Epigenome-wide association of DNA methylation markers in peripheral blood from Indian Asians and Europeans with incident type 2 diabetes: a nested case-control study

John C Chambers, Marie Loh*, Benjamin Lehne*, Alexander Drong*, Jennifer Kriebel*, Valeria Motta*, Simone Wahl, Hannah R Elliott, Federica Rota, William R Scott, Weihua Zhang, Sian-Tsung Tan, Gianluca Campanella, Marc Chadeau-Hyam, Loic Yengo, Rebecca C Richmond, Martyna Adamowicz-Brice, Uzma Afzal, Kiymet Bozaoglu, Zuan Yu Mok, Hong Kiat Ng, François Pattou, Holger Prokisch, Michelle Ann Rozario, Letizia Tarantini, James Abbott, Mika Ala-Korpela, Benedetta Albetti, Ole Ammerpohl, Pier Alberto Bertazzi, Christine Blancher, Robert Caiazzo, John Danesh, Tom R Gaunt, Simon de Lusignan, Christian Gieger, Thomas Illig, Sujeet Jha, Simon Jones, Jeremy Jowett, Antti J Kangas, Anuradhani Kasturiratne, Norihiro Kato, Navaratnam Kotea, Sudhir Kowlessur, Janne Pitkäniemi, Prakash Punjabi, Danish Saleheen, Clemens Schafmayer, Pasi Soininen, E-Shyong Tai, Barbara Thorand, Jaakko Tuomilehto, Ananda Rajitha Wickremasinghe, Soterios A Kyrtopoulos, Timothy J Aitman, Christian Herder, Jochen Hampe, Stéphane Cauchi, Caroline L Relton, Philippe Froguel, Richie Soong, Paolo Vineis, Marjo-Riitta Jarvelin*, James Scott*, Harald Grallert*, Valentina Bollati*, Paul Elliott*, Mark I McCarthy*, Jaspal S Kooner**

Summary

Background Indian Asians, who make up a quarter of the world's population, are at high risk of developing type 2 diabetes. We investigated whether DNA methylation is associated with future type 2 diabetes incidence in Indian Asians and whether differences in methylation patterns between Indian Asians and Europeans are associated with, and could be used to predict, differences in the magnitude of risk of developing type 2 diabetes.

Methods We did a nested case-control study of DNA methylation in Indian Asians and Europeans with incident type 2 diabetes who were identified from the 8-year follow-up of 25 372 participants in the London Life Sciences Prospective Population (LOLIPOP) study. Patients were recruited between May 1, 2002, and Sept 12, 2008. We did epigenome-wide association analysis using samples from Indian Asians with incident type 2 diabetes and age-matched and sex-matched Indian Asian controls, followed by replication testing of top-ranking signals in Europeans. For both discovery and replication, DNA methylation was measured in the baseline blood sample, which was collected before the onset of type 2 diabetes. Epigenome-wide significance was set at $p<1\times10^{-7}$. We compared methylation levels between Indian Asian and European controls without type 2 diabetes at baseline to estimate the potential contribution of DNA methylation to increased risk of future type 2 diabetes incidence among Indian Asians.

Findings 1608 (11·9%) of 13 535 Indian Asians and 306 (4·3%) of 7066 Europeans developed type 2 diabetes over a mean of 8·5 years (SD 1·8) of follow-up. The age-adjusted and sex-adjusted incidence of type 2 diabetes was 3·1 times (95% CI 2·8–3·6; p<0·0001) higher among Indian Asians than among Europeans, and remained 2·5 times (2·1–2·9; p<0·0001) higher after adjustment for adiposity, physical activity, family history of type 2 diabetes, and baseline glycaemic measures. The mean absolute difference in methylation level between type 2 diabetes cases and controls ranged from 0·5% (SD 0·1) to 1·1% (0·2). Methylation markers at five loci were associated with future type 2 diabetes incidence; the relative risk per 1% increase in methylation was 1·09 (95% CI 1·07–1·11; $p=1\cdot3\times10^{-17}$) for *ABCG1*, 0·94 (0·92–0·95; $p=4\cdot2\times10^{-11}$) for *PHOSPHO1*, 0·94 (0·92–0·96; $p=1\cdot4\times10^{-9}$) for *SOCS3*, 1·07 (1·04–1·09; $p=2\cdot1\times10^{-10}$) for *SREBF1*, and 0·92 (0·90–0·94; $p=1\cdot2\times10^{-17}$) for *TXNIP*. A methylation score combining results for the five loci was associated with future type 2 diabetes incidence (relative risk quartile 4 *vs* quartile 1 3·51, 95% CI 2·79–4·42; $p=1\cdot3\times10^{-26}$), and was independent of established risk factors. Methylation score was higher among Indian Asians than Europeans ($p=1\times10^{-34}$).

Interpretation DNA methylation might provide new insights into the pathways underlying type 2 diabetes and offer new opportunities for risk stratification and prevention of type 2 diabetes among Indian Asians.

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*Contributed equally

Department of Epidemiology and Biostatistics (J C Chambers PhD, M Loh PhD, B Lehne PhD, W R Scott MRCP, W Zhang PhD, G Campanella MSc, M Chadeau-Hyam PhD, U Afzal MRCP, Prof P Froguel PhD, Prof P Vineis MD, Prof M-R Jarvelin PhD, Prof P Elliott PhD), **MRC-PHE Centre for Environment and Health** (J C Chambers, Prof M-R Jarvelin, Prof P Elliott, Prof J S Kooner FRCP), **National Heart and Lung Institute** (W R Scott, S-T Tan MRCP, Prof J Scott PhD, Prof J S Kooner), **and Bioinformatics Support Service** (J Abbott PhD), **Imperial College London, London, UK; Ealing Hospital NHS Trust, Middlesex, UK** (J C Chambers, W Zhang, S-T Tan, U Afzal, Prof J S Kooner); **Imperial College Healthcare NHS Trust, London, UK** (J C Chambers, P Punjabi FRCS, Prof J S Kooner); **Translational Laboratory in Genetic Medicine, Agency for Science, Technology and Research (A*STAR), Singapore, Singapore** (M Loh); **High Throughput Genomics, Oxford Genomic Centre** (C Blancher PhD), **Wellcome**

