Supplements

Genetic identification of two novel loci associated with steroid-sensitive nephrotic syndrome

1 Stephanie Dufek*, 1 Chris Cheshire*, 1 Adam P Levine*, 1 Richard S Trompeter*, 1 Naomi Issler, 1 Matthew Stubbs, 1 Monika Mozere, 1 Sanjana Gupta, 1 Enriko Klootwijk, 1 Vaksha Patel, 2 Daljit Hothi, 2 Aoife Waters, 2 Hazel Webb, 2 Kjell Tullus, 2 Lucy Jenkins, 2 Lighta Godinho, 3 Elena Levtchenko, 4 Jack Wetzels, 5 Nine Knoers, 6 Nynke Teeninga, 6 Jeroen Nauta, 7 Mohamed Shalaby, 7 Sherif Eldesoky, 7 Jameela A Kari, 8 Shenal Thalgahagoda, 8 Randula Ranawaka, 8 Asiri Abeyagunawardena, 9 Adebowale Adeyemo, 10 Mark Kristiansen, 11 Rasheed Gbadegesin, 12 Nicholas J Webb, 1 Daniel P Gale*, 1 Horia C Stanescu*, 1 Robert Kleta*, 1 Detlef Bockenhauer*

* contributed equally

Affiliations:
1. Department of Renal Medicine, University College London, London, UK
2. Great Ormond Street Hospital for Children NHS Foundation Trust, London, UK
3. University Hospitals Leuven & University of Leuven, Leuven, Belgium
4. Nephrology, Radboud University Medical Center, Nijmegen, The Netherlands
5. Department of Genetics, UMC Groningen, Groningen, The Netherlands
6. Department of Pediatric Nephrology, Erasmus University Medical Centre-Sophia Children's Hospital, Rotterdam, The Netherlands
7. Pediatric Nephrology Center of Excellence, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia
8. Department of Paediatrics, University of Peradeniya, Sri Lanka
9. NHGRI, NIH, Bethesda, MD, USA
10. UCL Genomics, Institute of Child Health, UCL, London, UK
11. Department of Pediatrics, Duke University School of Medicine, Durham, NC, USA
12. Department of Paediatric Nephrology & NIHR Manchester Clinical Research Facility, Manchester Academic Health Science Centre, Royal Manchester Children's Hospital, Manchester, UK

Corresponding Authors:
Detlef Bockenhauer, d.bockenhauer@ucl.ac.uk & Robert Kleta, r.kleta@ucl.ac.uk
UCL Department of Renal Medicine
Rowland Hill Street, London NW3 2PF, UK
phone: +44 (0)20 7314 7554; fax: +44 (0)20 7472 6476

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Case definition

All cases had childhood onset of nephrotic syndrome and the criteria for diagnosis with nephrotic syndrome were defined as per standard guidelines: presence of proteinuria (40 mg/m^2/day or protein/creatinine ratio > 200 mg/mmol), hypoalbuminemia < 25 g/L and edema.[1] Steroid sensitive was defined as response to steroid treatment within four weeks of treatment, according to international guidelines.[1]

All patients who did not respond to steroid treatment within four weeks of therapy and therefore were by definition steroid resistant were excluded. Patients who had nephrotic syndrome secondary to infectious agents, malignancies, medications, and other conditions were also excluded.[1]

Case collection

A total of 712 cases were collected by the authors including from the PREDNOS (EudraCT 2010-022489-29) and PREDNOS2 (EudraCT 2012-003476-39) trials.[2] After selection for European ancestry, 422 cases remained for analysis of which 140 were female and 282 male, consistent with the reported 1 : 2 female : male ratio in childhood SSNS.[3] Of the 422 cases, 212 were from the PREDNOS and PREDNOS2 trials and the remainder submitted by the participating clinicians.

Genomic DNA was extracted from peripheral blood following a standard protocol. Full consent for DNA collection was obtained by each collaborator. Ethical approval was granted by host institutions according to local guidelines.

