Supplementary Table 13). From results, the binding protein was the most prominent in the molecular process group, followed by catalytic activity (mainly hydrolase and transferase enzymes), nucleic acid binding, and transcription process. Interestingly, the human phenotype group highlighted abnormalities of the vasculature, mainly aortic morphology and cardiac system. Moreover, abnormalities in the eye, digestive system, muscle-skeletal system (mainly hypotonia), immune system, and liver were predicted. A detailed GO analysis of significantly DMR-related genes in increasing hyperglycemia was summarized (Supplementary Table 14). At the biological process level, a major regulation of the immune system and neuron development was observed followed by hematopoiesis and response to lipid. KEEG database highlighted the involvement of the insulin signaling pathway, whereas REACTOME database pointed to signal transduction (cytokines, interleukins, receptor tyrosine kinases), immune system, metabolism, transcription regulation, and neural system, mainly protein-protein interactions (PPIs) at synapses.

3.3. Chromatin state discovery

We applied ChromHMM, a machine learning approach evaluating epigenomic information (called marks) across multiple cell types ($CD04^+$ and $CD08^+$ T cells) and multiple conditions (HS, Pre-Diab, T2D). As reported, this method allows us to recognize chromatin states, by identifying their genomic occurrences (see supplementary data). The combination of multiple marks and the relative genomic annotation can be highly informative of distinct biological functions. Starting from 18 emission states (Fig. 3A–C) (Supplementary Table 15), we selected only genomic regions associated with a decreased (state 1) or increased (states 7, 9) methylation level. We found 600 genomic regions associated with hypo-state 1 and 13,947 associated with hyper-states 7 and 9 from Pre-Diab to T2D. GO analysis is illustrated in Fig. 3C and D. Interestingly, we observed that most states were distinctly associated with hypermethylation status during increasing hyperglycemia. From REACTOME, we found 54 genes, such as *CD36*, *PPP2R5C*, and *SOS1* involved in the pathogenesis of "insulin resistance", "glycemic control of T2D" and "CVDs". Moreover, we noticed a lot of genes involved in "innate and adaptive immune system pathways". Finally, also through GO analysis, we found alterations in numerous "human phenotypes". A total of 51 genes characterized the increased "inflammatory response". In particular, some cytokine genes, such as *IL-1*, several complement cascade members, such as *C5* and *C4B* showed a pathogenic role in