Trust Centre for Human Genetics (A Drong PhD, Prof M I McCarthy MD), **and Oxford Centre for Diabetes, Endocrinology and Metabolism** (Prof M I McCarthy), **University of Oxford, Oxford, UK; Research Unit of Molecular Epidemiology** (J Kriebel PhD, S Wahl MSc, C Gieger PhD, H Grallert PhD), **Institute of Genetic Epidemiology** (C Gieger), **Institute of Epidemiology II** (J Kriebel, S Wahl, C Gieger, B Thorand PhD, H Grallert), **and Institute of Human Genetics** (H Prokisch PhD), **Helmholtz Zentrum München, German Research Centre for Environmental Health, Neuherberg, Germany; German Center for Diabetes Research, Munich, Germany** (J Kriebel, S Wahl, B Thorand, H Grallert); **Department of Clinical Sciences and Community Health, Università degli Studi di Milano, Milan, Italy** (V Motta PhD, F Rota PhD, L Tarantini PhD, B Albetti PhD, Prof P A Bertazzi MD, V Bollati PhD); **Computational Medicine** (Prof M Ala-Korpela PhD), **MRC Integrative Epidemiology Unit, School of Social and Community Medicine** (H R Elliott Dip Biol, R C Richmond BA, R Caiazzo PhD, T R Gaunt FRCP, Prof C L Relton PhD), **University of Bristol, Bristol, UK; European Genomic Institute for Diabetes, Lille, France** (L Yengo PhD, Prof F Pattou PhD, R Caiazzo, S Cauchi PhD, Prof P Froguel); **CNRS UMR8199, Pasteur Institute of Lille, Lille, France** (L Yengo, S Cauchi, Prof P Froguel); **Lille 2 University, Lille, France** (L Yengo, Prof F Pattou, S Cauchi, Prof P Froguel); **Physiological Genomics and Medicine Group, Medical Research Council Clinical Sciences Centre, Imperial College, Hammersmith Hospital, London, UK** (M Adamowicz-Brice PhD, Prof T J Aitman PhD); **Genomics and Systems Biology, Baker IDI Heart and Diabetes Institute, Melbourne, VIC, Australia** (K Bozaoglu PhD, R Caiazzo, J Jowett PhD); **Cancer Science Institute of Singapore** (Z Y Mok BSc, H K Ng BSc, M A Rozario BSc, R Soong PhD), **Department of Medicine, Yong**

Introduction

Type 2 diabetes is a major public health problem worldwide, particularly in rapidly urbanising countries such as India.[1,2] Indian Asians (ie, people originating from India, Pakistan, Bangladesh, or Sri Lanka), who comprise a quarter of the world's population, are at higher risk of type 2 diabetes than are North Americans and Europeans.[2–4] Type 2 diabetes is estimated to affect more than 100 million people in India alone by 2030.[1]

Diet, obesity, and physical inactivity are major risk factors for type 2 diabetes in Indian Asians, as they are in other populations, but differences in the prevalence of these behaviours between Indian Asians and Europeans do not seem to account for their increased risk of type 2 diabetes.[5,6] Genome-wide association studies among Indian Asians and Europeans have identified common genetic variants at about 80 genetic loci that affect the risk of type 2 diabetes,[4,7–10] although these only explain about 5% of type 2 diabetes risk in both populations.[11,12] Improved understanding of the mechanisms underlying the high incidence of type 2 diabetes among Indian Asians is needed to help reverse the epidemic of type 2 diabetes in this population.

DNA methylation at cytosine–guanine nucleotide pair (CpG) sites affects gene expression, cellular differentiation, and molecular response to environmental stressors.[13–16] Methylation at the *FTO* locus and other loci containing genetic variants linked to type 2 diabetes is associated with prevalent type 2 diabetes in Ashkenazi Jews, and baseline methylation of *FTO* was used as a marker to predict the likelihood of progressing from normal to impaired glucose metabolism in a follow-up study.[17] Additionally, disturbances in methylation at the *PPARG*, *KCNQ1*, *TCF7L2*, and *IRS1* loci have been reported in adipose and pancreatic tissue from people with prevalent type 2 diabetes.[18–20] These findings raise the possibility that alterations in DNA methylation might be involved in the biological pathways underlying development of type 2 diabetes.

Therefore, in this study, we aimed to investigate whether variations in DNA methylation are associated with future type 2 diabetes among Indian Asians, and whether differences in methylation patterns between Indian Asians and Europeans are associated with, and could be used to predict, differences in the magnitude of risk of developing type 2 diabetes.

Methods

Patients

In this nested case-control study, we did epigenome-wide association of DNA methylation in Indian Asians and Europeans with incident type 2 diabetes who were identified from the 8-year follow-up of 25 372 participants in the London Life Sciences Prospective Population (LOLIPOP) study. LOLIPOP[4] was a prospective population study of Indian Asian (n=17 606) and European (n=7766) men and women, recruited at age 35–75 years from the lists of 58 family doctors in west London, UK, between May 1, 2002, and Sept 12, 2008. Indian Asians had all four grandparents born on the Indian subcontinent (India, Pakistan, Sri Lanka, or Bangladesh); Europeans were of self-reported white ancestry.

At baseline, all participants completed a structured assessment of cardiovascular and metabolic health that included personal and family history, leisure time physical activity, and anthropometry. Participants were seen between 0800 h and 1200 h, after an overnight 8-h fast, for collection of fasting blood samples for measurement of complete blood count, HbA_{1c}, and glucose, insulin, and lipid concentrations. Aminoacid concentrations were measured by ^1H nuclear magnetic resonance spectroscopy.[21] Type 2 diabetes was defined as physician diagnosis, fasting glucose of at least 7 mmol/L, or HbA_{1c} of at least 6·5% (47·5 mmol/mol).[22] Physical activity was defined as engaging in at least 90 min of at least moderately vigorous leisure time physical activity (≥3 Metabolic Equivalent of Task) per week. Homoeostasis model assessments of insulin resistance (HOMA-IR) and β-cell function (HOMA-B) were calculated.[23] Samples of whole blood were stored at –80°C before extraction of genomic DNA.

The LOLIPOP study is approved by the National Research Ethics Service (07/H0712/150) and all participants gave written informed consent at enrolment.

Procedures

At follow-up, on Dec 31, 2013, electronic health records from primary care practitioners were extracted for each participant, and structured queries were used to identify individuals with incident type 2 diabetes. Additionally, a random subset of 7640 participants attended a clinical assessment between Jan 11, 2010, and Dec 31, 2013, during which they repeated the baseline questionnaire on cardiovascular and metabolic health and gave fasting blood samples for measurement of glucose concentration and HbA_{1c}. This assessment was done to identify people who did not have type 2 diabetes at the end of follow-up (ie, not receiving treatment for type 2 diabetes and with a fasting glucose concentration <7 mmol/L and HbA_{1c} <6·5% [47·5 mmol/mol]). Participants with incident type 2 diabetes were defined as those who did not have type 2 diabetes at baseline, but who developed the disease during follow-up. Controls were participants who did not have type 2 diabetes both at baseline and follow-up.

