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1 **(-)-Epicatechins promote vascular health through epigenetic reprogramming of**
2 **endothelial-immune cell signaling and reversing systemic low-grade inflammation**

3
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32 **Abstract**

33 Ingestion of (-)-epicatechin flavanols reverses endothelial dysfunction by increasing flow mediated
34 dilation and by reducing vascular inflammation and oxidative stress, monocyte-endothelial cell
35 adhesion and transendothelial monocyte migration *in vitro* and *in vivo*. This involves multiple
36 changes in gene expression and epigenetic DNA methylation by poorly understood mechanisms.
37 By *in silico* docking and molecular modeling we demonstrate favorable binding of different
38 glucuronidated, sulfated or methylated (-)-epicatechin metabolites to different DNA
39 methyltransferases (DNMT1/DNMT3A). In favour of this model, genomewide DNA methylation
40 profiling of endothelial cells treated with TNF and different (-)-epicatechin metabolites revealed
41 specific DNA methylation changes in gene networks controlling cell adhesion-extravasation
42 endothelial hyperpermeability as well as gamma-aminobutyric acid, renin-angiotensin and nitric
43 oxide hypertension pathways. Remarkably, blood epigenetic profiles of an 8 week (-)-epicatechin
44 diet intervention in male smokers revealed individual epigenetic gene changes targeting similar
45 pathways as the *in vitro* exposure experiments in endothelial cells. Furthermore, epigenetic changes
46 following (-)-epicatechin diet intervention oppose atherosclerosis associated epigenetic changes.
47 In line with biological data, the individual epigenetic response to an (-)-epicatechin diet is
48 associated with different vascular health parameters (glutathione peroxidase 1 and endothelin-1
49 expression, acetylcholine response), in part involving systemic shifts in blood immune cell types
50 which reduce the neutrophil-lymphocyte ratio (NLR). Altogether, our study suggests that different
51 (-)-epicatechin metabolites promote vascular health in part via epigenetic reprogramming of
52 endothelial-immune cell signaling and reversing systemic low-grade inflammation.

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57 **1. Introduction**

58 Flavonoids are a group of polyphenolic compounds and contribute to the beneficial health effects
59 of fruits and vegetables [1]. In the context of cardiovascular health, flavonoids are of interest,
60 because the habitual intake of flavanols, proanthocyanidins and theaflavins has been associated
61 with reduced coronary artery disease (CAD) risk and mortality [2, 3]. Flavanols consist of
62 monomeric catechin and epicatechin, and oligomeric procyanidins, among others, and have been
63 mainly found in cocoa, apples and tea. Only monomers and dimers are absorbed in the small
64 intestine, and undergo extensive metabolization in the gut and liver leading to glucuronidated,
65 sulphated and methylated epicatechin and γ -valerolactone metabolites [4-6].

66 Experimental studies and randomized controlled trials (RCTs) further confirm the cardioprotective
67 effects of flavanols [7] including effects on blood pressure [8, 9], vasodilation [9, 10], pulse wave
68 velocity [9] and LDL cholesterol [11]. The most consistent effect is on endothelial function
69 measured by flow-mediated vasodilation (FMD) [12], which is an independent risk factor for
70 cardiovascular diseases. Multiple RCTs showed an increase in FMD and reduction in blood
71 pressure, both in CVD high risk and low risk individuals [9, 13]. This effect is mainly due to the
72 enhancement of NO synthesis and levels in the vascularity [14]. Furthermore, (-)-epicatechin and
73 its circulating metabolite epicatechin-7-O-glucuronide were found the major compounds
74 responsible for this increase in FMD after the consumption of cocoa flavanol-rich diet [7].

75 Another way how flavanols may beneficial impact endothelial function and prevent cardiovascular
76 diseases is by reducing the recruitment and migration of monocytes into the arterial wall.
77 Accumulated oxidized LDL cholesterol in the intima leads to the expression of various cell adhesion
78 and chemokine proteins in endothelial cells. Circulating monocytes are subsequently recruited to
79 the endothelial wall and migrate into the intima where they differentiate into macrophages [15].
80 Previously, we have demonstrated that (-)-epicatechin metabolites decrease pro-inflammatory
81 interactions between monocytes and endothelial cells [16]. Moreover, we demonstrated that
82 monocyte-endothelial cell adhesion and leukocyte rolling induced by an inflammatory stimulus
83 was reduced upon pre-treatment with a mixture of 3 epicatechin metabolites *in vitro* and *in vivo*
84 [17]. Upon integration of transcriptome, miRNAome and DNA methylome profiles of endothelial
85 cells exposed to a mix of (-)-epicatechin metabolites, common targeted pathways were identified
86 related to endothelial cell adhesion and transmigration. These results indicate that (-)-epicatechin
87 may prevent monocyte recruitment and transendothelial migration through the modulation of gene

88 expression, miRNA expression and DNA methylation. Epigenetic effects of (-)-epicatechin
89 metabolites on endothelial DNA methylation provides an attractive working model explaining a
90 potential mechanism of action underlying their health properties because DNA methylation can be
91 seen as an important mediator in memorizing past adverse environmental effects. Indeed, previous
92 studies demonstrated that LDL cholesterol, homocysteine and disturbed blood flow induces DNA
93 methylation changes in endothelial cells [18-20]. It has been suggested that dietary polyphenols
94 promote cardiovascular health by targeting multiple classes of epigenetic writer-reader-eraser
95 enzymes related to histone acetylation-methylation and DNA methylation [21].
96 The aim of this study was to evaluate whether physiological concentrations of glucuronidated,
97 sulphated or methylated (-)-epicatechin metabolites trigger epigenetic DNA methylation changes
98 related to cardiovascular health *in vitro* and *in vivo* in a diet intervention study.

99
100

101 **2. Material and Methods**

102

103 **2.1 (-)-epicatechin flavanol metabolites**

104 (-)-epicatechin metabolites: 4'-*O*-methyl(-)-epicatechin (4'MEC), 4'-*O*-methyl(-)-epicatechin-7-
105 β -D-glucuronide (4'MEC7G) and (-)-epicatechin-4'-sulfate (EC4'S) were gifted by Mars Inc
106 (McLean, VA, USA). Conjugated metabolites were dissolved in 50% ethanol in double distilled
107 water and diluted to a final working concentration of 1 μ M in cell medium for cell culture
108 experiments. An 1 μ M (-) epicatechin mix was prepared containing equimolar fractions of the 3
109 metabolites. TNF α was obtained from R&D Systems (Lille, France) and was dissolved in 0.1%
110 bovin serum albumin (St. Louis, MA, USA).

111

112 **2.2 Cell culture conditions**

113 Human umbilical vein endothelial cells (HUVEC) (Lonza, Walkersville, MD, USA) were cultured
114 in a phenol-free endothelial growth medium (EGM) supplemented with 2% fetal bovine serum
115 (FBS), 0.4% fibroblast growth factor, 0.1% vascular endothelial growth factor, 0.1% heparin, 0.1%
116 insulin-like growth factor, 0.1% ascorbic acid, 0.1% epidermal growth factor and 0.04%
117 hydrocortisone (all from Lonza). HUVECs, at passage 4, were seeded into the 24-well plates (BD-
118 Falcon, Le Pont-De-Claix, France) and grown to reach 60-70% confluence. The medium was then
119 replaced to expose the cells for 3h to the culture medium containing either solvent alone (ethanol
120 0.5%, control wells, vehicle) or different metabolites: 4'-*O*-methyl(-)-epicatechin, 4'-*O*-methyl(-)-
121 epicatechin-7- β -D-glucuronide or (-)-epicatechin-4'-sulfate at 1 μ M. At the end of the incubation
122 period, the medium was replaced with flavanol free medium and left for further 18h until
123 confluence. The confluent monolayer was then stimulated for 4 hours with TNF α (R&D Systems
124 Lille, France) at 0.1 ng/mL or only incubated with PBS/BSA (0,001%, negative control).
125 Following gDNA isolation, genomewide DNA methylation profiles were generated by Illumina
126 450k BeadChip (Illumina, San Diego, CA, USA) arrays covering >450,000 CpG dinucleotides.