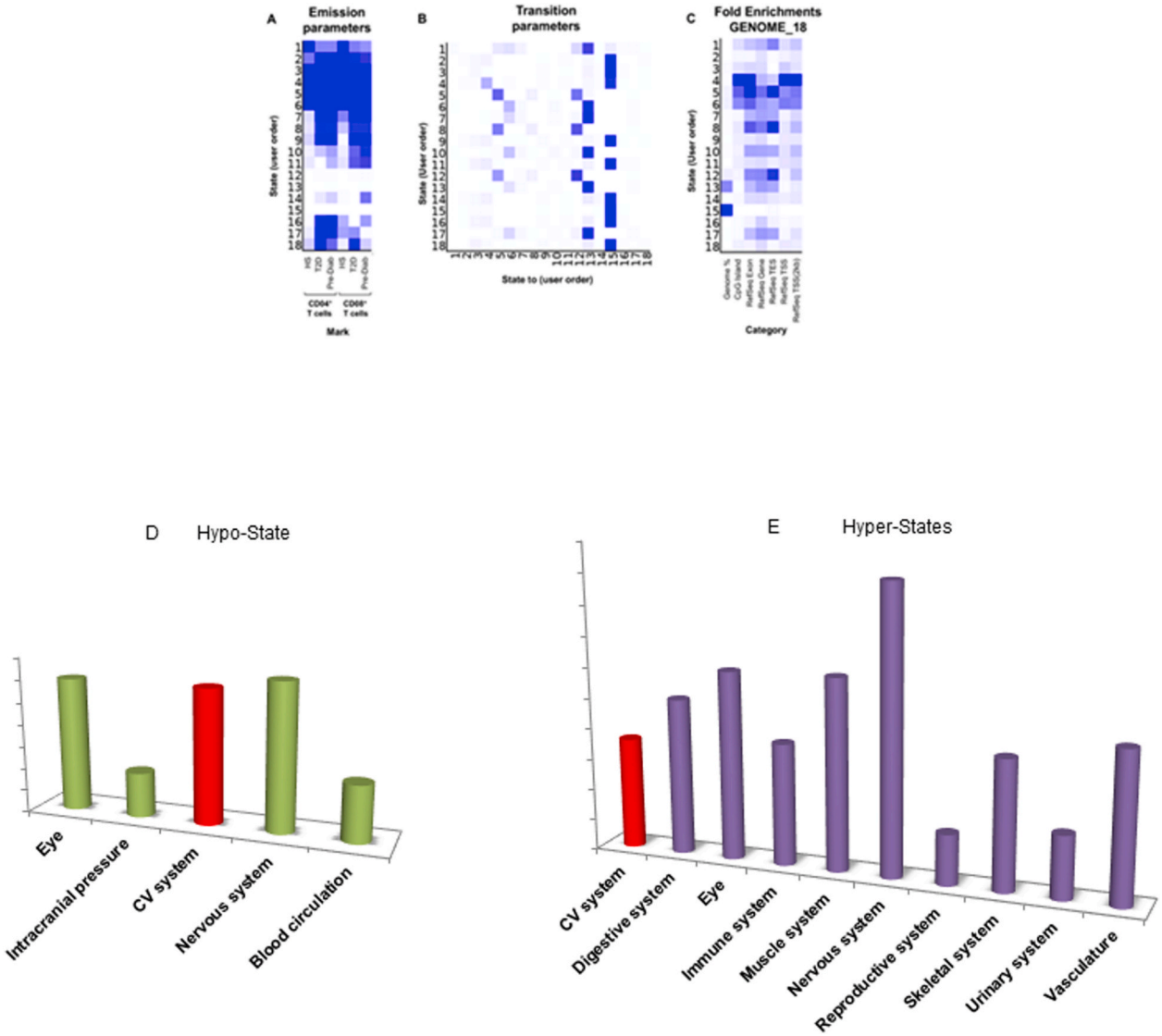

Fig. 3. ChromHMM analysis parameters. In the upper panel, we report the combination of multiple marks (Emission and Transition parameters) (**A-C**) and the relative genomic annotation (Fold Enrichments Genome_18) from 18 emission states. In the lower panel, the bar plots show the enrichment score ($-\log_{10}$Pvalue) for Human Phenotype GO terms from genes associated to hypo- (**D**) and hyper-**(E)** states characterizing hyperglycemic status.

"arterial hypertension", whereas *SMAD3* mediated "diabetic cardiac hypertrophy".

3.4. Gene prediction

We interrogated GeneMANIA to predict *SPARC* interactions, in particular PPIs, and function in the human interactome by selecting only physical interactions. We obtained a network of 21 nodes and 537 total links. We observed that SPARC protein interacts with twenty other proteins which are significantly annotated to an extracellular matrix organization, platelet activation, leukocyte migration, and differentiation as well as organ and embryonic development.

4. Discussion

Our study showed that: 1) the number of hypermethylated DMRs was greater than those hypomethylated in CD04$^+$ T cells during increasing hyperglycemia, whereas the opposite trend was observed in CD08$^+$ T cells; 2) functional analysis of DMR-related genes revealed that in CD04$^+$ T cells early modifications of DNA methylation were already evident in Pre-Diab patients leading to possible ocular damage. Moreover, we did not observe an association between DMRs and vascular damage for T2D patients in CD04$^+$ T cells. In contrast, we observed that different methylation profiles in CD08$^+$ T cells were involved both in micro- and macrovascular damage from Pre-Diab to T2D patients (Fig. 4A); and 3) circulating T cells showed a set of overlapping DMR-related genes with increasing or decreasing levels of DNA methylation from Pre-Diab to T2D patients. Pre-Diab represents a high risk for T2D onset and inflammatory-induced vascular damage in asymptomatic patients [1,2]; however, there are no stringent diagnostic criteria to define when and what pharmacotherapy may aid to prevent cardiac dysfunction at the individual level. [36]

The strengths of our study are that we evaluated and compared the differential DNA methylation profiles of CD04$^+$ and CD08$^+$ T cells focusing on the promoter regions which may contribute to early vascular damage in different stages of impaired glucose homeostasis. Recently, a DNA methylome analysis reported a predominant contribution from CD04$^+$ and CD08$^+$ T cells in regulating expression levels of *IL6R*, *FASLG*, and *CCL18* genes in ACS patients *vs* HS suggesting a significant role in disease pathogenesis [25]. Since the pathogenesis of ACS is related to vasculature damage and diabetes [37], we hypothesized that DNA methylation changes in both CD04$^+$ and CD08$^+$ T cells may reveal early molecular signals of cardiac dysfunction in Pre-Diab and T2D.