Epigenome-wide association was done among the first 1074 Indian Asian participants with incident type 2 diabetes and 1590 matched Indian Asian controls. Controls were matched to cases by age (5-year groups) and sex. DNA methylation was quantified in the baseline DNA samples collected at study enrolment. Samples were analysed in random order, masked to case-control status. Bisulfite conversion of genomic DNA from peripheral blood was done using the EZ DNA methylation

kit (Zymo Research, Orange, CA, USA). Epigenome-wide association was done using the Illumina HumanMethylation450 (450K) array according to the manufacturer's instructions (appendix). Replication testing was done among Europeans from the LOLIPOP study and KORA (Cooperative Health Research in the Region of Augsburg) S3 and S4 studies who had incident type 2 diabetes (appendix).[24] DNA methylation was quantified in the baseline DNA samples collected at study enrolment, when none of the participants had type 2 diabetes. Samples were matched for age and sex. Replication testing in samples from the LOLIPOP study was done by pyrosequencing of bisulphite-treated DNA (appendix), and in KORA samples using the 450K array. Levels of DNA methylation at the CpG sites of interest were compared between representative samples of Indian Asian and European controls (ie, without type 2 diabetes) in the LOLIPOP study using pyrosequencing.

The association of the identified DNA methylation markers with adiposity (quantified by dual-energy x-ray absorptiometry) phenotypes was studied among participants from the Avon Longitudinal Study of Parents and Children (ALSPAC) with DNA methylation measured using the 450K array (appendix).

To better understand the relation between DNA methylation markers and type 2 diabetes, we did fine-mapping of one of the identified loci (*TXNIP:* chr 1, bp 145,436,694-145,446,572) in 172 samples. We used a combination of next-generation sequencing and pyrosequencing (appendix). We did pyrosequencing of the CpG sites that showed close correlation (ie, $r>0\cdot5$) with the sentinel marker in Indian Asian participants with incident type 2 diabetes and Indian Asians controls, to quantify their association with type 2 diabetes both as single markers and in aggregate (ie, mean methylation across the sites assayed).

We examined cross-tissue patterns of methylation-paired peripheral blood and liver samples from obese individuals in the ABOS (Atlas Biologique de l'Obésité Sévère) study (NCT01129297; appendix). We separately investigated the relation between methylation and gene expression using peripheral blood leucocytes from Indian Asians and Europeans and liver tissue from Europeans (appendix).

Statistical analysis

We analysed epigenome-wide data in R (version 2.15) using minfi and other R scripts.[25] Marker intensities were normalised by quantile normalisation. A differential white blood cell (lymphocyte, monocyte, and granulocyte) count was available for all participants, and we used the epigenome-wide methylation scores to impute a further four lymphocyte subsets (CD4, CD8, natural killer, and B cells).[26] We did a principal components analysis to quantify latent structure in the data, including batch effects.[27]

We did single-marker tests using logistic regression to examine the association of each autosomal CpG site with type 2 diabetes incidence, adjusted for age and sex. We included intensity values from Infinium 450K assay control probes, bisulfite conversion batch, measured white cells and imputed white cell subsets, and the first five principal components as covariates in the regression models (appendix).[27] We corrected the association results for the genomic control inflation factor. Comparisons

	Europeans			Indian Asians		
	Controls (n=6760)	Incident type 2 diabetes (n=306)	p value	Controls (n=11 927)	Incident type 2 diabetes (n=1608)	p value
Age (years)	51·8 (11·2)	58·9 (9·5)	<0·0001	48·7 (10·8)	52·2 (10·0)	<0·0001
Sex						
Male	3975 (58·8%)	230 (75·2%)	<0·0001	6906 (57·9%)	1082 (67·3%)	<0·0001
Female	2785 (41·2%)	76 (24·8%)	..	5021 (42·1%)	526 (32·7%)	..
Family history of type 2 diabetes	1197 (17·7%)	71 (23·2%)	0·01	4198 (35·2%)	691 (43·0%)	<0·0001
Physical activity	3576 (52·9%)	122 (39·9%)	<0·0001	3876 (32·5%)	421 (26·2%)	<0·0001
BMI (kg/m^2)	27·0 (4·8)	31·1 (5·4)	<0·0001	26·8 (4·3)	28·9 (4·6)	<0·0001
Waist circumference (cm)	93·5 (13·0)	105·6 (13·0)	<0·0001	26·8 (4·3)	28·9 (4·6)	<0·0001
Waist:hip ratio	0·91 (0·08)	0·97 (0·07)	<0·0001	0·93 (0·08)	0·97 (0·07)	<0·0001
Glucose (mmol/L)	5·0 (0·5)	5·7 (0·6)	<0·0001	5·0 (0·5)	5·5 (0·6)	<0·0001
Insulin (pmol/L)	61·12 (51·39)	107·65 (74·31)	<0·0001	77·78 (56·95)	111·12 (72·23)	<0·0001
HbA_{1c}						
Percent	5·3 (0·4)	5·6 (0·5)	<0·0001	5·5 (0·4)	5·8 (0·5)	<0·0001
Mmol/mol	34 (5)	39 (4)	..	37 (4)	40 (5)	..
HOMA-IR	2·1 (1·9)	4·1 (3·1)	<0·0001	2·6 (2·0)	4·1 (2·8)	<0·0001
Impaired fasting glucose	277 (4·1%)	101 (33·0%)	<0·0001	406 (3·4%)	388 (24·1%)	<0·0001

Data are mean (SD) or number (%). HOMA-IR=homoeostasis model assessment of insulin resistance.