127

128 **2.3 Genomic DNA extraction**

129 Genomic DNA (gDNA) from HUVEC was extracted using DNeasy kit (Qiagen, Courtaboeuf,
130 France). DNA purity and concentrations were determined by UV-VIS spectrophotometry
131 (NanoDrop, Thermo Fisher Scientific Inc, Wilmington, DE, USA) and stored at -80°C until further

132 use. For each sample 1 μg of gDNA was bisulfite converted using the EZ DNA Methylation kit
133 (Zymo research, Irvine, CA, USA) according to manufacturer's instruction. Successful bisulfite
134 conversion was confirmed by performing a methylation-sensitive PCR in a region of the SALL3
135 gene. (see [22] for primer sequences).

136

137 **2.4 DNA-methylation arrays**

138 Bisulfite converted DNA from the HUVECs was hybridized to the Illumina HumanMethylation450
139 BeadChip arrays (Illumina, San Diego, CA, USA). Briefly, for each sample, 1 μg of genomic DNA
140 was bisulfite-converted using an EZ DNA methylation Kit (ZYMO research, Irvine, CA, USA)
141 according to the manufacturer's recommendations. Converted gDNA was eluted in 22 μl of elution
142 buffer. DNA methylation level was measured using the Illumina Infinium HD Methylation Assay
143 (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, 4 μl of
144 bisulfite-converted DNA (± 150 ng) was isothermally amplified overnight (20–24h) and fragmented
145 enzymatically. Precipitated DNA was resuspended in hybridization buffer and dispensed onto the
146 Infinium HumanMethylation450 BeadChips (12 samples/chip) using a Freedom EVO robot
147 (Tecan, Männedorf, Switzerland). The hybridization procedure was performed at 48°C overnight
148 (16–20 h) using an Illumina Hybridization oven. After hybridization, free DNA was washed away
149 and the BeadChips were processed through a single nucleotide extension followed by fluorescent
150 readout of the incorporated base using a Freedom EVO robot. Finally, the BeadChips were imaged
151 using an Illumina iScan (Illumina, San Diego, CA, USA). Raw Illumina450K data have been
152 deposited in the GEO-profile database as GSE133418 (in vitro methylation data), GSE107143
153 (atherosclerosis methylation data) [23] and GSE54643 (epicatechin diet intervention methylation
154 data) [24]. Detection p-values were calculated to identify failed probes as per Illumina's
155 recommendations. No arrays exceeded our quality threshold of >5% failed probes. Filtering of bad
156 quality probes and normalization of raw methylation beta values was conducted using RnBeads
157 package in R software [25]. Probes with detection p-values higher than 0.01, overlapping with
158 SNPs at the last 3 bases in its sequence or containing missing values were excluded. Beta mixture
159 quantile dilation (BMIQ) was used to normalize between the two different probe designs (Infinium
160 I and Infinium II) [26]. Differentially methylated probes were identified using moderated t-test in
161 the limma R package. Probes with a p-value below 0.01 and a methylation difference of at least
162 5% were defined as differentially methylated probes (DMPs). Probes were assigned to genes, CpG

163 island annotations (CpG island, CGI shores, CGI shelves, and open sea) and gene regions
164 (TSS1500, TSS200, 5'UTR, 1st exon, gene body and 3'UTR) based on the HumanMethylation450
165 v1.2 Manifest file from Illumina. Probes were also mapped to chromatin segmentation states
166 obtained from the UCSC genome browser. Enrichment of DMPs in different genomic regions was
167 measured by the Fisher's Exact test. Blood cell type composition was estimated using the
168 reference-based method of Houseman with the EpiDISH R package [27, 28]. All analyses were
169 performed in R and R Studio. Epigenetic age was estimated using the epigenetic clock of Horvath
170 [29].

171

172 **2.5 Bioinformatic analysis**

173 DMPs were mapped to genes according the HumanMethylation450 v1.2 Manifest file from
174 Illumina. Corresponding gene lists were used to perform pathway enrichment analysis using
175 Ingenuity Pathway Analysis (IPA) software, KEGG (Kyoto Encyclopedia of Genes and Genomes)
176 (http://www.genome.jp/kegg/tool/map_pathway2.html) and Metacore software packages
177 Metacore™ (GeneGo, St Joseph, MO, USA; <https://portal.genego.com>). Enrichment statistics were
178 calculated for these data sets assuming a hypergeometric distribution to identify significantly
179 overrepresented pathways. Canonical pathways were considered significant overrepresented when
180 they had a Fisher's Exact p-value < 0.05. Venn diagrams were created using the VennDiagram R
181 package. Partial Least Squares Discriminant Analysis (PLS-DA) multivariate analysis was
182 performed using the mixOmics R package. Gene network analyses were searched using text-
183 mining algorithm of MetaCore software from Clarivate Analytics (<https://portal.genego.com>). To
184 identify pathways that are significantly associated with the genes, we used the web tool GeneTrail2
185 (<https://genetrail2.bioinf.uni-sb.de/>) [30], version 1.6, as a platform to access KEGG and BioCarta
186 databases. Interactions between functional groups of genes were searched using the online tool
187 Metascape (<http://metascape.org>) [31]. The obtained network was further visualized using
188 Cytoscape platform for molecular interaction networks visualization (<https://cytoscape.org/>) [32]

189

190 **2.6 mm-GBSA binding affinity calculations**

191 *2.6.1 Preparation of proteins and ligands:* The atomic coordinates of both human DNMT1 and
192 DNMT3A proteins were retrieved from the Protein Data Bank (PDB, <https://www.rcsb.org>). Chain
193 A of 4WXX crystal structure was chosen for the docking simulations of DNMT1. In the absence

194 of a full protein crystal structure of DNMT3A, 3LLR and 4U7T crystal structures were chosen to
195 study amino acids 275-427 and 476-912, respectively. Proteins were prepared for docking using
196 the default parameters of Protein Preparation Wizard implemented in Maestro, 2017-4 version
197 (Schrödinger, New York, NY, USA). Ligand 2D structure was retrieved from PubChem database
198 (<https://pubchem.ncbi.nlm.nih.gov>, NIH, Bethesda, MD, USA) as SDF. (-)-epicatechin, 4'MEC,
199 4'MEC7G and EC4'S, sucrose and naphthalene have a PubChem CID of 72276, 11420256,
200 131750802 71579043, 5988 and 931, respectively. Ligands' 3D structure was generated using the
201 LigPrep command in Maestro.