Previously, DNA methylome analysis was focused on PBMNCs [11,13] and tissue biopsies [12,38,39] isolated from HS *vs* T2D patients, without considering the Pre-Diab state. Moreover, PBMNCs and tissue biopsies are characterized by cell heterogeneity which does not fit with the cell-specific DNA methylation patterns limiting the identification for starting sites of disease pathogenesis. Another strength of our RRBS analysis is that we specifically analyzed DMRs rather than the methylation level of single CpG dinucleotides. In fact, DMRs can control spatiotemporal gene expression, have the most statistical power and by-pass putative effects of genetic polymorphisms during epigenome-wide association studies [40].

Our epigenetic trajectories suggested that hyperglycemia might early affect the interactome of both CD04$^+$ and CD08$^+$ T cells already in Pre-Diab patients by regulating DNA methylation at a different set of genes. Interestingly, *SPARC* was the most hypomethylated gene in Pre-Diab and its methylation level gradually decreased in CD08$^+$ of T2D patients. *SPARC* gene encodes for a multifunctional protein modulating the interaction between cells and the extra-cellular matrix (ECM) by the regulation of collagen and vitronectin [41]. SPARC protein is involved in many processes including wound healing, inflammation, angiogenesis, cardiac remodeling, and modulation of growth factor signaling [41]. Moreover, *SPARC* is expressed in adipocytes and pancreatic cells with profibrotic effects [41].

Since the negative correlation between DNA methylation promoter and gene expression, we would expect gradually increased levels of SPARC protein from Pre-Diab to T2D patients. Previous studies reported that increased plasma levels of SPARC protein were correlated to inflammation, insulin resistance, and dyslipidemias in gestational diabetes [42]. Moreover, SPARC protein was increased in plasma of hyperglycemic patients, also correlating with early nephropathy in T2D [43]. This evidence fits with our expected trend, for which DNA hypomethylation of *SPARC* promoter could increase levels of gene expression in increasing hyperglycemia. Besides, our network analysis predicted an