Table 1: **Demographics and baseline clinical characteristics of incident type 2 diabetes cases and controls**

Loo Lin School of Medicine (E-S Tai PhD), **and Saw Swee Hock School of Public Health** (E-S Tai), **National University of Singapore, Singapore; Inserm UMR859, Claude-Huriez Hospital, Lille, France** (Prof F Pattou); **Institute of Human Genetics, Technical University Munich, München, Germany** (H Prokisch); **Computational Medicine** (Prof M Ala-Korpela, A J Kangas MSc, P Soininen PhD), **Institute of Health Sciences** (M Loh, Prof M-R Jarvelin) **and Biocenter Oulu** (Prof M-R Jarvelin), **University of Oulu, Oulu, Finland; NMR Metabolomics Laboratory, School of Pharmacy, University of Eastern Finland, Kuopio, Finland** (Prof M Ala-Korpela, P Soininen); **Unit of Primary Care** (Prof M-R Jarvelin), **Oulu University Hospital** (Prof M Ala-Korpela), **Oulu, Finland; Institute of Human Genetics** (Prof O Ammerpohl PhD) **and Department of Visceral and Thoracic Surgery** (Prof C Schafmayer MD), **University Hospital Schleswig-Holstein, Kiel Campus, Kiel, Germany; Department of Public Health and Primary Care, University of Cambridge, Cambridge, UK** (Prof J Danesh FRCP); **University of Surrey, Guildford, UK** (Prof S de Lusignan PhD, Prof S Jones PhD); **Hannover Medical School, Hannover Unified Biobank, Hannover, Germany** (Prof T Illig PhD); **Department of Endocrinology, Diabetes and Obesity, Max Healthcare, New Delhi, India** (S Jha MRCP); **Department of Public Health, Faculty of Medicine, University of Kelaniya, Ragama, Sri Lanka** (A Kasturiratne MD, Prof A R Wickremasinghe PhD); **Department of Gene Diagnostics and Therapeutics, Research Institute, National Center for Global Health and Medicine, Tokyo, Japan** (N Kato PhD); **Department of Health Sciences, University of Mauritius, Reduit, Mauritius** (N Kotea PhD); **Ministry of Health and Quality of Life, Port louis, Mauritius** (S Kowlessur Dip Pub Health Admin); **Hjelt Institute, School of Medicine** (J Pitkäniemi PhD) **and Department of Public Health** (Prof J Tuomilehto PhD),

University of Helsinki, Finland; Finnish Cancer Registry, Institute for Statistical and Epidemiological Cancer Research, Helsinki, Finland (J Pitkäniemi); Center for Non-Communicable Diseases, Karachi, Pakistan (D Saleheen PhD); Department of Public Health and Primary Care, University of Cambridge Strangeways Research Laboratory, Cambridge, UK (D Saleheen); Cardiovascular Institute, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA (D Saleheen); Duke National University of Singapore Graduate Medical School, Singapore, Singapore (E-S Tai); National Hellenic Research Foundation, Institute of Biology, Pharmaceutical Chemistry and Biotechnology, Athens, Greece (Prof S A Kyrtopoulos PhD); Institute for Clinical Diabetology, German Diabetes Center, Leibniz Center for Diabetes Research at Heinrich Heine University Düsseldorf, Düsseldorf, Germany (C Herder PhD); German Center for Diabetes Research, Düsseldorf, Germany (C Herder); Medical Department 1, University Hospital of the Technical University Dresden, Dresden, Germany (Prof J Hampe MD); Department of Pathology, National University Hospital, Singapore, Singapore (R Soong); HuGeF Foundation, Torino, Italy (Prof P Vineis); and Oxford National Institute for Health Research Biomedical Research Centre, Churchill Hospital, Oxford, UK (Prof M I McCarthy)

between 36 samples measured in duplicate confirmed high reproducibility for quantification of DNA methylation, with no evidence for confounding by batch effect (appendix).[27]

We also used logistic regression to test the association between DNA methylation and type 2 diabetes in the replication stage. Results were combined across the discovery and replication stages by inverse variance meta-analysis. Epigenome-wide significance was set at $p<1\times10^{-7}$ providing Bonferroni correction for the 466 186 autosomal markers tested. Our choice of threshold was supported by the results of permutation testing (appendix). The prespecified criterion for taking markers through from the discovery stage to replication testing was $p<5\times10^{-7}$. Markers were considered to be associated with type 2 diabetes if they reached epigenome-wide significance overall and $p<0\cdot05$ in the replication stage. Markers on the sex chromosomes were tested similarly for association with type 2 diabetes, but separately in men and women.

Role of the funding source

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

Results

The appendix lists baseline characteristics of the 13 535 Indian Asians and 7066 Europeans who did not have type 2 diabetes at enrolment into the LOLIPOP study. Despite lower BMI ($p=0\cdot01$) and younger age ($p<0\cdot0001$), Indian Asians had higher waist circumference ($p<0\cdot0001$), waist:hip ratio ($p<0\cdot0001$), HbA_{1c} ($p<0\cdot0001$), and glucose ($p=0\cdot01$), insulin ($p<0\cdot0001$), and triglyceride ($p<0\cdot0001$) concentrations, and lower HDL cholesterol concentrations ($p<0\cdot0001$) compared with Europeans. Family history of type 2 diabetes was more common and physical activity was lower among Indian Asians compared with Europeans (both $p<0\cdot0001$).

1608 (11·9%) of 13 535 Indian Asians and 306 (4·3%) of 7066 Europeans had incident type 2 diabetes over a mean of 8·5 years (SD 1·8) of follow-up. Indian Asians who developed type 2 diabetes during follow-up had higher baseline BMI, waist circumference, waist:hip ratio, HbA_{1c}, HOMA-IR, and fasting glucose and insulin concentrations than did Indian Asian controls (all $p<0\cdot0001$; table 1).

The age-adjusted and sex-adjusted risk of type 2 diabetes was 3·1 times (95% CI 2·8–3·6; $p<0\cdot0001$) higher among Indian Asians than among Europeans, and remained 2·5 times (2·1–2·9; $p<0\cdot0001$) higher after further adjustment for the major type 2 diabetes risk factors of family history of type 2 diabetes, physical activity, BMI, waist:hip ratio, HbA_{1c}, and glucose and insulin concentrations (table 2).

The appendix lists characteristics of the 1074 Indian Asians who had incident type 2 diabetes and 1590 Indian Asian controls investigated by epigenome-wide association. Epigenome-wide association revealed an excess of association across a range of p value thresholds (appendix). Methylation markers at seven genetic regions were associated with incident type 2 diabetes at $p<5\times10^{-7}$ (table 3; appendix); these markers were assessed in replication testing among 1141 Europeans from the LOLIPOP study (181 with incident type 2 diabetes and 568 controls) and KORA S3 and S4 studies (196 with incident type 2 diabetes and 196 controls; appendix). Five of the seven methylation markers were associated with incident type 2 diabetes at $p<0\cdot05$ (appendix) in the replication samples. In the combined analysis of epigenome-wide discovery and replication data, all five markers reached epigenome-wide significance ($p<1\times10^{-7}$) for association with type 2 diabetes ($p=4\cdot7\times10^{-10}$ to $p=1\cdot5\times10^{-18}$; table 3). The appendix shows regional plots; the five loci are identified by nearest gene (*ABCG1, PHOSPHO1, SOCS3, SREBF1,* and *TXNIP*). Present knowledge regarding function of these genes is summarised in the appendix.

