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Published in:
Metabolism

DOI:
10.1016/j.metabol.2014.05.014

Publication date:
2014

Citation for published version (APA):
Translational

DNA methylation is altered in B and NK lymphocytes in obese and type 2 diabetic human☆

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ABSTRACT

Objective. Obesity is associated with low-grade inflammation and the infiltration of immune cells in insulin-sensitive tissues, leading to metabolic impairment. Epigenetic mechanisms control immune cell lineage determination, function and migration and are implicated in obesity and type 2 diabetes (T2D). The aim of this study was to determine the global DNA methylation profile of immune cells in obese and T2D individuals in a cell type-specific manner.

Material and methods. Fourteen obese subjects and 11 age-matched lean subjects, as well as 12 T2D obese subjects and 7 age-matched lean subjects were recruited. Global DNA methylation levels were measured in a cell type-specific manner by flow cytometry. We validated the assay against mass spectrometry measures of the total 5-methylcytosine content in cultured cells treated with the hypomethylation agent decitabine (r = 0.97, p < 0.001).

Results. Global DNA methylation in peripheral blood mononuclear cells, monocytes, lymphocytes or T cells was not altered in obese or T2D subjects. However, analysis of blood fractions from lean, obese, and T2D subjects showed increased methylation levels in B cells from obese and T2D individuals and in natural killer cells from T2D patients. In these cell types, DNA methylation levels were positively correlated with insulin resistance, suggesting an association between DNA methylation changes, immune function and metabolic dysfunction.

Keywords: Epigenetic changes, Metabolic diseases, Leukocytes, Flow cytometry

Abbreviations: PBMCs, peripheral blood mononuclear cells; T2D, type 2 diabetes; SmeC, 5-methylcytosine; BMI, body mass index; HOMA-IR, Homeostasis Model Assessment; FBS, fetal bovine serum; D-PBS, Dulbecco’s phosphate-buffered saline; BSA, bovine serum albumin; PFA, paraformaldehyde; NK, natural killer; MFI, median fluorescence intensity; LC-MS/MS, liquid chromatography tandem mass spectrometry; 5mdC, 5-methyl-2′-deoxycytidine.

† Conflict of interest: The authors have no competing financial interests to declare.
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1. Introduction

The immune system is composed of a wide variety of cell types, each possessing a specific gene expression signature determined by epigenetic processes such as DNA methylation and histone modification [1]. Through the control of chromatin structure, epigenetic processes modulate the access of DNA-binding transcription factors, thereby selecting for transcriptional activation or repression of, for example, lineage-specific genes. Studies investigating the DNA methylation of peripheral blood mononuclear cells (PBMCs) have shown specific methylation patterns at genes important for immune function and further suggested that alteration in DNA methylation is likely to be an important mechanism contributing to the inter-individual variation in immune cells function [2,3].

Obesity, characterized by an increased adiposity over lean mass, is associated with epigenetic variability in blood [4–9]. It is now clear that immune cells participate to the chronic, low-grade systemic inflammation observed in obesity, with infiltration of macrophages in adipose tissue promoting inflammation [10]. Adipose tissue macrophages contribute to the etiology of insulin resistance through the secretion of pro-inflammatory cytokines, such as tumor necrosis factor-α or interleukin-6, known down-regulators of insulin signaling [11]. The role of immune cells in the development of insulin resistance is not limited to macrophages. Both B and T lymphocytes have also been shown to be important contributors to the inflammatory process and the resulting insulin resistance in metabolically active tissues, and in particular in adipose tissue [12,13].

In studies investigating DNA methylation in blood from subjects with metabolic dysfunction, DNA methylation was exclusively analyzed in whole PBMC fractions, which are composed of numerous cell types. However, global DNA methylation measurements made from whole PBMC fractions could be obscured by potential changes in blood cell types counts [14]. Given that obesity and type 2 diabetes (T2D) were found to be associated with a remodeling of blood cell type counts [15,16] and that leukocyte sub-populations exhibit a specific global DNA methylation signature [17], altered DNA methylation in whole PBMC fractions could simply mirror a change in the frequency of certain leukocytes’ sub-populations.