Control cohorts

Ethnically matches controls were obtained from three difference sources:

1. An “Oxford” dataset available through the European Genome Archive (EGAD00010000144 and EGAD00010000520) provided data from 432 individuals. Genotyping was performed as described.[4, 5]

2. “CEU Illumina ethnicity” controls were obtained from Illumina and provided data from 90 individuals genotyped as described previously (http://www.illumina.com).[6]

3. Wellcome Trust Case Control Consortium (WTCCC) controls. This is a combined dataset comprising the 1958 UK birth cohort controls and the UK blood service control group controls (Consortium #251), a total of 5,604 individuals. Genotyping was performed as described previously and data are available through the WTCCC website (https://www.wtccc.org.uk).[7]

Genotyping

Genotyping of all cases was performed at UCL Genomics (Institute of Child Health, UCL, London, UK) on the Illumina Infinium Multi-Ethnic Global BeadChip v.A1 with 1,779,818

markers. Sample processing was carried out in accordance with the Infinium HD Ultra Assay (Part # 15023140 Rev. A) protocol (Illumina Inc, San Diego, CA, USA). Briefly, 200ng DNA was whole genome amplified (37°C for 20 hours), fragmented (37ºC for 1 hour and 15 mins), precipitated and resuspended in hybridization buffer. Samples were hybridized onto Illumina array beadchips using a liquid handling robot (Freedom Evo, Tecan Ltd, Switzerland) and incubated at 48°C for 16 hours. Staining of the beadchips was also performed using a liquid handling robot (Freedom Evo, Tecan Ltd, Switzerland). Finally, the beadchips were scanned using the iScan scanner with autoloader (Illumina, San Diego, CA, USA). Data was collected in raw IDAT format.

Post genotyping processing

In-house software was utilized to re-encode genotypes uniformly to the genomic forward encoding scheme following internal quality control (QC) and matching to dbSNP, based on the genotyping chip manifest and excluding indels and CNV. The final number of markers for the case dataset was 1,565,259 of which 1,513,983 were autosomal.

Quality control

Standard procedures were used for QC of samples and markers.[8] In summary, QC per sample included removal of individuals with a call rate < 90%, removal of duplicates or related individuals (identity by descent (IBD) >0.1875), removal of samples with average identity by state distance (DST) (i.e., IBS distance (IBS2 + 0.5*IBS1) / (N SNP pairs)) <0.66 and heterozygosity rates of more than three standard deviations below or above the mean for the overall samples. QC steps for samples and markers were carried out

independently in the cases and in each control dataset as genotyping had been performed on different SNP chips. The per sample and per SNP QC steps were subsequently repeated on the combined case-control set.

- For the cases (n=712), samples were removed because of call rate (CR) <90% (n=3), IBD >0.1875 (n=58), average DST <0.66 (n=0) and heterogeneity >3SD (n=26). Remaining for principal component analysis (PCA) were 625 individuals.

- For the Oxford controls (n=432), samples were removed because of CR <90% (n=0), IBD >0.1875 (n=6), average DST <0.66 (n=0) and heterogeneity >3SD (n=6). Remaining for PCA were 420 individuals.

- For the Illumina CEU controls (n=90), samples were removed because of CR <90% (n=0), IBD >0.1875 (n=30), average DST <0.66 (n=0) and heterogeneity >3SD (n=1). Remaining for PCA were 59 individuals.

- For the WTCCC controls (n=5604), samples were removed because of CR <90% (n=55), IBD >0.1875 (n=67), average DST <0.66 (n=0) and heterogeneity >3SD (n=82). Remaining for PCA were 5400 individuals.

After QC of the samples, the total number of cases was 625 and the total number of combined controls was 5879.

QC for markers included the removal of markers with CR <99%, markers with more than one alternate allele and markers with a minor allele frequency of <1%.[9] Markers in the control datasets with a significant (p-value <0.001) deviation from Hardy-Weinberg equilibrium (HWE) were removed. For controls, QC per marker was performed on each dataset separately before the control datasets were combined.