The mean absolute difference in methylation level between participants with type 2 diabetes and controls ranged from 0·5% (SD 0·1) to 1·1% (0·2; appendix). The relative risk for incident type 2 diabetes per 1% increase in methylation was 1·09 (95% CI 1·07–1·11; $p=1\cdot3\times10^{-17}$) for *ABCG1,* 0·94 (0·92–0·95; $p=4\cdot2\times10^{-11}$) for *PHOSPHO1,* 0·94 (0·92–0·96; $p=1\cdot4\times10^{-9}$) for *SOCS3,* 1·07 (1·04–1·09; $p=2\cdot1\times10^{-10}$) for *SREBF1,* and 0·92 (0·90–0·94; $p=1\cdot2\times10^{-17}$) for *TXNIP*. Relative risk of type 2 diabetes between the top and bottom quartiles of methylation at the five identified markers ranged from 1·73 (95% CI 1·39–2·16; $p=1\cdot2\times10^{-6}$) to 2·41 (1·93–3·02; $p=1\cdot2\times10^{-14}$; figure 1; appendix) in Indian Asians and 1·77 (1·45–2·15; $p=1\cdot7\times10^{-8}$) to 2·14 (1·76–2·61; $p=3\cdot5\times10^{-14}$) in the combined analysis with Europeans (appendix).

DNA methylation at the five identified loci was associated with BMI, waist:hip ratio, glucose concentrations, HOMA-IR, and other metabolic measures of insulin resistance (appendix). Methylation at *SREBF1, PHOSPHO1,* and *ABCG1* was also associated ($p<0\cdot05$)

	Relative risk (95% CI)	p value
Model 1: adjusted for age and sex	3·1 (2·8–3·6)	<0·0001
Model 2: as for model 1, plus family history of type 2 diabetes and physical activity	2·7 (2·4–3·1)	<0·0001
Model 3: as for model 2, plus BMI and waist:hip ratio	2·9 (2·5–3·3)	<0·0001
Model 4: as for model 3, plus glucose and insulin concentrations, and HbA_{1c}	2·5 (2·1–2·9)	<0·0001
Model 5: as for model 3, plus HOMA-IR	2·9 (2·5–3·4)	<0·0001

HOMA-IR=homoeostasis model assessment of insulin resistance.

Table 2: **Relative risk of new-onset type 2 diabetes in Indian Asians versus Europeans after various adjustments**

	Chromosome	Position	Locus	Discovery		Replication		Combined		$P_{heterogeneity}$†
				RR (95%CI)*	p value	RR (95%CI)*	p value	RR (95%CI)*	p value	
cg19693031	1	145 441 552	*TXNIP*	0·92 (0·91–0·94)	$1·0×10^{-13}$	0·96 (0·94–0·98)	$2·5×10^{-5}$	0·92 (0·90–0·94)	$1·5×10^{-18}$	0·98
cg09152259	2	128 156 114	*PROC*	0·95 (0·93–0·97)	$9·3×10^{-8}$	0·99 (0·97–1·01)	0·32	0·95 (0·93–0·97)	$4·8×10^{-7}$	0·04
cg04999691	7	150 027 050	*C7orf29*	0·95 (0·93–0·96)	$1·4×10^{-8}$	1·00 (0·98–1·02)	0·71	0·96 (0·94–0·98)	$4·8×10^{-5}$	0·004
cg11024682	17	17 730 094	*SREBF1*	1·06 (1·04–1·08)	$8·4×10^{-9}$	1·03 (1·01–1·05)	0·0054	1·07 (1·04–1·09)	$3·0×10^{-10}$	0·07
cg02650017	17	47 301 614	*PHOSPHO1*	0·94 (0·92–0·96)	$2·1×10^{-9}$	0·97 (0·95–0·99)	0·0012	0·94 (0·92–0·95)	$4·1×10^{-12}$	0·48
cg18181703	17	76 354 621	*SOCS3*	0·95 (0·93–0·97)	$2·1×10^{-7}$	0·97 (0·95–0·99)	0·0016	0·94 (0·92–0·96)	$4·7×10^{-10}$	0·76
cg06500161	21	43 656 587	*ABCG1*	1·08 (1·06–1·10)	$2·2×10^{-13}$	1·04 (1·02–1·06)	0·00012	1·09 (1·07–1·11)	$1·1×10^{-17}$	0·32

RR=relative risk. *Associated with a 1% increase in respective methylation marker in the discovery phase (1074 Indian Asians with incident type 2 diabetes and 1590 controls), in replication testing among 1141 Europeans (377 with incident type 2 diabetes) and in combined analysis. †Heterogeneity of effect between discovery and replication.

***Table 3*: Association of methylation markers with future type 2 diabetes incidence**

with quantitative measures of total and regional body fat distribution, and with lean mass, as assessed by dual-energy x-ray absorptiometry among participants of the ALSPAC study (appendix). The association of *PHOSPHO1* with lean mass remained after adjustment for BMI (appendix).

In multivariable analyses, the relation between methylation at the *TXNIP* locus and incident type 2 diabetes remained significant at $p<1×10^{-7}$ after adjustment for the measured known risk factors for type 2 diabetes of baseline BMI, waist:hip ratio, HOMA-IR, HOMA-B, and branched-chain and aromatic aminoacid concentrations (appendix). By contrast, associations of markers at *ABCG1, PHOSPHO1, SOCS3,* and *SREBF1* with type 2 diabetes were not significant after adjustment for adiposity or HOMA-IR.

In a combined analysis, the five methylation markers identified were each associated with incident type 2 diabetes among Indian Asians (appendix). A methylation score combining results for the five markers, weighted by effect size, was associated with risk of future type 2 diabetes incidence among Indian Asians (relative risk for quartile 4 *vs* quartile 1 3·51, 95% CI 2·79–4·42, $p=1·3×10^{-26}$; per 1 SD 1·68, 1·55–1·83, $p=1·1×10^{-33}$; appendix) and was not accounted for by the known type 2 diabetes risk factors of adiposity and HOMA-IR (appendix). Methylation score was replicated in the independent sample of Europeans with incident type 2 diabetes (relative risk for quartile 4 *vs* quartile 1 2·49, 95% CI 1·50–4·15, p=0·00046; per 1 SD 1·88, 1·56–2·26, $p=2·5×10^{-11}$; appendix), with no evidence for heterogeneity of effect with Indian Asians (p=0·54 and p=0·58, respectively).