In the present study, we aimed to use a cell-specific assay to determine the global DNA methylation profile of the different PBMCs’ sub-types in obese and T2D individuals. Using a monoclonal antibody to 5-methylcytosine (5meC) and flow cytometry, we provide the first measurement of relative global DNA methylation levels in individual cell types within PBMCs of lean, obese and T2D subjects.

2. Material and methods

2.1. Study population

This project was approved by the Ethics Committee from the Capital Region of Denmark (reference number H-1-2011-077). Forty-four male subjects, aged 18–60 were recruited by local advertisement on university campuses or local hospitals and informed consent was obtained from the participants. Participants were free of recent illness, and infectious or inflammatory diseases, other than metabolic disorders, that might affect their immune system. Body mass index (BMI) and diagnosis of T2D were used to generate 4 groups: 14 obese males (OBESE, age = 35.1 ± 6.7 yr, BMI = 36.3 ± 6.3 kg/m², non-diabetic/healthy) and 11 age-matched lean males (LEAN, age = 34.8 ± 3.0 yr, BMI = 23.0 ± 1.4 kg/m², non-diabetic/healthy) as well as 12 patients with type 2 diabetes (T2D, age = 44.1 ± 6.5 yr, BMI = 34.9 ± 4.8 kg/m²) or 7 age-matched lean subjects (LEAN, age = 44.1 ± 6.0 yr, BMI = 23.7 ± 2.2 kg/m², non-diabetic/healthy) were recruited for this study.

2.2. Sample collection

Blood samples were obtained by venipuncture from an antecubital vein and collected on ethylenediaminetetraacetic acid-treated tubes (BD vacutainer, USA) or acid-citrate-dextrose treated tubes (ACD, BD vacutainer, USA) followed by PBMCs isolation by Ficoll gradient centrifugation as previously described [18].

2.3. Full blood cell count and insulin resistance index

In small subgroups, differential cell count was performed using a Sysmex XE-2100 automated hematology analyzer (Sysmex, USA) for the determination of the frequency of neutrophils, monocytes, lymphocytes and T helper (CD3/CD4 double positive), T cytotoxic cells (CD3/CD8 double positive), B cells (CD45/CD19/CD20 triple positive) and NK cells (CD3 negative, CD45/16/56 triple positive). Fasting glucose and insulin levels were measured using automated analyzers (Beckman LX analyzer, Beckman Coulter, USA and Immulite 2000, Diagnostic Products Corporation, USA respectively) and the level of insulin resistance was calculated using the Homeostasis Model Assessment (HOMA-IR) [19].

2.4. Cell culture

The colorectal cancer cell line RKO (ATCC, USA) was maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Life Technologies, USA) supplemented with 25 mM glucose, 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, 100 ug/
ml streptomycin and 2 mM glutamate (all from Life Technologies, USA) and grown at 37 °C in 5% CO2. Cells were treated every 24 h for a period of 72 h by replacing media supplemented with 2.5 μM decitabine (5-aza-2′-deoxycytidine, Sigma-Aldrich, Australia) freshly prepared in 50% filter sterilized acetic acid (Sigma-Aldrich, Australia). Cells were harvested at daily time points before and during treatment and at 3 days following drug withdrawal.

The human T lymphocyte cell line Jurkat (ATCC, USA) was maintained in RPMI 1640 (Gibco, Life Technologies, USA) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), L-glutamine (1 mmol/mL) and 10% FBS. The culture medium was refreshed every third day and the cell density was maintained below 1 million cells/mL. Twenty-four hours after the last passage, cells were treated with 2.5 μM decitabine as described above and harvested at different time points up to 52 h.

2.5. Global methylation quantification by flow cytometry

Global methylation in PBMCs, RKO and Jurkat cells was quantified as previously described with slight modifications [20]. Cryopreserved PBMCs were quickly thawed at 37 °C, transferred into culture medium and washed twice in RPMI 10% FBS. For RKO cells, cells were detached from the culture vessel by incubating with trypsin (0.25%, Gibco, Life Technologies, USA) at 37 °C for 5 min. Cells were then washed twice in Dulbecco’s phosphate-buffered saline (D-PBS, Sigma-Aldrich, Australia) with 1% BSA. For Jurkat cells, cells were transferred to a new sterile tube and washed twice in D-PBS with 1% BSA.