- For the cases (autosomal markers n=1,512,983), markers were removed because of CR <99% (n=222,889), minor allele frequency (MAF) <1% (n=700,397) and multiallelic (n=0). Remaining were 669,943 markers.

- For the Oxford controls (autosomal markers n=653,959), markers were removed because of CR <99% (n=13,310), MAF <1% (n=67,724), multiallelic (n=0), HWE p<0.001 (n=2,889). Remaining were 571,616 markers.

- For the Illumina CEU controls (autosomal markers n=653,959), markers were removed because of CR <99% (n=79,777), MAF <1% (n=68,185), multiallelic (n=0), HWE p<0.001 (n=1,795). Remaining were 516,372 markers.

- For the WTCCC controls (autosomal markers n=1,025,437), markers were removed because of CR <99% (n=133,778), MAF <1% (n=135,992), multiallelic (n=0), HWE p<0.001 (n=16,941). Remaining were 788,849 markers.

The number of markers in the combined control dataset was 372,137.

After combining with the case dataset, data were available for 158,314 overlapping markers. Subsequently, QC was performed on the combined case-control dataset with HWE testing on controls only. The final number of markers in the combined dataset was 158,217.

Analysis was done using SNP & Variation Suite (SVS) v8.8.1 (Golden Helix Inc., Bozeman, MT, USA; www.goldenhelix.com).[10] The identification of duplicates and related individuals was undertaken using PRIMUS (https://primus.gs.washington.edu/primusweb/index.html)[11] following linkage disequilibrium (LD) pruning of markers.[12] LD pruning was performed in SVS using standard settings.

Ancestry selection

PCA was used to evaluate population stratification and to select individuals of European ancestry only. Analyses were performed in SVS using genotyped data after LD pruning. Based on the distribution of cases and controls along principal component axes, European ancestry outliers were visualized and those with a standard deviation >3 from the combined dataset (cases and controls) were excluded (Supplementary Figure 1).

After QC and ancestry selection, the final dataset comprised 422 cases and 5642 controls genotyped for 158,217 markers. PCA was repeated on the final dataset to retrieve eigenvectors for the first ten (based on manual inspection of Eigen values) principal

components for use as covariates in the association analysis to adjust for finer resolution population substructure.

Imputation

Imputation analysis was performed using Beagle 5.0 (https://faculty.washington.edu/browning/beagle/beagle.html) with the 1000 Genomes Project Phase 3 data (version 5a) as a reference panel (ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/release/20130502/).[13] Cases and controls were imputed together using only samples and markers passing the aforementioned stringent QC. Post imputation filter was performed using bcftools (https://samtools.github.io/bcftools/bcftools.html) to remove those markers with Dosage R-Squared (DR2) <80%. INDELS and markers with a MAF <1%, CR <99% and those showing significant deviation from HWE (p<0.001) in the control individuals were removed using PLINK v1.90 beta (https://www.cog-genomics.org/plink/1.9).[14] The final dataset included 5,216,266 markers and was imported into SVS for further analysis.

GWAS methods and conditional analysis

Genome-wide association analysis was performed only on those samples and markers which passed the stringent QC. For association analysis, a numerical association test under a logistic regression model was used. Association analysis was performed using both the best guess genotypes and using allelic dosages. The methods delivered comparable results and only those using the best guess genotypes are reported. The first ten principal components were included as covariates. The widely accepted p-value <

$5.0x10^{-8}$ was used to declare genome-wide significance.[15] Association testing and the generation of the Manhattan plot were performed using SVS. The genomic inflation factor was calculated to quantify potential inflation of type I error due to stratification or technical artefacts. No substantial inflation was noted (lambda = 1.027).

Conditional analysis was performed using a numerical regression analysis model as per the discovery GWAS but incorporating the test SNP genotypes, in numerical form, as a covariate.