As a sensitivity analysis, we excluded Indian Asians with prediabetes at baseline (HbA_{1c} ≥6% [42 mmol/mol] or fasting glucose ≥6 mmol/L); DNA methylation score remained independently associated with future type 2 diabetes incidence (relative risk for quartile 4 *vs* quartile 1 3·1, 95% CI 2·3–4·1, $p=6·1×10^{-14}$; per 1 SD 1·65, 1·48–1·84, $p=2·3×10^{-19}$; appendix).

We found evidence for an interaction between adiposity and DNA methylation. Among the 1932 normoglycaemic

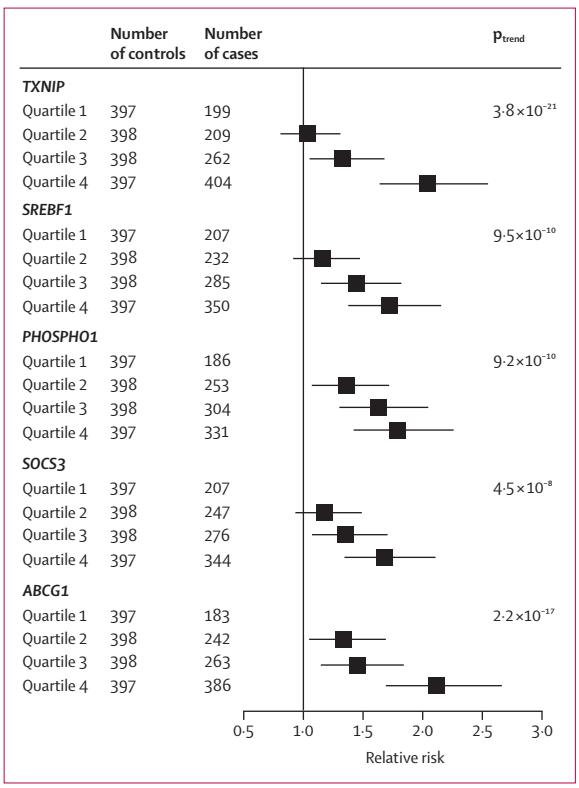

***Figure 1*: Relative risk of type 2 diabetes by quartile of methylation score**
Relative risk is for the comparison with quartile 1.

(HbA_{1c} <6% [42 mmol/mol] and fasting glucose <6 mmol/L) Indian Asians in the LOLIPOP study, future risk of type 2 diabetes incidence was up to four times higher in the highest quartile versus the lowest quartile of methylation among obese and overweight Indian Asians, but not among normal weight individuals ($p_{interaction}=0·0003$; figure 2).

Levels of DNA methylation at the CpG sites of interest were compared between 186 Indian Asian and 192 European controls. Methylation at the *ABCG1* and *SREBF1* loci was higher, and at *PHOSPHO1* and *SOCS3* loci was lower, among Indian Asian than among

Correspondence to:
Dr John C Chambers, Department of Epidemiology and Biostatistics, Imperial College London, London W2 1PG, UK
john.chambers@ic.ac.uk
or
Prof Jaspal S Kooner, National Heart and Lung Institute, Imperial College London, London W12 0NN, UK
j.kooner@ic.ac.uk
See Online for appendix

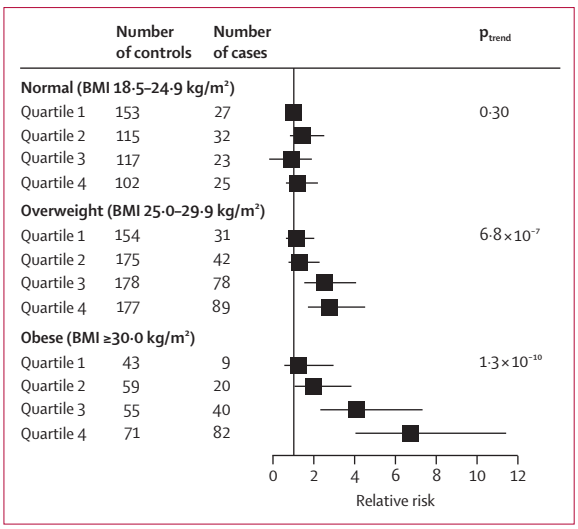

Figure 2: **Incidence of type 2 diabetes by quartile of methylation marker among Indian Asians with normoglycaemia**
Interaction between adiposity and DNA methylation on risk of type 2 diabetes $p_{interaction}$=0·0003.

European controls (appendix). At each of the loci, the amount of methylation noted among Indian Asians compared with Europeans was predictive of increased risk of type 2 diabetes (appendix).

In the multivariable analysis, the DNA methylation score was 0·86 SD (95% CI 0·74–0·98; p=1×10⁻³⁴) higher among Indian Asians than Europeans after adjustment for age, sex, BMI, waist:hip ratio, and glucose and insulin concentrations. Based on the relation between methylation score and type 2 diabetes (relative risk of type 2 diabetes incidence 1·41 per 1 SD change in methylation score), an 0·86 SD higher methylation score is associated with a 1·34 times (ie, exp[ln(1·41)×0·86]) increased risk of future type 2 diabetes incidence among Indian Asians. Thus, an estimated 32% (ln[1·34]/ln[2·5]) of the unexplained increased risk of type 2 diabetes among Indian Asians was associated with a higher methylation score.

Resequencing of the *TXNIP* locus revealed a cluster of eight CpG sites in the 3′untranslated region of *TXNIP*, which showed methylation that correlated closely with methylation at the sentinel marker (r>0·5; figure 3). Mean methylation across these eight CpG sites was associated with risk of future type 2 diabetes, and this regional association was stronger than for any individual CpG site (relative risk per 1 SD change for the discovery marker [cg19693031] 1·29, 95% CI 1·15–1·43; p=0·0052; regional score 1·38, 1·24–1·52, 0·00079; figure 3).

To test whether DNA methylation in blood correlates with methylation in a metabolically relevant tissue, we compared methylation at the five sentinel CpG sites in blood (2201 samples) and liver (116 samples) using paired samples from 175 obese European individuals. A relation was noted between methylation in peripheral blood and methylation in liver at the *TXNIP* (p=0·02) and *SOCS3* loci (p=5·3×10⁻⁵; appendix) in this group.