Cell viability was assessed by visual inspection using trypan blue staining (Sigma-Aldrich, Australia) and density was normalized to a maximum of 5 million cells per mL. After 30 min at 37 °C, 5% CO2, cells were fixed in 2% paraformaldehyde (PFA, Sigma-Aldrich, Australia) in D-PBS for 10 min at 37 °C. After two washes using D-PBS with 1% bovine serum albumin (BSA, Sigma-Aldrich, Australia) and 0.1% Tween 20 (Sigma-Aldrich, Australia), cells were permeabilized in 100% methanol (Sigma-Aldrich, Australia) at −20 °C for 30 min. After three additional washes, cells were incubated at 37 °C in 2 N HCl for 30 min and then transferred into 0.1 M of borate buffer for 5 min. After three additional washes the PBMCs were stained with a combination of anti CD3-phycocerythrin conjugated, CD8- and CD14-peridinin chlorophyll conjugated, CD4- and CD19-allophycocyanin conjugated (all from BD, USA). Stained PBMCs, RKO and Jurkat cells were further stained with unconjugated anti-5-methylcytosine (Smec, AbD serotec, Bio-Rad, USA) or its corresponding isotype control (Mouse IgG1, BD, USA). Both unconjugated antibodies were labeled using a labeling kit according to the manufacturer’s instructions (Zenon Alexa Fluor 488 Mouse IgG1 labeling kit, Molecular Probes, Life Technologies, Australia). Cells were incubated with the different antibodies or their corresponding isotype controls for 20 min at room temperature in the dark. After two more washes, they were resuspended in 400 uL of 1% PFA in D-PBS and acquired immediately on a flow cytometer (FACS Calibur, BD, USA).

All data were analyzed using FlowJo version 10 (Tree Star, USA) and Cytobank (Cytobank Inc., USA). Lymphocytes and monocytes were gated based on their size and granularity. Monocytes were further gated based on CD14 positivity. Lymphocytes CD3+CD4+ were considered as T helper cells, CD3+CD8+ as T cytotoxic cells, CD3−CD19+ as B cells and CD3−CD8+ as natural killer (NK) cells. For RKO or Jurkat cells, the population was gated based on size and granularity. In each population the median fluorescence intensity (MFI) was measured and normalized by the MFI from the isotype control.

2.6. Global methylation quantification by LC-MS/MS

Global methylation in RKO cells was quantified as previously described using liquid chromatography tandem mass spectrometry (LC-MS/MS) [21]. The absolute quantity of 5-methyl-2′-deoxycytidine (5mC; global methylation) was determined as a percentage of 2′-deoxycytidine plus 5mC in genomic DNA extracted from RKO cells by phenol-chloroform extraction using LC-MS/MS as previously published [22]. This involved the in-house biosynthesis of [15N3]-dC and [15N3]-5mC internal standards and a one-step digestion of 1 ug genomic DNA spiked with [U-15N]-internal standards into deoxyribonucleosides. Analyses were performed using an Ultra High Performance Liquid Chromatography Accela Pump (Thermo Finnigan, USA) and HTC PAL (CTC Analytics, USA) autosampler coupled directly to a TSQ Quantum Access triple quadrupole mass spectrometer (Thermo Finnigan, USA) in the positive ion mode via an electrospray interface. Data were processed using the Quanbrowser function of the Xcalibur Software package v2.0.7 (Thermo Finnigan, USA).