202 *2.6.2 Docking model and mm-GBSA calculation:* The sitemap algorithm (Schrödinger, New York,
203 NY, USA) was used to determine five possible docking sites of DNMT1, which were later
204 identified by comparing to domain information from UniProt (<https://www.uniprot.org>). 3PTA a
205 crystal structure of DNMT1 in complex with DNA was used as a reference to the DNA binding
206 domain of the DNMT1. Three of the predicted binding sites, a site at the DNA binding domain and
207 two sites at the catalytic domain were chosen for further analysis. Four binding sites were
208 determined For DNMT3A. 3LLR is the crystal structure of the histone binding PWWP domain. A
209 grid of the histone binding site was generated based on the centroid of histone H3 peptide from the
210 5CIU crystal structure of DNMT3B PWWP domain in complex with H3. In addition, three binding
211 sites of DNMT3A were determined by the protein interactions in the 4U7T crystal structure of the
212 heterotetramer DNMT3L-DNMT3A-DNMT3A-DNMT3L in complex with histone H3. A grid of
213 the histone binding site was generated based on the centroid of histone H3 peptide. Grids of
214 DNMT3A-DNMT3L and DNMT3A-DNMT3A binding sites were generated as a centroid of
215 DNMT3L (chain b) and DNMT3A (chain c) amino acids in a range of 3Å from DNMT3A (chain
216 a), respectively. In each site, default Glide options for extra precision were used for the docking
217 model with the exception of using 30 poses for the number of poses to include and ten poses for
218 the number of poses to write out. For each ligand, docking pose with the lowest Glide emodel score
219 was selected for further analysis. Binding free energy was calculated using the Prime mm-GBSA
220 package (Schrödinger, New York, NY) as follows: $\Delta G(\text{bind}) = E(\text{complex}) - [E(\text{ligand}) +$
221 $E(\text{receptor})]$

222 3 Results

223 3.1 In silico modeling of different (-)-epicatechin metabolites to DNMT1 and DNMT3A 224 reveals favorable binding modes.

225 Based on *in silico* models of (-)epicatechin binding to DNMTs in nutriepigenetic studies with green
226 tea [33, 34], we compared DNMT binding properties of different (-)-epicatechin metabolites
227 identified in cocoa flavanol diet intervention studies, i.e. 4'-*O*-methyl(-)-epicatechin (4'MEC), 4'-
228 *O*-methyl(-)-epicatechin-7- β -D-glucuronide (4'MEC7G) and (-)-epicatechin-4'-sulfate (EC4'S)
229 (**Figure 1A**). Using the sitemap algorithm, we were able to predict five potential docking sites in
230 DNMT1 (**Figure 1B-G**), of which only those located at regions with defined function were selected
231 for the docking simulation: one site located at the DNA binding site of the protein and two sites
232 located at the catalytic domain. DNMT3A is active as a hetero-tetrameric (DNMT3L-DNMT3A-
233 DNMT3A-DNMT3L) protein. Four docking sites were determined from the 3D structure: the
234 PWWP domain, usually found in DNA-binding proteins, the H3 histone binding site and two
235 protein-protein interaction sites (DNMT3A-DNMT3A and DNMT3A-DNMT3L) (**Figure 1E-H**).
236 Using docking simulation, we propose possible binding modes of of EC4'S in both active sites and
237 regulatory regions of DNMT1 and DNMT3A (shown in enlarged rectangle). Hydrogen bonds are
238 represented as light blue dashed lines (**Figure 1B-H**).

239 The mm-GBSA method serves for ranking ligands with similar scaffold according to their
240 calculated binding affinity [35]. One way to validate the calculation-based predictions is to
241 demonstrate their specificity to the active molecules in question. We have calculated the binding
242 potential of sucrose, an H-bond donor-rich molecule, and of naphthalene, a hydrophobic molecule,
243 both without effect on the mechanisms discussed here, as a benchmark for metabolites' binding
244 potency. Our predictions demonstrate the highest binding affinities to most of the tested binding
245 sites for one metabolite, i.e., 4'MEC7G (**Table 1**). The calculated binding energy (ΔG) of
246 4'MEC7G was 12.83-34.76 kcal/mol lower than of the control molecules, in all sites except for
247 one site located in the DNMT1 catalytic domain. However, both 4'MEC and MEC4'S presented
248 potential affinities for DNA binding site but not for catalytic domain I and II. Interestingly,
249 epicatechin metabolites present higher affinities for DNMT1 than the parent compound. As for
250 DNMT1, 4MEC7G was also predicted to have highest affinity for DNMT3A, with calculated
251 binding energy (ΔG) of 4MEC7G estimated to be 13.27-29.61 kcal/mol lower than of the control

252 molecules for all 4 tested sites. In our analyses, 4'MEC presented the highest binding affinity for
253 PWWP domain and EC4'S H3 peptide binding site (**Table 1**).

254

255 **3.2 (-)-Epicatechin metabolites trigger common endothelial DNA methylation changes** 256 **opposite to TNF treatment**

257 Previously, (-)-epicatechin specific inhibition of DNMT activity has been demonstrated *in vitro* for
258 Ss1 DNMT and human DNMT1 enzymes, with IC50 values of respectively 8.1 and 8.4 μM [34].
259 Here, we further compared epigenetic DNA methylation changes in TNF treated HUVEC cells,
260 either or not, following pretreatment with each of the 3 different (-)-epicatechin metabolites.
261 Briefly, subconfluent HUVEC cells were exposed for 3 h to control medium (i.e. culture medium
262 with 0.5%, ethanol solvent) or culture medium containing 1 μM of individual flavanol metabolites
263 4'-O-methyl(-)-epicatechin (4'MEC), 4'-O-methyl(-)-epicatechin-7- β -D-glucuronide (4'MEC7G)
264 or (-)-epicatechin-4'-sulfate (EC4'S), which corresponds with maximum plasma concentrations of
265 metabolites detected in cocoa diet intervention studies [17, 36-38]. After 3h (-)-epicatechin
266 treatment, the medium was replaced with flavanol free medium and left for 18h untreated, before
267 adding TNF α for 4h. Following gDNA isolation and bisulfite treatment, genomewide DNA
268 methylation profiles were generated by Illumina 450k BeadChip arrays covering >450,000 CpG
269 dinucleotides (GSE133418) [17]. Reliability of our DNA methylation profiling approach was
270 confirmed upon comparing the endothelial DNA methylation profile of HUVEC control cells with
271 independent HUVEC DNA methylation profiles available in the ENCODE dataset GSE82234,
272 which revealed highly significant correlation (Pearson correlation > 0.98) [17] (data not shown).
273 Next, partial least squares discriminant multivariate analysis (PLS-DA) of epigenetic profiles
274 revealed clear separation between untreated cells (control), TNF treated cells, and TNF treated cells
275 following (-)-epicatechin metabolite pretreatment (**Figure 2A**). More specifically, 4h TNF α
276 treatment elicits significant DNA methylation. changes ($P < 0.01$ and $\Delta \beta\text{-value} > 0.05$) of 559
277 hypermethylated and 198 hypomethylated CpG probes (**Figure 2B**). Since the profiles of the 3 (-
278)-epicatechin metabolites 4'MEC, 4'MEC7G and EC4'S clustered together, this suggests clear
279 similarities in DNA methylation changes by the different epicatechin metabolites.
280 Pretreatment of endothelial cells to 4'MEC, 4'MEC7G or EC4'S followed by TNF treatment
281 (**Figure 3B**) triggered respectively hypomethylation of 393, 740 and 548 CpG and
282 hypermethylation of 313, 902 and 848 CpG probes as compared to TNF treatment alone (**Figure**

283 **2B**). Analyses of the methylation changes across chromosomes in a Manhattan plot showed a
284 widespread distribution across the genome (**Figure 2C**). More particularly, we observed an
285 enrichment of differentially methylated probes (DMPs) in intergenic regions, and a depletion in
286 regions around the transcription start site (TSS) of genes when compared to all the Illumina CpG
287 probes (**Figure 3A**). Along the same line, mapping DMPs to chromatin states confirmed a depletion
288 in promoter states. Especially hypo-DMPs were slightly enriched in gene bodies and 3'UTRs. Both
289 hypo- and hyper-DMPs were found to be depleted in CpG islands, and were therefore mainly
290 located in CpG-poor regions. DMPs were weakly enriched in enhancer regions, although
291 significance ($p < 0.05$) could not be reached in all contrasts. An enrichment of DMPs in
292 heterochromatin states was found for all contrasts.