In the same group, we also investigated the relations between the methylation at the five loci associated with type 2 diabetes and expression of the nearest gene in blood and liver. In blood, methylation was associated with expression of *ABCG1* and *SREBF1* among both Indian Asians and Europeans (p=0·0038 to 3·8×10⁻²¹), and also showed some evidence for association with expression of *PHOSPHO1* and *SOCS3* in Europeans (appendix). In liver, methylation was associated with *TXNIP* expression (p=0·039 to 0·00074; appendix).

Discussion

Methylation of DNA at CpG sites regulates gene expression and mediates the biological response to environmental exposures.[13–16] Although previous studies have investigated the association between methylation and type 2 diabetes, these have been largely limited to the study of patients with established disease.[17–20,28,29] In this large, prospective, nested case-control study, we identified an association between differential methylation at five genetic loci (*ABCG1, PHOSPHO1, SOCS3, SREBF1,* and *TXNIP*) and risk of future type 2 diabetes incidence among Indian Asians and Europeans. We found an about four times higher risk of future type 2 diabetes between upper and lower quartiles of a DNA methylation score, which was independent of known major risk factors for type 2 diabetes. The association of DNA methylation score with risk of type 2 diabetes was particularly evident in normoglycaemic Indian Asians, among whom high levels of methylation in metabolically unhealthy obese individuals were associated with a high risk of future type 2 diabetes. Although further validation of our findings is needed to confirm generalisability to non-migrant Indian Asians, our findings raise the possibility that assessment of DNA methylation could be used to identify Indian Asians who would benefit from early pharmacological or lifestyle interventions to prevent development of type 2 diabetes.

In this study, risk of type 2 diabetes incidence was three times higher among Indian Asians than among Europeans; this risk was not accounted for by differences in adiposity, glycaemic measures, or physical activity. Using epigenome-wide association, we found that differential methylation of genomic DNA at five genetic loci was associated with incident type 2 diabetes in both Indian Asians and Europeans. Fine-mapping of the top-ranking locus revealed several additional methylation markers that were associated with type 2 diabetes. Methylation levels differ between Indian Asians and Europeans, suggesting that measurement of DNA methylation might help explain the increased risk of type 2 diabetes among Indian Asians (panel).

The reasons underlying the disturbances in methylation at the *ABCG1, PHOSPHO1, SOCS3, SREBF1,* and *TXNIP* loci before type 2 diabetes onset are not known. The five methylation sites are in or near

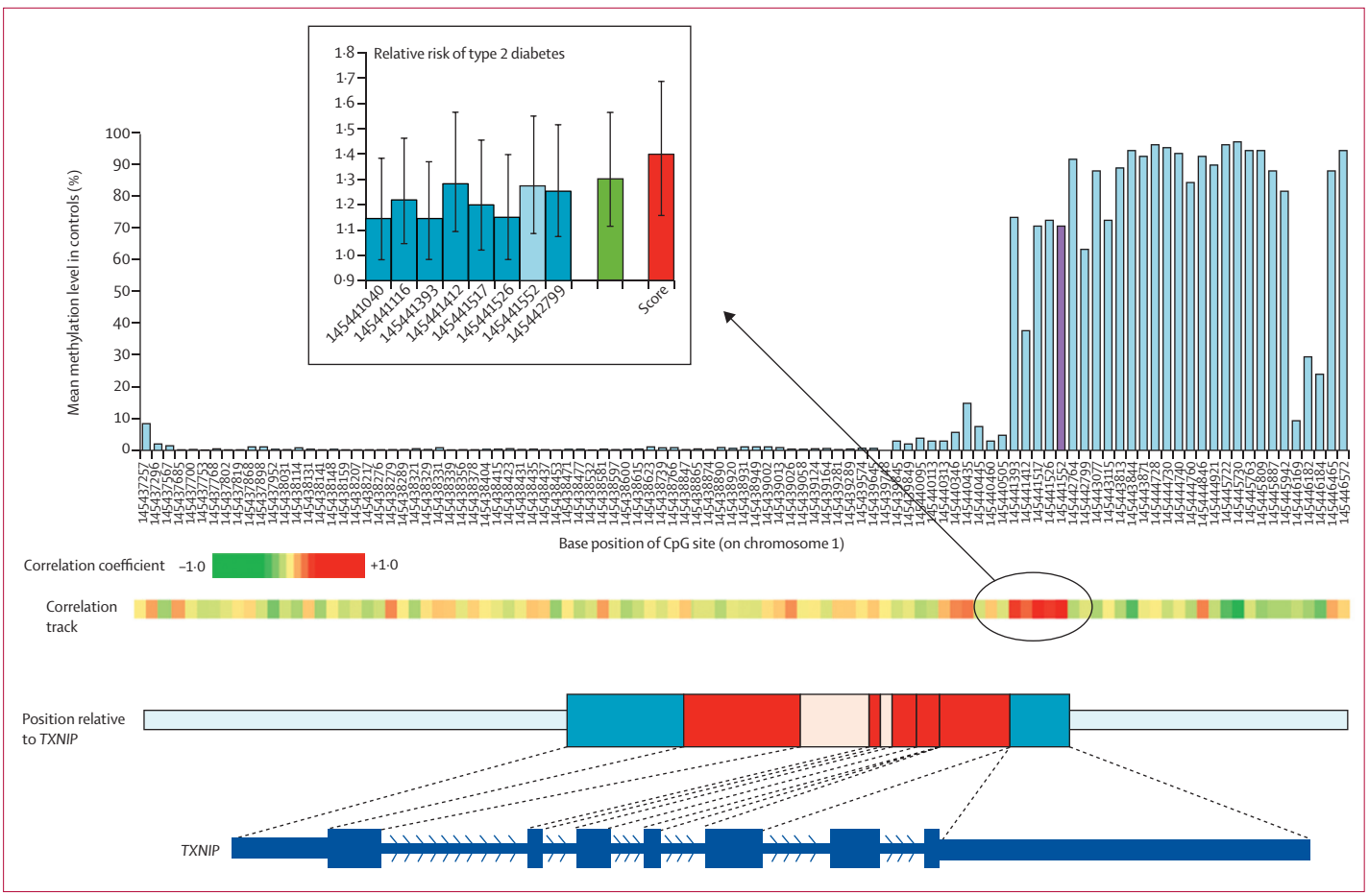

Figure 3: **Targeted resequencing of the *TXNIP* locus by next-generation sequencing**
Bars show mean methylation at the CpG sites assessed. The purple bar is the sentinel marker, as identified by epigenome-wide association analyses. The correlation track shows the correlation between methylation at each CpG site with the sentinel marker. The inset graph shows the relative risk for type 2 diabetes associated with a 1 SD reduction in methylation or methylation score for the methylation markers at the *TXNIP* locus identified by targeted resequencing. Results are shown for the eight individual CpG sites assayed by pyrosequencing (blue; light blue for the sentinel marker); the sentinel marker by microarray (green); and the sum of all eight methylation markers (orange). CpG=cytosine–guanine nucleotide pair.