HLA type imputing and association testing

HLA imputation was performed using SNP2HLA v1.0.3 (http://software.broadinstitute.org/mpg/snp2hla/) with default parameters.[16] As input a subset of 1,189 SNPs from those selected for GWAS (post QC) and overlapping with the SNP2HLA imputation HapMap European reference dataset were used. Post imputation filtering included removal of imputed HLA types with a quality score R2<80%. A total of 100 HLA types were carried forward for further analysis. Logistic regression with adjustment for the first ten PCs of ancestry was used to test for association of each HLA allele with SSNS. If the higher resolution and lower resolution allele frequencies were the same in cases and controls, only the higher resolution results were reported. Conditional analysis of the lead HLA alleles was performed using a logistic regression model.

eQTL Analysis

The following publicly available expression quantitative trait loci (eQTL) databases were queried (December 2018) to ascertain whether variants significantly associated with SSNS were known to influence the expression levels of corresponding gene products in multiple tissues: Gtex[17] (https://gtexportal.org/home/), eQTLgene[18] (http://www.eqtlgen.org), NephQTL[19] (http://nephqtl.org) and Human Kidney eQTL Atlas[20] (http://susztaklab.com/eqtl).

Calculation of genetic risk scores

Genetic risk scores were calculated using Mangrove software[21] (https://cran.r-project.org/web/packages/Mangrove/index.html) for all individuals based on their genotype at the two replicated loci using the odds ratios and allele frequency, using a logistic regression model. The proportion of phenotypic variation explained by the genetic risk score was calculated using Nagelkerke's pseudo R^2 with the fmsb package in R (https://cran.r-project.org/web/packages/fmsb/index.html).

Supplementary results

Conditional HLA analysis

After conditioning on HLA-DQA1*02:01 the strongest signal came from HLA-DQA1*01 ($P=1.24x10^{-31}$, OR=0.31, 95% CI=0.25-0.38). After conditioning on both, HLA-DQA1*02:01 and HLA-DQA1*01 only HLA-DQB1*03:03 ($P=1.22x10^{-8}$, OR=0.38, 95% CI=0.26-0.54) and HLA-DQB1*03 ($P=1.69x10^{-8}$, OR=0.64, 95% CI=0.55-0.75) remained independently significant.

GWAS power calculation

A power calculation was performed using the Michigan Genetic Association Study power calculator (https://csg.sph.umich.edu/abecasis/gas_power_calculator/index.html).[22] Comparing 422 cases with 5642 controls using alpha = $5x10^{-8}$ under a multiplicative model, the power to detect association of an allele with a frequency of 0.1 in controls exceeds 0.8 at a genotype relative risk (GRR) of 1.6 and power to detect the effect of more common alleles exceeds 0.8 at smaller GRRs.

Calculation of genetic risk scores

We calculated that the two replicated loci (Chr 6p21.3 and 6q22.1) together explain approximately 11% of the risk of SSNS.

Acknowledgements

PREDNOS Trial Management Group	
Professor Nicholas Webb Central Manchester University Hospitals NHS Foundation Trust (Royal Manchester Children's Hospital)	Chief Investigator, Applicant
Dr Richard Trompeter Centre for Nephrology, University College, London	Co-Chief Investigator, Co-Applicant Nephrology
Professor Keith Wheatley University of Birmingham (Cancer Research UK Clinical Trials Unit)	Co-Applicant Clinical Trials
Dr Carole Cummins University of Birmingham (Institute of Applied Health Research)	Co-Applicant Clinical Trials & Statistics
Miss Natalie Ives University of Birmingham (Birmingham Clinical Trials Unit)	Co-Applicant Clinical Trials & Statistics
Dr Emma Frew University of Birmingham (Institute of Applied Health Research)	Co-Applicant Health Economics

PREDNOS Trial Management and Day to Day Coordination **Birmingham Clinical Trials Unit, University of Birmingham**	
Elizabeth Brettell	Team Leader
Emma Barsoum	Senior Trial Coordinator
Helen Bodenham-Chilton	Trial Coordinator
Noreen Akhtar	Trial Administrator
Charmaine Hunt (previously Meek)	Trial Administrator
Terry Hughes	Data Manager
Adam Khan	Data Manager
Nicholas Hilken	Programming Team Leader
Neil Winkles	Senior Analyst Programmer
Adrian Wilcockson	Senior Analyst Programmer
Mark Hunter	Analyst Programmer