293 To check whether the hyper- or hypomethylation changes were similar between the 3 different
294 flavanol metabolites and TNF versus TNF treatment alone, the $-\log_{10}(p\text{-values})$ of each probe were
295 calculated for each contrast and made negative when hypomethylated and kept positive when
296 hypermethylated. Upon comparing these $-\log_{10}(p\text{-values})$, strong positive Pearson correlations
297 were found across the different metabolites (r between 0.47 and 0.51) (**Figure 3B**), indicating that
298 the 3 different flavanol metabolites induce similar epicatechin specific DNA
299 hyper/hypomethylation changes in line with the PLS-DA analysis (**Figure 2A**). In contrast, when
300 comparing epigenetic changes of each (-)-epicatechin metabolite and TNF versus TNF treatment
301 with epigenetic changes of TNF treatment versus control cells, a negative Pearson correlation (r
302 between -0.26 and -0.33) was found between the $-\log_{10}(p\text{-values})$. This indicates that in general
303 the 3 (-)-epicatechin metabolites trigger opposite epigenetic changes in comparison to TNF induced
304 DNA methylation effects (**Figure 3C**).

305

306 **3.3. (-)-Epicatechin metabolites trigger common endothelial DNA methylation changes** 307 **in genes involved in cell adhesion, cytoskeletal organization, renin angiotensin, nitric oxide** 308 **hypertension and axonal guidance signaling pathways**

309 We further applied respectively Metacore gene network, KEGG and Ingenuity pathway
310 enrichment analysis on DMP associated gene lists of the 3 different (-)-epicatechin metabolite
311 treatments and TNF combination treatments relative to TNF treatment alone, which identified 81
312 common differentially methylated target genes (**Figure 4A**). The enriched gene networks (**Figure**
313 **4B**) and pathways (**Figure 4C-D**) were ranked again based on the sum of the $-\log_{10}$ enrichment

314 p-values, in each treatment contrast. In this way, networks or pathways which are enriched in more
315 than one contrast are therefore in common across the treatments and ranked first. Among the top
316 ranked pathways, we found interesting pathways including cell adhesion, axonal guidance, netrin,
317 ephrin and integrin signaling, actin cytoskeletal signaling, vascular morphogenesis, hedgehog and
318 wnt signaling. These enriched pathways are involved in various aspects of atherogenesis via
319 eliciting endothelial dysfunction, monocyte attraction, leukocyte infiltration, monocyte-
320 macrophage retention, platelet hyperreactivity, and neovascularization [39-43]. Moreover, the
321 results are in line with previously obtained *in vitro* and *in vivo* biological data, showing that
322 epicatechin metabolites mediate their vasculoprotective effects through dynamic regulation of
323 endothelial cell monocyte adhesion and permeability [17]. IPA pathway analysis (**Figure 4D**)
324 also identified significant gamma-aminobutyric acid (GABA) and pathway enrichment in response
325 to the 3 (-)-epicatechin metabolites. Recent evidence revealed a neuro-cardioprotective role for
326 GABA against oxidative endothelial injury and regulation of endothelial nitric oxide eNOS [44,
327 45]. Also renin-angiotensin and nitric oxide signaling pathways are enriched which play critical
328 roles in hypertension and may contribute in cardioprotective blood pressure-lowering effects of (-
329)-epicatechins [12, 46-48]. Endocrine flavanol functions are suggested via regulation of
330 gonadotropin receptor hormone (GnRH) and androgen receptor (AR) signaling, involved in plaque
331 stabilization and/or rupture [49-51]. Finally, integrated Metascape protein network analysis
332 revealed various links between the different (-)-epicatechin specific biological processes related
333 to focal adhesion, signal transduction, cancer development, G-alpha protein signaling and axon
334 guidance (**Figure 4E**).

335 336 **3.4. (-)-Epicatechin metabolites and TNF trigger opposite endothelial DNA methylation** 337 **changes in cell junction associated genes**

338 Out of 81 epicatechin specific gene associated DMPs (**Figure 4A**), we identified 26 genes for
339 which opposite methylation changes were detected by at least one (-)-epicatechin metabolite in
340 comparison to TNF treatment alone (USP35, PCDHGA1, SMOC2, SDK1, SKI, GLUD1P3, SOX1,
341 DOCK5, AKAP12, PTPRN2, NLGN1, FLJ38576, TNXB, DLGAP2, SLC38A4, BANP, LAS1L,
342 XKR4, DACT1, SYNPR, FTCDNL1, GLI3, PCDH9, PTCHD3, ERICH1-AS1, KDM2B) (**Figure**
343 **5A**). Besides, TNF induces also multiple DNA methylation changes which are not responsive to
344 (-)-epicatechin treatment (**Figure 5A**). Heatmap analysis of DNA methylation changes of these 26

345 genes identified 2 major clusters of opposite DNA methylation changes by TNF and the 3 (-)
346)epicatechin metabolites (**Figure 5B**). Cluster 1 reveals 5 hypomethylated TNF responsive genes,
347 which remain hypermethylated upon pretreatment with the 3 (-) epicatechin metabolites (**Table 2**).
348 The opposite pattern can be seen in cluster 2, showing 11 hypermethylated TNF responsive genes,
349 which remain hypomethylated upon pretreatment with the 3 (-)-epicatechin metabolites, as
350 illustrated for the gene USP35 (**Table 3**) (**Figure 5C**). Pathway enrichment analysis of the shortlist
351 of 26 genes, reveals significant enrichment of cell junction regulation (GO:0030054, P-
352 value= 9.470×10^{-3}), which is critically involved in endothelial permeability and transendothelial
353 monocyte migration [16, 17]. Endothelial cell–cell junctions are critical for vascular integrity,
354 whereas their dysfunction and endothelial hyperpermeability represents an initiating step in early
355 atherosclerosis and coronary artery disease [52-54]. In support of our results, TNF has previously
356 been shown to increase endothelial permeability and promote monocyte transendothelial migration,
357 whereas (-)-epicatechin (pre)treatment antagonizes inflammation associated endothelial
358 hyperpermeability [17, 55-57].