genes in key pathways underlying type 2 diabetes and related metabolic defects. *TXNIP* is a key component of pancreatic β-cell biology, nutrient sensing, energy metabolism, and regulation of cellular redox. *TXNIP* expression is highly induced by glucose through activation of the carbohydrate response element-binding protein, which binds the *TXNIP* promoter.[35] TXNIP downregulates GLUT1, a major transmembrane glucose transporter, thereby acting as a negative feedback loop to regulate glucose entry and mitochondrial oxidative stress. *TXNIP* is one of the most glucose-responsive genes expressed in human islets; in animal models, Txnip is a mediator of glucotoxic β-cell death, whereas *Txnip* downregulation protects against obesity-induced diabetes by preventing β-cell apoptosis and preserving β-cell mass.[36] TXNIP might also contribute to regulation of adiposity and energy expenditure through hypothalamic pathways.[37] ABCG1 is involved in cholesterol and phospholipid transport and in insulin secretion.[38] *Abcg1*$^{-/-}$ mice have impaired glucose tolerance and insulin secretion, but normal insulin sensitivity.[39] Methylation at *ABCG1* is associated with fasting insulin and HOMA-IR.[33] SREBF1 is a key transcriptional regulator of hepatic lipogenesis.[40] Insulin activates *SREBF1*, and SREBF1 contributes to the dyslipidaemia and hepatic steatosis that occurs in obesity, insulin resistance, and type 2 diabetes.[41] Our results pave the way for functional studies to define the pathways linking DNA methylation at these sites to adiposity, type 2 diabetes, and their related metabolic disturbances.

DNA methylation is affected by both genetic and environmental factors, including adverse intra-uterine and early-life exposures, and might also show transgenerational inheritance.[42] *TXNIP* expression is highly sensitive to glucose concentration, which is consistent with abnormal *TXNIP* methylation being an early marker for impaired glucose homoeostasis.[35] By contrast, we found that methylation at *ABCG1*,

Panel: Research in context

Systematic review

Indian Asians are at high risk of developing type 2 diabetes; this increased risk is not explained by adiposity, physical inactivity, adverse diet, or known genetic susceptibility factors. DNA methylation is a major mechanism in genomic regulation, and has been implicated in adiposity and insulin resistance. We searched PubMed on Nov 27, 2014, for the terms ("epigenome-wide association study" OR "EWAS") AND "type 2 diabetes". We found five articles, none of which were epigenome-wide association studies with incident type 2 diabetes as the phenotype of interest. Three were environment-wide association studies.[30–32] One study[33] was an epigenome-wide association study of fasting measures of glucose, insulin, and HOMA-IR among 837 non-diabetic participants, and the other[34] was a study of the association between methylation profiles from peripheral blood samples and obesity in 48 obese and 48 lean African–American youths. Hypomethylation at *FTO* is associated with prevalent type 2 diabetes and progression to impaired glucose metabolism.[17] Other studies have investigated DNA methylation and type 2 diabetes in adipose, muscle, and pancreatic tissue from small numbers of people.[18–20] We found no other epigenome-wide study investigating whether differences in DNA methylation in peripheral blood predict future type 2 diabetes incidence.

Interpretation

In this large prospective nested case-control study of Indian Asians and Europeans, we found an about three times higher risk of type 2 diabetes incidence among Indian Asians than among Europeans, which was not explained by differences in the prevalence of conventional risk factors. Using epigenome-wide association analyses, we identified and replicated an independent association between DNA methylation and future type 2 diabetes incidence. We found a four times higher risk of future type 2 diabetes between upper and lower quartiles of methylation, and found that methylation patterns among Indian Asians compared with Europeans are associated with increased risk of developing type 2 diabetes. Our findings of differences in DNA methylation underlying type 2 diabetes will be of interest to researchers and clinicians worldwide, and might provide the basis for development of new strategies for risk stratification and personalised approaches to prediction and prevention of type 2 diabetes.

PHOSPHO1, SOCS3 and *SREBF1* was associated with BMI, waist circumference, insulin concentrations, and HOMA-IR; our findings suggest that DNA methylation at these loci provides additional information about type 2 diabetes susceptibility beyond routine clinical measures of adiposity, and that DNA methylation might be a biomarker of metabolically unfavourable patterns of adiposity and insulin resistance.

Contributors

JCC, PAB, JD, SJh, JJ, NKa, JP, DS, E-ST, JT, ARW, M-RJ, JS, VB, PE, MIM, and JSK conceived and designed the study. JCC, JK, HRE, WRS, S-TT, RCR, UA, KB, RC, JD, TRG, SdL, CG, TI, SJh, SJo, AK, NKo, SK, PP, BT, ARW, CH, SC, CLR, PF, PV, JS, HG, PE, and JSK were involved in participant recruitment and characterisation. JCC, ML, AD, VM, SW, HRE, FR, WRS, WZ, S-TT, GC, MC-H, LY, RCR, MA-B, ZYM, HKN, FP, HP, MAR, LT, JA, MA-K, BA, OA, PAB, CB, TRG, CG, TI, JJ, AJK, NKo, SK, CS, PS, BT, SAK, TJA, CH, JH, SC, CLR, PF, RS, PV, M-RJ, JS, HG, VB, and JSK generated molecular phenotype data. JCC, ML, BL, AD, JK, SW, HRE, WZ, GC, LY, RCR, JP, CLR, M-RJ, PE, MIM, and JSK were involved in statistical analyses. JCC, ML, BL, AD, CG, SJh, NKa, E-ST, ARW, RS, PV, M-RJ, JS, HG, VB, PE, MIM, and JSK wrote the manuscript draft. All authors contributed to, read, and approved the final version of the manuscript.

Declaration of interests

We declare no competing interests.

Acknowledgments

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