PREDNOS Trial Statistics **Birmingham Clinical Trials Unit, University of Birmingham**	
Natalie Ives	Statistics Team Leader
Rebecca Woolley	Trial Statistician
Andrew Howman	Trial Statistician
Natalie Marchevsky	Statistician

PREDNOS Trial Health Economics	
Emma Frew University of Birmingham (Institute of Applied Health Research)	Health Economics Lead
Tosin Lambe	Health Economics

University of Birmingham (Institute of Applied Health Research)	
Associated Professor Billingsley Kaambwa Flinders University (School of Medicine)	Health Economics (original Health Economics Lead and co-applicant)

Participating centres and PREDNOS collaborative group members	
Abertawe Bro Morgannwg University Health Board Morriston Hospital	
Dr Michelle James-Ellison	Principal Investigator
Hilary Marie Williams	Research Nurse
Airedale NHS Foundation Trust Airedale General Hospital	
Dr Pronab Bala	Principal Investigator
Alder Hey Children's NHS Foundation Trust Alder Hey Children's Hospital	
Dr Caroline Jones	Principal Investigator
Dr Richard Holt	Clinician
Dr Henry Morgan	Clinician
Elizabeth Bailey	Research Nurse
Lauren Flanagan	Data Manager
Ashford and St Peter's Hospitals NHS Foundation Trust St Peter's Hospital	
Dr Tariq Bhatti	Principal Investigator
Dr Shailini Bahl	Clinician
Lorna Walding	Research Nurse
Barnsley Hospital NHS Foundation Trust Barnsley Hospital	
Dr Ezzedin Gouta	Principal Investigator
Dr Rajeev Gupta	Clinician
Dr Diarmuid Kerrin	Clinician
Barts Health NHS Trust Newham University Hospital, The Royal London Hospital, Whipps Cross University Hospital	
Dr Ami Parikh	Principal Investigator
Dr Ann Duthie	Previous Principal Investigator; Clinician (Whipps Cross University Hospital)
Dr Nimze Gadong	Previous Principal Investigator; Clinician (Newham University Hospital)
Dr Bahadur Anjum	Clinician (Newham University Hospital)
Bessie Crone	Research Nurse
Hilarious De Jesus	Research Nurse (The Royal London Hospital)
Ivone Lancoma-Malcolm	Research Nurse (Newham University Hospital)
Nicolene Plaatjies	Research Nurse (Newham University Hospital, The Royal London Hospital)
Basildon and Thurrock University Hospitals NHS Foundation Trust Basildon University Hospital	
Dr Khorshid Khalifa	Principal Investigator
Belfast Health and Social Care Trust The Royal Belfast Hospital for Sick Children	

Dr Karl McKeever	Principal Investigator
Dr Grace McCall	Clinician
Patricia McCreesh	Research Nurse
Muriel Millar	Research Nurse
Betsi Cadwaladr University Health Board	
Glan Clwyd Hospital, Ysbyty Gwynedd	
Dr Markus Hesseling	Principal Investigator (Glan Clwyd Hospital)
Dr Madalitso Kubwalo	Principal Investigator (Ysbyty Gwynedd)
Annette Bolger	Research Officer
Lucie Hobson	Research Nurse
Birmingham Women's and Children's NHS Foundation Trust	
Birmingham Children's Hospital	
Dr David Milford	Principal Investigator
Dr Larissa Kerecuk	Clinician
Claire Norton	Central Pharmacist
Narinder Rahania	Central Pharmacist
Helen Williamson	Central Pharmacist
Fatima Bibi	Central Pharmacy Clinical Trials Assistant
Tracy Gazeley	Central Pharmacy Technician
Julie Williams	Central Pharmacy Technician
Bolton NHS Foundation Trust	
Royal Bolton Hospital	
Dr Fiona Watson	Principal Investigator
Claire Abbot	Research Nurse
Bradford Teaching Hospitals NHS Foundation Trust	
Bradford Royal Infirmary	
Dr Simon Frazer	Principal Investigator
Louise Akeroyd	Research Nurse
Burton Hospitals NHS Foundation Trust	
Queen's Hospital	
Dr Mansoor Ahmed	Principal Investigator
Dr Dominic Muogbo	Clinician
Claire Backhouse	Research Nurse
Stephanie Boswell	Research Nurse
Calderdale and Huddersfield NHS Foundation Trust	
Calderdale Royal Hospital, Huddersfield Royal Infirmary	
Dr Eilean Crosbie	Principal Investigator
Cambridge University Hospitals NHS Foundation Trust	
Addenbrooke's Hospital	
Dr Peter Heinz	Principal Investigator
Dr Birgit Ulbrich	Clinician
Cardiff and Vales University Health Board	
University Hospital of Wales	
Dr Shivaram Hegde	Principal Investigator
Pauline Jones	Research Nurse
Central Manchester University Hospitals NHS Foundation Trust	
Royal Manchester Children's Hospital	
Professor Nicholas JA Webb	Chief Investigator; Principal Investigator
Dr Rachel Lennon	Clinician; Laboratory