2.7. Bisulfite sequencing of DNA from isolated B-cells

PBMCs were resuspended in PBS 1% BSA and stained with anti-CD19 APC (BD Biosciences, Europe). Stained cells were washed twice in PBS 1% BSA and resuspended in 400 uL of PBS 1% BSA prior to sorting on a FACS-Aria (BD Biosciences, Europe). Genomic DNA was purified from B-cells using DNeasy Blood & Tissue Kit (Qiagen, Denmark). Bisulfite conversion was performed using the EZ DNA methylation-lightning kit (ZymoResearch, Germany) according to the manufacturer’s protocol. Regions corresponding to TFAM (Transcription factor A, mitochondria), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), UBASH3a (ubiquitin-associated and SH3 domain-containing protein A) and TRIM3 (tripartite motif-containing 3) were amplified using the respective primers: Forward GGGGATAGAGGTGGTTTAATAG and Reverse TCCCTAACCCTCGGAGATTTCCTC, Forward ATAGATAGGATTGGAATTAG and Reverse CAATTTTCAAAATTCTATT and Reverse TCCCTAACCCTCGGAGATTTCCTC, Forward ATAGATAGGATTGGAATTAG and Reverse CAATTTTCAAAATTCTATT and Reverse TCCCTAACCCTCGGAGATTTCCTC, Forward ATAGATAGGATTGGAATTAG and Reverse CAATTTTCAAAATTCTATT. Purified PCR products were prepared for sequencing using the TruSeq DNA preparation protocol adapted to low input concentrations (Illumina, USA). Samples were sequenced using a MiSeq sequencer (Illumina, USA). Fastq files were analyzed by FastQC version 0.9, and adapters/primers identified were removed by Trimomatic 0.30. Read ends were clipped to remove nucleotides with a score below 3, and an average score above 15 across 4 bases. The QCed reads were mapped by Bismark version 10.0 using bowtie2 version 2.2.2, allowing 1 mismatch in
the seed, and a seed length of 10 bases. The aligner was set to a very sensitive mode using -D 50 and -R 10. Bismark was also used to process the resulting alignment files, generating a report on all methylated cytosines in the 4 sequences (using --CX and --cytosine_report). Subsequent analyses were done in R version 3.1.0. For every sample, positions covered by less than 1000 reads were excluded. For plotting, the mean and the 95% confidence interval were calculated using the adjusted bootstrap percentile method. Plots were generated using the ggplot2 package.

2.8. Statistical analysis

Results are reported as mean ± standard deviation. All statistical analyses were performed on the SPSS Statistics Package Version 21 (IBM, USA) and statistical significance was set at p < 0.05. The normality of the distribution was tested using the Skewness and the Kurtosis tests. When the assumption of normality was violated, log transformation was performed to achieve normal distribution. The difference between groups (Lean vs Obese and Lean vs T2D) for anthropometric and hematological values or global methylation levels was tested using a Student t test. Correlation levels were assessed using the Pearson’s correlation coefficient. Bisulfite sequencing results were analyzed for every cytosine position, where differences between lean and obese or T2D were tested using the non-parametric Kolmogorov–Smirnov test.

3. Results

To determine the global DNA methylation profile of individual cell types within PBMCs, we used a method previously developed in cell lines [20]. We tested this method, that relies on the assessment of DNA methylation using a monoclonal antibody to 5mC detected by flow cytometry, against high-resolution mass spectrometry, which represents, to our knowledge, the assay having the highest sensitivity and precision for the quantification of 5mC [23]. DNA methylation analysis was performed on untreated RKO cells as well as cells exposed to the DNA hypomethylating agent decitabine for 24, 48, 72 h and 3 days after drug withdrawal. Pearson’s correlation analysis of the total cytosine methylation levels measured using both techniques revealed a tight association (r = 0.97, p = 0.001, Fig. 1A and B). This was confirmed in Jurkat cells where decitabine effect was successfully detected by flow cytometry (Fig. 1C), further validating the use of flow cytometry to monitor global DNA methylation.

We next used this assay to determine global DNA methylation levels in PBMCs from obese and T2D individuals. Importantly, because blood global DNA methylation has been

### Table 1 – Blood cell counts.

<table>
<thead>
<tr>
<th></th>
<th>Lean (n = 4)</th>
<th>Obese (n = 12)</th>
<th>T2D (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils (%)</td>
<td>51.8 ± 4.5</td>
<td>52.7 ± 8.2</td>
<td>58.7 ± 10.6</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>8.9 ± 2.1</td>
<td>7.7 ± 1.7</td>
<td>6.6 ± 1.3</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>35.5 ± 4.7</td>
<td>35.6 ± 6.5</td>
<td>31.2 ± 10.5</td>
</tr>
<tr>
<td>T helper cells (%)</td>
<td>16.6 ± 3.6</td>
<td>16.2 ± 3.8</td>
<td>15.8 ± 8.4</td>
</tr>
<tr>
<td>T cytotoxic cells (%)</td>
<td>6.4 ± 1.8</td>
<td>10.1 ± 4.1</td>
<td>8.1 ± 3.0</td>
</tr>
<tr>
<td>B cells (%)</td>
<td>4.6 ± 1.4</td>
<td>5.4 ± 2.1</td>
<td>4.0 ± 2.9</td>
</tr>
<tr>
<td>NK cells (%)</td>
<td>5.9 ± 2.5</td>
<td>4.7 ± 2.8</td>
<td>3.8 ± 1.7</td>
</tr>
</tbody>
</table>