359

360 **3.5. Individual DNA methylation response of (-)-epicatechin diet intervention targets genes** 361 **involved in cell adhesion, cytoskeletal organization, renin angiotensin, nitric oxide** 362 **hypertension and axonal guidance signaling pathways**

363 A double-blind, randomized, placebo-controlled intervention study revealed that the daily
364 consumption of monomeric and oligomeric (-)-epicatechin flavanols (MOF) derived from seeds of
365 grapes (*Vitis vinifera* L.) for 8 weeks accomplishes a vascular health benefit in male
366 smokers [58]. Corresponding DNA methylation levels of blood samples were measured before
367 and after the diet intervention using the 450k Illumina array platform, but no common diet
368 responsive CpG probe was found to be differentially methylated due to strong interindividual
369 epigenetic variation in baseline methylation levels and variable response to a (-)-epicatechin rich
370 diet intervention [24] (GEO-data available at GSE54690). Upon reanalysis, we now selected probes
371 that changed in methylation by at least 0.1 in beta-value (corresponding with >10% methylation
372 change) after epicatechin diet intervention for every individual participant of the study. We noticed
373 that some individuals (SN_110, SN_109 and SN_114) showed a strong epigenetic response with a
374 total number of DMPs between 5,469 and 15,189, while other individuals showed much less
375 difference in methylation changes after the (-)-epicatechin intervention (**Figure 6A**). We could not

376 identify a common CpG probe which was differentially methylated in every individual and only a
377 small fraction of the probes was affected in more than one individual (**Figure 6B**). Next, we
378 performed IPA pathway analysis for every individual for the corresponding CpG probes that
379 showed a change in methylation of at least 10%. To compare the enriched pathways among the
380 individuals, we ranked the pathways based on the sum of the $-\log_{10}$ enrichment pvalues in each
381 individual. In this way, the pathways which were enriched in more than one individual were ranked
382 first (**Figure 6C**). In addition, $-\log_{10}$ enrichment pvalues of pathway analysis of the *in vitro*
383 experiments with the different (-)-epicatechin metabolites or a mix thereof were included and
384 cluster analysis was formed (**Figure 6C**) Remarkably, among the top ranked pathways, we could
385 find several common enriched pathways of interest, including leukocyte extravasation signaling,
386 axonal guidance signaling, netrin signaling, thrombin signaling, GABA, GnRH and nitric oxide
387 signaling, similar to the *in vitro* treatment experiments. Despite the significant enrichment ($p <$
388 0.05) of various pathways across different individuals, no common differentially methylated genes
389 could be identified in response to the diet intervention (data not shown) [24]. This suggests highly
390 redundant gene networks to control cell adhesion and leukocyte extravasation.

391

392 **3.6. Individual DNA methylation response of (-)-epicatechin diet intervention associates** 393 **with cardioprotective vascular health biomarkers.**

394 Since the cardioprotective role of (-)-epicatechin flavanols is well established (see introduction),
395 we first evaluated whether atherosclerosis and flavanol diet intervention associated DNA
396 methylation profiles show opposite methylation direction. We therefore compared the mean DNA
397 methylation differences after the diet intervention with the DNA methylation signature of
398 atherosclerotic patients (athDMPs) [23]. Interestingly, based on the mean delta betas (indicates
399 degree of DNA Methylation difference) of all individuals, a negative correlation ($r=-0.41$) was
400 found with the delta betas of the athDMPs, suggesting that (-)-epicatechin treatment elicits
401 opposite DNA methylation changes than atherosclerosis progression (**Figure 7A**).
402 Hypomethylated athDMPs were on average increased in methylation after the (-)-epicatechin diet
403 intervention, whereas hypermethylated athDMPs were mainly decreased in methylation.
404 Furthermore, when calculating the correlation between delta betas in every individual separately,
405 we found more individuals with negative correlation (i.e. cardioprotection) than with positive
406 correlations (i.e. proatherogenic) (**Figure 7B**). Especially individuals SN_114, SN_109 and

407 SN_110 demonstrated a strong negative cardioprotective correlation. Interestingly, the three
408 samples (SN_114, SN_109 and SN_110) with the strongest DNA methylation response (highest
409 number of DMPs) (SN_114, SN_109 and SN_110) were also the most negatively correlated with
410 the athDMPs.

411 Since blood is a heterogeneous collection of different cell types, each characterized by unique DNA
412 methylation profile, we next applied the houseman algorithm to estimate blood cell type
413 composition before and after diet intervention in every individual [27]. This algorithm allows
414 mathematical deconvolution of relative immune cell type composition of blood samples based on
415 Illumina 450k data [59]. In line with this prediction model, increased fraction of neutrophil
416 granulocytes and reduced fraction of CD4+T lymphocyte cells have already been reported in
417 atherosclerosis patients in comparison to control individuals [23]. Interestingly, the (-)-epicatechin
418 diet intervention promotes predominantly opposite shifts in cell type composition, based on the
419 Pearson correlation coefficients of (-)-epicatechin DMPs of the different individuals versus
420 atherosclerosis athDMPs (**Figure 7C**). For example, DMPs of individual SN_114 with the
421 strongest negative athDMP correlation coefficient showed the biggest reduction in granulocyte
422 fraction but highest increase of CD4+T cell fraction. This houseman prediction could be further
423 experimentally confirmed with the vascular health biomarkers which have been monitored in these
424 individuals [58] (**Figure 8A**). When comparing the individual pearson correlation coefficient of
425 epicatechin DMPs versus athDMPs, the strongest positive correlation was found for the neutrophil
426 counts and the most negative correlation was found for the lymphocyte counts. In case of a
427 negative pearson correlation efficient of epicatechin diet DMPs versus athdMPs , this corresponds
428 with reduced neutrophil counts and increased lymphocyte counts, in line with the houseman
429 algorithm prediction. This suggests that epigenetic reprogramming of adaptive immunity of
430 neutrophil and T cell populations in response to (-)-epicatechin might also significantly contribute
431 to its cardioprotective properties.

432 We next checked to what extent epigenetic responders of the (-)-epicatechin diet could be
433 correlated with additional vascular health parameters. For this, correlations were performed
434 between the total number of DMPs and all the micro and macro vascular biomarkers which have
435 been monitored in these individuals [58] (**Figure 8B**). The most significant positive correlations
436 were found for GPX1 expression values and acetylcholine response which increased with higher
437 number of DMPs following (-)-epicatechin diet intervention. Epicatechin is known to improve the

438 impaired endothelium-dependent relaxation response to acetylcholine [60]. GPX1 is involved in
439 oxidative stress defense mechanisms [61]. GPX1 deficiencies play a critical role in cardiac
440 dysfunctions and AngII-dependent hypertension, but GPX1 activity can be restored upon
441 epicatechin treatment [62, 63]. Besides, GPX1 also regulates epigenetic DNA hydroxymethylation
442 following oxidative stress [64]. The most negative correlations were found for endothelin-1 (ET1)
443 levels which decreased with increasing number of (-)-epicatechin responsive DMPs. ET1 is playing
444 a critical role in cardiovascular hypertension. Of special note, epicatechin is known to lower ET-1
445 release [60]. How changes in GPX1 and ET1 expression modulate DNA (hydroxy)methylation
446 changes (or vice versa) needs further investigation. Finally, no significant association could be
447 found between the (-)-epicatechin specific DNA methylation response and the total vascular health
448 index, which is an integrative measure of vascular health combining the results from all the vascular
449 biomarkers measured (**Figure 8A-B**) [58]. This suggests that not all measured vascular health
450 parameters are under epigenetic DNA methylation control.