Dr Malcolm Lewis	Clinician
Dr Nicholas Plant	Clinician
Dr Mohan Shenoy	Clinician
Dr Shaila Sukthankar	Clinician
Charlotte Bryant	Research Coordinator
Sarah Douglas	Research Coordinator
Helen Sumner	Research Coordinator
Jane Howell	Research Nurse
Chelsea and Westminster Hospital NHS Foundation Trust	
West Middlesex University Hospital	
Dr Nour Elhadi	Principal Investigator
Irene Bishton	Research Nurse
Chesterfield Royal Hospital NHS Foundation Trust	
Chesterfield Royal Hospital	
Dr Oyekunle Ayonrinde	Principal Investigator
Colchester Hospital University NHS Foundation Trust	
Colchester General Hospital	
Dr Andrea Turner	Principal Investigator
Dr Jonathan Campbell	Clinician
Aine Turner	Research Nurse
Countess of Chester Hospital NHS Foundation Trust	
Countess of Chester Hospital	
Dr John Gibbs	Principal Investigator
Dr Elizabeth Newby	Clinician
Dr Satyanarayana Saladi	Clinician
Dr Alison Timmis	Clinician
Caroline Burchett	Research Nurse
Sarah De-Beger	Research Assistant
County Durham and Darlington NHS Foundation Trust	
Darlington Memorial Hospital, University Hospital of North Durham	
Dr Asha Nair	Principal Investigator
Dr Antima Banerjee	Clinician
Croydon Health Services NHS Trust	
Croydon University Hospital	
Dr Theo Fenton	Principal Investigator
Dartford and Gravesham NHS Trust	
Darent Valley Hospital	
Dr Selwyn D'Costa	Principal Investigator
Derby Teaching Hospitals NHS Foundation Trust	
Royal Derby Hospital	
Dr Richard Bowker	Principal Investigator
Coral Smith	Research Nurse
Vanessa Unsworth	Research Nurse
Doncaster and Bassetlaw Teaching Hospitals NHS Foundation Trust	
Bassetlaw Hospital, Doncaster Royal Infirmary	
Dr Anuja Natarajan	Principal Investigator
Dr Lai Men Wong	Clinician
Dorset County Hospital NHS Foundation Trust	
Dorset County Hospital	