T2D: type 2 diabetes group, NK: natural killer. No significant difference was observed between the different groups for any of the sub-populations reported here.
Fig. 2 – Analysis of 5-methylcytosine levels in peripheral blood mononuclear cells (PBMCs), lymphocytes and monocytes between lean and obese or type 2 diabetic subjects. A — Representative quantification of 5-methylcytosine levels in PBMCs, lymphocytes and monocytes (obese group in dark line and lean group in filled gray). B — Comparison of 5-methylcytosine median fluorescence intensity (MFI) between the lean group (gray) and the obese group (black). C — Representative quantification of 5-methylcytosine levels in PBMCs, lymphocytes and monocytes (lean group in filled gray and type 2 diabetic group (T2D) in dark line). D — Comparison of 5-methylcytosine MFI between the lean group (gray) and T2D group (black).
Fig. 3 – Comparison of global methylation in lymphocyte sub-populations between lean and obese or type 2 diabetic patients. A — Representative 5-methylcytosine levels in T helper, T cytotoxic, B and Natural Killer (NK) cells (lean group in filled gray and obese group in dark line). B — Comparison of 5-methylcytosine median fluorescence intensity (MFI) between the lean group (gray) and the obese group (black), *Significantly different from the obese group (p < 0.05). C — Representative 5-methylcytosine levels in T helper, T cytotoxic, B and NK cells (lean group in filled gray and T2D in dark line). D — Comparison of 5-methylcytosine MFI between the lean group (gray) and the T2D group (black), *significantly different from the T2D group (p < 0.05).
shown to vary with age [24], we age-matched obese and T2D participants to two separate control cohorts of lean and normal glucose tolerant subjects. In addition, it has previously been hypothesized that a variation in blood cell counts could potentially mask an alteration in global methylation in a cell-type-specific fashion [14]. We thus investigated leukocyte subtypes frequency. However, in our population, blood cell counts showed similar profile across groups (Table 1, p > 0.05).

We then compared the obese and T2D subjects to their respective control groups and found no difference in global DNA methylation levels in the PBMCs fraction (comprising both lymphocytes and monocytes) or in the lymphocyte or monocyte populations assessed separately (Fig. 2A and B for lean vs obese, n = 11 and n = 14 respectively, Fig. 2C and D for lean vs T2D, n = 7 and n = 12 respectively, p > 0.05).

We further analyzed global DNA methylation in individual cell sub-types (T helper, T cytotoxic, B and NK cells). We found that B cells from obese subjects showed a marked increase in methylation levels compared to their lean controls (Fig. 3A and B, n = 14 for obese and n = 11 for lean, p = 0.012), but not T helper, T cytotoxic or NK cells (Fig. 3A and B, p > 0.05). In T2D patients we detected increased methylation levels in B and NK cells (Fig. 3C and D, n = 12 for T2D and n = 7 for lean, p = 0.022 and p = 0.004 for B cells and NK cells, respectively) but no significant methylation difference in T helper and T cytotoxic cells (Fig. 3C and D, p > 0.05). Moreover, we found that global DNA methylation

Fig. 4 – Association between global DNA methylation in B and NK lymphocytes and metabolic indices. Correlation between 5-methylcytosine levels (median fluorescence intensity or MFI) in B lymphocytes and glucose A-, insulin B- and HOMA-IR C-. Correlation between 5-methylcytosine levels (MFI) in natural killer (NK) lymphocytes and glucose D-, insulin E- and HOMA-IR F-.
Fig. 5 – Analysis of gene specific DNA methylation in B-cells of lean, obese and type 2 diabetic subjects. Percent methylation of cytosines within the GAPDH, TFAM, UBASH3A and TRIM3 genes in B-cells from the Lean (Red), Obese (Green) or T2D (Blue) subjects, relative to transcription start site (TSS). Data are shown as mean with 95% confidence interval. Filled symbols — CpG, Empty symbol — non-CpG, Squares — significantly different from lean group (p < 0.05).
in B cells was correlated with fasting insulin (Fig. 4B, r = 0.64, p = 0.008, n = 16) and insulin resistance (Fig. 4C, HOMA-IR, r = 0.63, p = 0.009, n = 16) but not with fasting glucose (Fig. 4A), further favoring a link between B cells and the development of insulin resistance [12]. A similar trend was also observed in NK cells, but only reaching near significance for insulin resistance (Fig. 4F, r = 0.49, p = 0.057, n = 16), whereas no association was observed with fasting glucose or insulin (Fig. 4D and E).