451

452 **4. Discussion**

453 (-)-Epicatechins are protective against cardiovascular diseases and improve vascular health,
454 however, the underlying mechanism is not fully elucidated. Using molecular modeling and in
455 silico docking, energetically favorable binding was demonstrated of 3 different (-)-epicatechin
456 metabolites with potential docking sites in DNMT1 and DNMT3A/B enzymes. Moreover, in
457 endothelial HUVEC cells, the 3 (-)-epicatechin metabolites induced similar DNA methylation
458 changes, across the different flavanol metabolites. Surprisingly, the CpG sites that changed after (-)
459)-epicatechin treatment were rather enriched in intergenic regions and gene bodies, as compared to
460 promoter regions. Since few promoters changed their methylation status following (-)-epicatechin
461 treatment, only a limited number of genes were found to be both differentially expressed and
462 methylated [17]. Although additional chromatin dependent promoter regulation cannot be excluded
463 [65], (-)-epicatechin specific DNA methylation changes may alternatively modulate long-term
464 transcriptional responses upon repetitive exposure to accumulate a particular DNA methylation
465 threshold [66]. Besides, it also needs to be further investigated whether (-)-epicatechin specific
466 DNA methylation may have additional roles in alternative RNA splicing or regulation of ncRNAs
467 [67-70]. We found that the intergenic and gene body localized DMPs are enriched for genes
468 involved in cell adhesion related pathways, including axonal guidance signaling, paxillin

469 signaling, netrin signaling, integrin signaling, and actin cytoskeleton signaling. Of particular
470 interest, the role of alternative RNA splicing and noncoding microRNAs in regulation of cell
471 adhesion-motility is increasingly recognized [17, 71-73]. These results are in accordance with
472 previous functional studies showing reduced monocyte-endothelial cell-cell adhesion after
473 epicatechin treatment *in vitro* and *in vivo* [17]. The recruitment and migration of monocytes into
474 the arterial wall is one of the key initial processes during the development of an atherosclerotic
475 plaque. Furthermore, our data also identified (-)-epicatechin specific epigenetic regulation of
476 GABA, renin-angiotensin and nitric oxide pathways controlling blood pressure, as well as
477 endocrine responses via GnRH and AR, which may prevent plaque ruptures [44, 45]. Furthermore,
478 we found that the 3 (-)-epicatechin metabolites can counteract TNF induced epigenetic regulation
479 of 2 gene clusters involved in cell junction regulation, controlling endothelial permeability. This is
480 a critical determinant of leukocyte extravasation and transendothelial migration and can be
481 inhibited by (-)-epicatechins [17, 52-57]. Altogether, we identified TNF-dependent as well as
482 TNF-independent epigenetic changes in multiple pathways associated with cardioprotective
483 properties.

484 When further trying to identify an (-)-epicatechin specific DNA methylation signature *in vivo* in
485 blood samples of male smokers following an eight-week (-)-epicatechin diet intervention, we
486 presumably failed because of strong interindividual epigenetic variation in lifestyle factors
487 (nutrition, stress, exercise, smoking behavior, age), the limited duration of the diet intervention (8
488 weeks intervention is short relative to atherosclerosis development over years) and the relative
489 small sample sizes analyzed, (too limited statistical power) [24]. However, upon reanalyzing (-)-
490 epicatechin specific DNA methylation responses at the individual level, we observed significant
491 gene enrichment of common pathways related to cell adhesion and leukocyte extravasation, GnRH,
492 GABA, renin-angiotensin and nitric oxide signaling across all individuals, similar to the *in vitro*
493 results of (-)-epicatechin exposure in the endothelial cell model. Of particular interest, heatmap
494 clustering of these pathway enrichment results of individual epigenetic responses to (-)-epicatechin
495 *in vitro* and *in vivo* reveals that some individual profiles are more similar to 4'MEC and EC4'S
496 metabolite treatment, whereas other individuals are more similar to treatment with 4'MEC7G (-)-
497 metabolite or an equimolar mixture (1 μ M) of the 3 metabolites. Indeed, interindividual variation
498 in (-)-epicatechin metabolization in the gut-microbiome [6, 9, 74] and/or metabolite specific
499 pharmacodynamics [75] may further expand variation of epigenetic gene responses to (-)-

500 epicatechin diet intervention [33, 34]. Remarkably, the common pathways identified seem to be
501 robust and tolerate strong interindividual epigenetic variation through redundant gene networks.
502 Upon analyzing vascular health benefits of (-)-epicatechin specific epigenetic changes, Pearson
503 correlation studies showed that individual DMP profile of an (-)-epicatechin diet intervention
504 oppose adverse epigenetic marks associated with atherosclerosis (ath-DMP signature). Moreover,
505 the houseman deconvolution algorithm revealed that part of the observed DNA methylation
506 changes correspond with decreased neutrophil granulocyte and increased CD4+T-lymphocyte cell
507 counts. The neutrophil-lymphocyte ratio (NLR) is a measure of systemic inflammation and is a
508 known prognostic and predictive marker for cardiovascular diseases. A higher NLR has been
509 associated with increased cardiovascular risk and hypertension [76-79]. As such DNA methylation
510 based estimation of small shifts in immune cell counts and activation status could become an
511 attractive immunomethylomic measure for chronic systemic inflammation, cardiovascular risk and
512 hypertension, similar to NLR based cancer prediction markers [78, 80]. Along the same line, C-
513 reactive protein (CRP), another systemic inflammation marker, was found associated with blood
514 DNA methylation changes of 218 CpGs, of which at least 51 CpGs are related to cardiometabolic
515 health [81]. From a cardioprotective perspective, a polyphenol rich (Mediterranean) diet was
516 reported to lower NLR and chronic inflammatory markers, including CRP [82-88]. Similarly, our
517 DNA methylation results indicate that an (-)-epicatechin diet intervention lowers NLR in various
518 individuals, in favour of cardioprotection. These results could also experimentally be confirmed by
519 vascular health measurements, showing decreased neutrophil granulocyte and increased T-
520 lymphocyte counts following (-)-epicatechin diet intervention. It can therefore be concluded that
521 changes in systemic low grade inflammation or its reduction by (-)-epicatechin can be detected in
522 blood DNA methylation profiles and NLR-specific methylation DMPs may become an attractive
523 blood surrogate biomarker for cardiovascular health.

524 Besides, we also found significant Pearson correlations for total number of epicatechin responsive
525 DMPs with Gpx1 and ET1 expression levels and acetylcholine response. Experimental data
526 suggest that antioxidative GPx-1 activity modulates cardiovascular risk associated with
527 homocysteine plasma levels, which is linked to methyl donor metabolism supplying S-
528 adenylnmethionine donors for DNA methylation [89]. Similarly, homocysteine exerts deleterious
529 effects on endothelial function and vascular tone via release of ET1 [90, 91]. In this respect,
530 epicatechins may promote indirect DNA methylation effects by increasing homocysteine

531 catabolism (reduced hyperhomocysteinemia) and reshaping the methyl donor metabolism [92].
532 Associations with other vascular health markers of interest (such as fmd, aggregation, VCAM, CRP)
533 did not reach significance ($P > 0.05$), presumably due to the small sample cohort size and
534 underpowered statistics and may become significant in larger cohort studies.

535 Because all study participants were heavy smokers, we also tested whether the epicatechin diet
536 could reverse adverse smoking-induced DNA methylation [93]. However, no clear reversal of the
537 smoking methylation pattern could be observed when comparing average methylation changes of
538 the diet intervention and smoking related methylation changes (data not shown). Finally, we also
539 checked whether the (-)-epicatechin diet could rejuvenate the epigenetic age measured by the
540 epigenetic clock of Horvath [29]. However, besides some weak individual rejuvenation effects, no
541 statistically significant change in epigenetic age could be observed at the population level after 8
542 week diet intervention (data not shown). Similarly, in a recent study, benefits of dietary
543 intervention are limited when started in old age, owing to the development of an inflexible
544 nutritional memory [94].

545 In conclusion, our results provide solid biochemical evidence that (-)-epicatechins elicit DNA
546 methylation changes in endothelial cells *in vitro* and in a diet intervention study *in vivo*.
547 Characterisation of the corresponding DMPs revealed targeting of common cell adhesion-
548 extravasation signaling pathways, axonal guidance, GABA, renin-angiotensin and nitric oxide
549 hypertension pathways, associated with various cardiovascular health parameters (Gpx1, ET1,
550 acetylcholine response and a decreased neutrophil/lymphocyte ratio). Prolonged longitudinal diet
551 intervention studies in larger study cohorts may allow to evaluate longterm persistence of
552 epicatechin specific epigenetic changes in relation to its cardioprotective properties.