Dr Phil Parslow	Principal Investigator
East and North Hertfordshire NHS Trust Lister Hospital	
Dr Vijay Kandala	Principal Investigator
Dr Salman Imran	Previous Principal Investigator
East Cheshire NHS Trust Macclesfield District General Hospital	
Dr Surendran Chandrasekaran	Principal Investigator
Dr Sheng-Ang Ho	Previous Principal Investigator
Dr Ignatius Losa	Clinician
Dr Katrina Marinaki	Clinician
Natalie Keenan	Research Nurse
Jessica Nichols	Research Nurse
Joanne Shippey	Research Nurse
East Lancashire Hospitals NHS Trust Royal Blackburn Hospital	
Dr Javed Iqbal	Principal Investigator
Epsom and St Helier University Hospitals NHS Trust Epsom Hospital	
Dr Kirsty Watts	Principal Investigator
Frimley Health NHS Foundation Trust Wexham Park Hospital	
Dr Angela Yannoulias	Principal Investigator
Dr Zilla Huma	Previous Principal Investigator
Gloucestershire Hospitals NHS Foundation Trust Gloucestershire Royal Hospital	
Dr Adamu Sambo	Principal Investigator
Dr Lyda Jadresic	Previous Principal Investigator
Susan Beames	Research Nurse
Great Ormond Street Hospital for Children NHS Foundation Trust Great Ormond Street Hospital	
Professor Detlef Bockenhauer	Principal Investigator; Laboratory
Dr Daljit Hothi	Previous Principal Investigator
Great Western Hospitals NHS Foundation Trust Great Western Hospital	
Dr Nick West	Principal Investigator
Dr Helen Price	Previous Principal Investigator
Guy's and St Thomas' NHS Foundation Trust Evelina London Children's Hospital	
Dr Manish Sinha	Principal Investigator
Emma Neal	Research Nurse
Elisabeth Reus	Research Nurse
Paula Sofocleous	Research Nurse
Harrogate and District NHS Foundation Trust Harrogate District Hospital	
Dr Ian Cannings	Principal Investigator
Homerton University Hospital NHS Foundation Trust Homerton University Hospital	
Dr Rajiv Sood	Principal Investigator

Hull and East Yorkshire Hospitals NHS Trust Hull Royal Infirmary	
Dr Verghese Mathew Dr Elsadeg Sharif	Principal Investigator Previous Principal Investigator
Kettering General Hospital NHS Foundation Trust Kettering General Hospital	
Dr Harsha Bilolikar Sonia White	Principal Investigator Research Nurse
Kingston Hospital NHS Foundation Trust Kingston Hospital	
Dr Titi Ayeni	Principal Investigator
Lancashire Teaching Hospitals NHS Foundation Trust Royal Preston Hospital	
Dr Ruth O'Connor	Principal Investigator
Luton and Dunstable Hospital NHS Foundation Trust Luton and Dunstable University Hospital	
Dr Michael Eisenhut Dr Tomasz Rajkowski Samantha Clough Rebecca Dixon Karen Reep	Principal Investigator Clinician Research Nurse Research Nurse Research Nurse
Maidstone and Tunbridge Wells NHS Trust Maidstone Hospital, Tunbridge Wells Hospital	
Dr Krishnan Balasubramanian Dr Hamudi Kisat	Principal Investigator Clinician
Medway NHS Foundation Trust Medway Maritime Hospital	
Dr Janette Cansick	Principal Investigator
Mid Cheshire Hospitals NHS Foundation Trust Leighton Hospital	
Dr Julie Ellison Sally Smith Samantha Tapscott	Principal Investigator Research Nurse Research Nurse
NHS Ayrshire and Arran University Hospital Crosshouse	
Dr Bridget Oates Claire Bell	Principal Investigator Research Nurse
NHS Dumfries & Galloway Dumfries and Galloway Royal Infirmary	
Dr Rajendran Shyam	Principal Investigator
NHS Fife Victoria Hospital	
Dr Evelyn Menzies Pamela Cruickshanks	Principal Investigator Clinical Nurse Specialist
NHS Forth Valley Forth Valley Royal Hospital	
Dr John Schulga	Principal Investigator
NHS Grampian Royal Aberdeen Children's Hospital	