To determine if global changes in DNA methylation affect every single cytosine in the genome or are gene-specific, we analyzed the methylation of a housekeeping gene and genes shown in other studies to be differentially methylated in PBMCs from obese (TRIM3, UBASH3A [8]) or insulin resistant subjects (TFAM). In the regions tested, only few cytosines residues were found differentially methylated, notably cytosine –235 relative to the transcription start site of the UBASH3A gene, which was hypermethylated in T2D (Fig. 5). These data suggest that the global alteration in the DNA methylome of B lymphocytes is gene-specific.

4. Discussion

Numerous studies have reported that global and gene-specific DNA methylation profiles are altered in blood from obese individuals, without focusing on specific blood cell subpopulations [3,6–9,14]. Here, we have used a flow cytometry-based assay to further determine, in a cell type-specific manner, the global DNA methylation profile of blood obtained from obese and T2D subjects. We provide evidence that obesity and T2D are associated with a dramatic remodeling of the epigenome of specific immune cell types.

Previously, Zhao et al. reported that global DNA methylation in peripheral blood leukocytes, as assessed by bisulfite pyrosequencing on Alu elements, was associated with insulin resistance [9]. In our study we did not find any difference in global DNA methylation for PBMCs, lymphocytes or monocytes between lean, obese or type 2 diabetic individuals. Given that our analysis was conducted in pure B cell fractions, the discrepancy could be due to monocytes and other lymphocytes subpopulations accounting for the changes detected in whole PBMCs fractions [8].

In the present study, we found that both obese and type 2 diabetic individuals were characterized by increased global DNA methylation in B cells. Previously, B cells have been shown to play a central role in the development of insulin resistance [12], through the production of IgG antibodies and the activation of T cells and macrophages [13]. It is well documented that several functions of B cells are epigenetically regulated and that epigenetic dysregulation in immune cells has been associated to altered immune function in disease [26]. Similarly, we found that NK cells, though only in type 2 diabetes, showed increased global methylation. NK cells' functions can also be epigenetically regulated [27] and NK cells have been associated with diet-induced obesity and inflammation further leading to insulin resistance [28,29]. Our results showing that global DNA methylation is increased in B and NK cells suggest that epigenetic changes are involved in the altered immune function described in obesity and T2D. This is further supported by the positive correlation we observed between the level of insulin resistance and global DNA methylation in B cells and to a certain extent in NK cells.

Collectively, our results show that insulin resistance is associated with dramatic epigenetic changes in specific lymphocyte subpopulations, potentially contributing to the altered immune function reported in metabolic disorders. Interestingly, the differences in global DNA methylation were not found at the whole PBMCs level but in specific cell types. This stresses the relevance of using cell type-specific assays when investigating epigenetic signatures in clinical tissue samples characterized by a high heterogeneity in cell types frequency and phenotype, such as blood.

Authors’ contribution

This study was designed by DS and RB. Data were acquired by DS, SV, ID, JL, LH, VN and AF; analyzed by DS, SV, ID, JL, LH and RB; and interpreted by DS, SV, ID, LH and RB. The manuscript was written by DS and RB; and reviewed and approved by all authors.

Funding

This study was funded by the Novo Nordisk Foundation to RB and a Start Up Fund from UNSW Australia to DS.

The Novo Nordisk Foundation Centre for Basic Metabolic Research is an independent Research Centre at the University of Copenhagen partially funded by an unrestricted donation from the Novo Nordisk Foundation (www.metabol.ku.dk).
Conflict of interest

The authors have no competing financial interests to declare.

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