553

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556

557 **Figure legends**

558
 559 **Figure 1: In silico docking analyses of epicatechin metabolites to DNMT1 and DNMT3A.** A) Chemical structures of 4'MEC, EC4'S and 4'MEC7G metabolites B) DNA binding site of DNMT1. C,D) catalytic domains of DNMT1. E) PWWP domain of DNMT3A. F) H3 peptide binding site of DNMT3A. G) DNMT3A dimerization site. H) DNMT3L-DNMT3A binding site. The pose of EC4'S in each binding site is shown in enlarged rectangle. Hydrogen bonds are represented as light blue dashed lines.

565
 566 **Figure 2: DNA methylation changes (DMPs) of HUVEC cells upon exposure to epicatechin metabolites and TNF relative to TNF treatment alone.** A) Multivariate PLS-DA plot of the first two components showing clear separation of the different treatment conditions. B) Volcano plots revealing CpG probes with significant changes in DNA methylation status. The x-axis shows the mean DNA methylation difference ($\Delta \beta$ -value), whereas the y-axis shows the $-\log_{10}$ of the P-value for each probe, representing the strength of association. Green and red dots represent the hypo- and hypermethylated probes, respectively. The numbers of hyper- and hypomethylated sites are indicated in the plot. C) Manhattan plots showing the differentially methylated sites in each treatment contrast. Red means hypermethylated, green means hypomethylated.

575
 576 **Figure 3: Comparison of DMPs in response to different epicatechin metabolites and TNF relative to TNF treatment alone** A) Genomic enrichment of differentially methylated sites. In the heatmap, the \log_2 enrichment ratio is given comparing the sig-DMPs with all the probes on the Illumina array for different genomic regions (left: gene elements, middle: CpG island elements, right: chromatin state segmentations). Enrichment of the genomic regions was calculated using the Fisher's Exact test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. B) Scatterplots comparing the $-\log_{10}$ (pvalues) of each DMP across the different epicatechin metabolites (4'MEC, 4'MEC7G and EC4'S) and TNF relative to TNF treatment alone. Spearman's correlation coefficients are given. C) Scatterplots of the $-\log_{10}$ (pvalues) of each DMP comparing epicatechin metabolite effect (4'MEC, 4'MEC7G and EC4'S) and TNF against TNF treatment alone versus DMPs of TNF treatment against untreated control. Spearman's correlation coefficients are given.

587

588 **Figure 4: Pathway enrichment analysis of common gene associated DMPs in response to 3**
 589 **different epicatechin metabolites and TNF versus TNF treatment alone.** A) Venn diagram of
 590 the overlapping gene associated DMPs across the 3 (-)-epicatechin metabolite contrasts. B)
 591 Metacore enriched gene networks C) KEGG enriched pathways C) Metascape protein network
 592 analysis network. Circles present different pathways, color represent metabolites that affected
 593 methylation level, red for 4'MEC, blue for 4'MEC7G, green for EC4'S. D) Ingenuity pathway
 594 enrichment analysis. Top 40 most significantly enriched IPA canonical pathways across the 3 (-)-
 595 epicatechin metabolites. The heatmap represents the $-\log_{10}(\text{p-value})$ of the enriched pathways.
 596 Pathways were ranked by the sum of $-\log_{10}(\text{p-values})$.

597
 598 **Figure 5: Epicatechin metabolites counteract TNF induced DNA methylation changes.** A)
 599 Venn diagram showing the overlapping differentially methylated genes across the different
 600 treatment contrasts. B) Heatmap showing the methylation differences of the 26 common genes,
 601 including 2 gene clusters. Green means hypomethylation, while red means hypermethylation. C)
 602 Example plot of DMP in USP35.

603
 604 **Figure 6: Blood DMPs and corresponding pathway enrichment analysis of common gene**
 605 **associated DMPs after an 8-week (-)-epicatechin diet intervention in male smokers.** A)
 606 Number of DMPs ($|\text{delta beta-value}| > 0.1$, i.e. DNA methylation change $> 10\%$). **B)** Number of
 607 probes differentially methylated in one or multiple individuals. **C)** Ingenuity pathway enrichment
 608 analysis. Top 40 most significantly enriched IPA canonical pathways across the 10 individuals (in
 609 vivo) and 3 (-)-epicatechin metabolites or its equimolar mix (in vitro). The heatmap represents the
 610 $-\log_{10}(\text{p-value})$ of the enriched pathways. Pathways were ranked by the sum of $-\log_{10}(\text{p-values})$.

611
 612
 613 **Figure 7: Comparison (-)-epicatechin diet intervention DMPs with atherosclerosis related**
 614 **athDMP.** A) Comparison of delta beta values atherosclerosis/healthy (athero-DMPs, X-axis) and
 615 delta beta values after (-)-epicatechin diet intervention (Y-axis). Red CpG probes are those athero-
 616 DMPs which were reversed by the (-)-epicatechin diet intervention. **B)** Correlation of the delta beta
 617 values between atherosclerosis and (-)-epicatechin diet for every individual. **C)** Comparison of the
 618 correlation coefficient between atherosclerosis and diet effect (X-axis) and estimated granulocyte
 619 (left) and CD4+ T-cell fraction (Y-axis).

620 **Figure 8: Pearson correlation of individual epigenetic response to (-)-epicatechin diet**
621 **intervention versus corresponding vascular health biomarker measurements.** A) Pearson
622 correlation of individual (-)-epicatechin diet DMPs vs athDMPs against corresponding vascular
623 health biomarker measurements. B) Pearson correlation of individual number of (-)-epicatechin
624 diet DMPs against corresponding vascular health biomarker measurements. Vascular health
625 markers were grouped in different categories (blood.cell.counts, GE.inflammatory, etc).

626

627

628 **Table legends**

629

630 **Table 1: mm-GBSA binding energy (ΔG , kcal/mol) of molecules to (A) DNMT1 and (B)**
631 **DNMT3A**

632 **Table 2: Reciprocal TNF and (-)-epicatechin gene associated DMPs cluster 1**

633

634 **Table 3: Reciprocal TNF and (-)-epicatechin gene associated DMPs cluster 2**

635

636

637 **Conflict of interest**

638 The authors have no conflict of interest

639

640

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646

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962 **Table 1:** mm-GBSA binding energy (ΔG , kcal/mol) of molecules to (A) DNMT1 and (B)
 963 DNMT3A

(A)	DNA binding site	Catalytic domain (I)	Catalytic domain (II)
(-)-epicatechin	-31.82	-32.07	-37.95
3'MEC	-32.82	-23.79	-36.18
EC4'S	-35.14	-24.56	-34.79
4MEC7G	-46.24	-38.72	-55.43
Sucrose	-24.47	-36.82	-36.97
Naphthalene	-13.62	-8.3	-20.67

964

(B)	PWWP domain	H3 peptide binding site	DNMT3A binding site	DNMT3L binding site
(-)-epicatechin	-48.34	-26.50	-31.24	-29.76
3'MEC	-38.23	-28.81	-29.70	-22.93
EC4'S	-42.10	-17.35	-26.70	-31.34
4MEC7G	-51.72	-36.81	-55.42	-33.10
Sucrose	-30.37	-9.20	-34.01	-20.27
Naphthalene	-30.77	-8.11	-22.52	-19.33