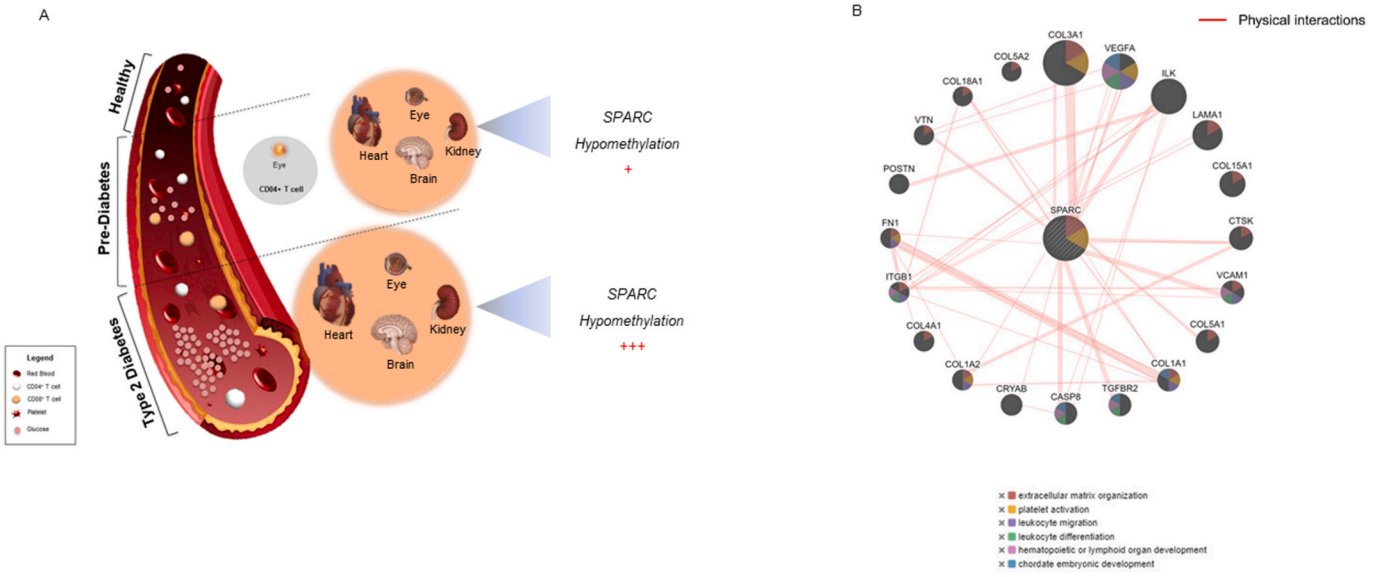

Fig. 4. A) Association between DNA methylation profile and vascular damage in increasing hyperglycemia. Early modifications of DNA methylation underlying microvascular damages appear in CD04$^+$ T cells of Pre-Diab patients . Otherwise, CD08$^+$ T cells undergo to changes in DNA methylation already in Pre-Diab and persist in T2D state patients leading to abnormalities of micro- and macro-domains in vasculature of hyperglycemic patients. **B) GeneMANIA network.** GeneMANIA PPI network of the *SPARC* gene predicted 21 nodes and a total of 537 total links representing physical interactions mainly involved in extracellular matrix organization, platelet activation, and leukocyte migration.

interesting physical interaction between SPARC and VEGFA. Since VEGFA is commonly deregulated in diabetic-related microvascular damage [44], we emphasized that a possible regulatory interaction between SPARC and VEGFA proteins should be investigated in Pre-Diab.

Targeted quantification of *SPARC* mRNA levels should be performed to confirm whether these changes of DNA methylation lead to modulation of gene expression in $CD08^+$ T cells and/or other tissues isolated from Pre-Diab and T2D patients.

5. Conclusion

The adaptive immune response is strongly involved during progression from HS to Pre-Diab, T2D, and insulin therapy; thus, novel pathogenic mechanisms may improve primary prevention of CVDs [7,36] This is the first molecular-bioinformatic approach combining RRBS DNA methylome analysis and clinical data in different stages of impaired glucose homeostasis in 2 subtypes of cells (e.g., $CD04^+$ and $CD08^+$ T cells). Our pilot study established that the differential methylation of genes involved in T2D pathogenesis, such as *SPARC*, correlated with DBP, Creatinine, LVDd, LVSD, LAD, LVPWd, and AODd. Our data needs to be confirmed in large multicenter studies. This evidence suggested a putative biomarker useful to early diagnose Pre-Diab patients and predict the presence/absence of vascular damage and kidney complications. Further longitudinal clinical trials combining network-oriented analysis and liquid-based assays should be performed to identify the precise epigenetic-sensitive pathways involved in transition from normoglycemia to Pre-diab state which may increase the individual risk for vascular damage later in life [7,24,30,45,46].

Ethical approval

This study was approved by the local Ethical Committee of the Department of Advanced Medical and Surgical Sciences (DAMMS), University of Campania “Luigi Vanvitelli”, Italy (Naples) (Protocol N. 114).

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Author contribution

G.B., M.F., C.S., R.M. and C.N. contributed to the design of the study, analyzed data, contributed to the discussion, and wrote the manuscript. M.F., M.Z., and O.A. performed informatic/statistical analysis. G.B., M. F. and C.S analyzed bioinformatic data. M.M., and T.I. performed experimental procedures, P.P., C.S., and R.M. recruited patients. G.B is principal investigator of PIRAMIDE. G.M., L.S., R.M., M.F., G.P., G.D.V, G.F., G.F.N., M.S., G.P. and C.N. supervised the manuscript. All authors approved the final version of the manuscript. R.M., M.F., and G.B. are the guarantors of this work and, as such, had full access to all of the data in the study and take the responsibility for the integrity of the data and the accuracy of the data analysis.

Conflicts of interest

All Authors declare no conflict of interest.

Consent

Written informed consent was obtained from the patient for publication of this study. A copy of the written consent is available for review by the Editor-in-Chief of this journal on request.

Registration of research studies

Name of the registry: ClinicalTrials.gov.

Unique Identifying number or registration ID: NCT03792607.

Hyperlink to your specific registration (must be publicly accessible and will be checked): https://clinicaltrials.gov/ct2/show/NCT03792607?cond=NCT03792607&draw=2&rank=1.

Guarantor

G.B. and C.N. are the Guarantors of this work.

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Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.amsu.2020.10.016.

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