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967 **Table 2**

Genes cluster 1	treatment	$\Delta\beta$ versus TNF	$\Delta\beta$ versus Ctrl	P.Value	cgProbe_ID	β_{control}	β_{TNF}	$\beta_{\text{4'MEC}}$	$\beta_{\text{4'MEC7G}}$	$\beta_{\text{EC4'S}}$
PCDH9	4'MEC	0,08		0,009	cg20212077	0,38	0,34	0,42	0,42	0,39
	4'MEC7G+TNF	0,08		0,008	cg20212077	0,38	0,34	0,42	0,42	0,39
	EC4'S	0,06		0,008	cg16184802	0,81	0,78	0,79	0,84	0,85
	TNF		-0,06	0,007	cg15566921	0,98	0,92	0,97	0,93	0,95
DLGAP2	4'MEC	0,08		0,003	cg06452558	0,49	0,47	0,56	0,53	0,53
	4'MEC7G+TNF	0,06		0,004	cg15961904	0,86	0,86	0,88	0,92	0,88
	4'MEC7G+TNF	0,06		0,009	cg05455971	0,71	0,72	0,72	0,78	0,75
	EC4'S	0,07		0,006	cg00579531	0,68	0,65	0,66	0,69	0,72
	EC4'S	0,06		0,008	cg02095946	0,90	0,89	0,92	0,92	0,94
	TNF		-0,05	0,006	cg03308704	0,95	0,90	0,92	0,94	0,93
SDK1	4'MEC	0,06		0,009	cg25783987	0,84	0,83	0,90	0,85	0,83
	4'MEC7G+TNF	0,06		0,009	cg04534231	0,81	0,81	0,82	0,88	0,87
	EC4'S	0,07		0,006	cg19569074	0,65	0,63	0,66	0,64	0,70
	TNF		-0,06	0,001	cg11998431	0,92	0,86	0,87	0,88	0,89
BANP	4'MEC	0,06		0,002	cg06173520	0,93	0,87	0,93	0,93	0,91
	4'MEC7G+TNF	0,06		0,007	cg06173520	0,93	0,87	0,93	0,93	0,91
	EC4'S	0,07		0,002	cg00935421	0,80	0,82	0,84	0,85	0,89
	TNF		-0,06	0,003	cg06173520	0,93	0,87	0,93	0,93	0,91
KDM2B	4'MEC	0,06		0,009	cg16062733	0,75	0,69	0,75	0,77	0,78
	4'MEC7G+TNF	0,08		0,001	cg16062733	0,75	0,69	0,75	0,77	0,78
	EC4'S	0,09		0,001	cg16062733	0,75	0,69	0,75	0,77	0,78
	TNF		-0,05	0,010	cg16062733	0,75	0,69	0,75	0,77	0,78

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979 **Table 3**

Genes cluster 2	treatment	$\Delta\beta$ versus TNF	$\Delta\beta$ versus Ctrl	P.Value	cgProbe_ID	$\beta_{control}$	β_{TNF}	$\beta_{4'MEC}$	$\beta_{4'MEC7G}$	$\beta_{EC4'S}$
AKAP12	4'MEC	-0,06		0,003	cg21047322	0,86	0,92	0,86	0,85	0,86
	4'MEC7G+TNF	-0,07		0,001	cg21047322	0,86	0,92	0,86	0,85	0,86
	EC4'S	-0,06		0,006	cg21047322	0,86	0,92	0,86	0,85	0,86
	TNF		0,06	0,002	cg21047322	0,86	0,92	0,86	0,85	0,86
LAS1L	4'MEC	-0,06		0,002	cg17299883	0,13	0,18	0,12	0,12	0,12
	4'MEC7G+TNF	-0,07		0,001	cg17299883	0,13	0,18	0,12	0,12	0,12
	EC4'S	-0,06		0,002	cg17299883	0,13	0,18	0,12	0,12	0,12
	TNF		0,05	0,004	cg17299883	0,13	0,18	0,12	0,12	0,12
USP35	4'MEC	-0,07		0,002	cg15243034	0,78	0,86	0,80	0,79	0,80
	4'MEC7G+TNF	-0,08		0,001	cg15243034	0,78	0,86	0,80	0,79	0,80
	EC4'S	-0,06		0,003	cg15243034	0,78	0,86	0,80	0,79	0,80
	TNF		0,09	0,000	cg15243034	0,78	0,86	0,80	0,79	0,80
GLUD1P3	4'MEC	-0,07		0,008	cg02005425	0,73	0,82	0,75	0,73	0,72
	4'MEC7G+TNF	-0,09		0,002	cg02005425	0,73	0,82	0,75	0,73	0,72
	EC4'S	-0,10		0,001	cg02005425	0,73	0,82	0,75	0,73	0,72
	TNF		0,09	0,001	cg02005425	0,73	0,82	0,75	0,73	0,72
PTCHD3	4'MEC	-0,09		0,003	cg10599633	0,85	0,91	0,83	0,79	0,85
	4'MEC7G+TNF	-0,12		0,000	cg10599633	0,85	0,91	0,83	0,79	0,85
	EC4'S	-0,07		0,009	cg10599633	0,85	0,91	0,83	0,79	0,85
	TNF		0,06	0,008	cg10599633	0,85	0,91	0,83	0,79	0,85
NLGN1	4'MEC	-0,08		0,006	cg06097077	0,54	0,63	0,54	0,56	0,56
	4'MEC7G+TNF	-0,06		0,004	cg04981409	0,88	0,91	0,89	0,85	0,88
	EC4'S	-0,10		0,007	cg03417233	0,83	0,83	0,79	0,81	0,74
	TNF		0,09	0,002	cg06097077	0,54	0,63	0,54	0,56	0,56
SKI	4'MEC	-0,12		0,000	cg11191979	0,89	0,95	0,83	0,89	0,86
	4'MEC7G+TNF	-0,05		0,004	cg11191979	0,89	0,95	0,83	0,89	0,86
	EC4'S	-0,09		0,000	cg11191979	0,89	0,95	0,83	0,89	0,86
	TNF		0,06	0,001	cg11191979	0,89	0,95	0,83	0,89	0,86
XKR4	4'MEC	-0,10		0,000	cg14552904	0,77	0,83	0,73	0,79	0,70
	4'MEC7G+TNF	-0,07		0,002	cg19887883	0,89	0,93	0,90	0,86	0,91
	EC4'S	-0,13		0,000	cg14552904	0,77	0,83	0,73	0,79	0,70
	TNF		0,06	0,004	cg14552904	0,77	0,83	0,73	0,79	0,70
FLJ38576	4'MEC	-0,15		0,000	cg10631544	0,86	0,94	0,80	0,81	0,85
	4'MEC7G+TNF	-0,13		0,000	cg10631544	0,86	0,94	0,80	0,81	0,85
	EC4'S	-0,09		0,002	cg10631544	0,86	0,94	0,80	0,81	0,85
	TNF		0,08	0,002	cg10631544	0,86	0,94	0,80	0,81	0,85
DACT1	4'MEC	-0,07		0,001	cg01617520	0,89	0,92	0,85	0,81	0,86
	4'MEC	-0,14		0,003	cg00677272	0,86	0,92	0,78	0,88	0,85
	4'MEC7G+TNF	-0,10		0,000	cg01617520	0,89	0,92	0,85	0,81	0,86
	EC4'S	-0,06		0,003	cg01617520	0,89	0,92	0,85	0,81	0,86
	TNF		0,07	0,005	cg00869962	0,62	0,70	0,67	0,69	0,70