Dr Craig Oxley	Principal Investigator
NHS Greater Glasgow and Clyde Royal Alexandra Hospital, Royal Hospital for Children	
Dr David Hughes	Principal Investigator (Royal Hospital for Children)
Dr Amita Sharma	Principal Investigator (Royal Alexandra Hospital)
Dr Leah Krischock	Previous Principal Investigator (Royal Hospital for Children)
Dr Jamie Houston	Clinician (Royal Alexandra Hospital)
Diane Carroll	Research Nurse (Royal Hospital for Children)
Sheryl Goddard	Research Nurse (Royal Hospital for Children)
Elizabeth Waxman	Research Nurse
NHS Highland Raigmore Hospital	
Dr Alan Webb	Principal Investigator
NHS Lanarkshire Wishaw General Hospital	
Dr Thin Thin Saing	Principal Investigator
Dr Colin Cunningham	Clinician
Angela Brown	Research Nurse
Victoria Causer	Clinical Nurse Specialist
NHS Lothian Royal Hospital for Sick Children	
Dr David Hughes	Principal Investigator
Dr Sepideh Taheri	Previous Principal Investigator
Alyson Macdonald	Clinical Nurse Specialist
Tracey McGregor	Clinical Nurse Specialist
Emma Carson	Research Nurse
Kay Riding	Research Nurse
Norfolk and Norwich University Hospitals NHS Foundation Trust Norfolk and Norwich University Hospital	
Dr Nandu Thalange	Principal Investigator
Dr Chris Upton	Previous Principal Investigator; Clinician
Louisa Fear	Research Nurse
North Cumbria University Hospitals NHS Trust Cumberland Infirmary	
Dr Glyn Jones	Principal Investigator
North Tees and Hartlepool NHS Foundation Trust University Hospital of North Tees	
Dr Vijay Tandle	Principal Investigator
Gabrielle Osborne	Research Nurse
North West Anglia NHS Foundation Trust Peterborough City Hospital	
Dr Mona Aslam	Principal Investigator
Northampton General Hospital NHS Trust Northampton General Hospital	
Dr Imogen Norton	Principal Investigator
Northern Devon Healthcare NHS Trust	

North Devon District Hospital	
Dr Dermot Dalton	Principal Investigator
Dr Andrew Arend	Previous Principal Investigator
Dr Krishnakumar Thattakkat	Previous Principal Investigator
Northern Lincolnshire and Goole NHS Foundation Trust	
Diana, Princess of Wales Hospital	
Dr Bukar Wobi	Principal Investigator
Dr Bemigho Etuwewe	Clinician
Tara Walker	Data Manager
Julie Browning	Research Nurse
Marjorie Carling	Research Nurse
Emma Killingbeck	Research Nurse
Dorothy Ryan	Research Nurse
Consolata Tsvangirai-Mahachi	Research Nurse
Nottingham University Hospitals NHS Trust	
Nottingham Children's Hospital	
Dr Andrew Lunn	Principal Investigator
Dr Martin Christian	Clinician
Oxford University Hospitals NHS Foundation Trust	
John Radcliffe Hospital	
Dr Janet Craze	Principal Investigator
Plymouth Hospitals NHS Trust	
Derriford Hospital	
Dr Catherine Derry	Principal Investigator
Dr Robert Jones	Previous Principal Investigator
Sarah-Jane Sharman	Research Nurse
Sally Harwood	Trial Administrator
Poole Hospital NHS Foundation Trust	
Poole Hospital	
Dr Steve Wadams	Principal Investigator
Heather Barham	Research Nurse
Susan Power	Research Nurse
Portsmouth Hospitals NHS Trust	
Queen Alexandra Hospital	
Dr Catherine Tuffrey	Principal Investigator
Dr Simon Birch	Clinician
Dr Judith Scanlan	Clinician
Andrew Gribbin	Research Nurse
Sharon McCready	Research Nurse
Royal Berkshire NHS Foundation Trust	
Royal Berkshire Hospital	
Dr Ann Gordon	Principal Investigator
Dr Gregory Boden	Previous Principal Investigator
Susan Hallett	Research Nurse
Royal Cornwall Hospitals NHS Trust	
Royal Cornwall Hospital	
Dr Chris Williams	Principal Investigator
Gillian Craig	Research Nurse
Royal Devon and Exeter Hospital NHS Foundation Trust	

Royal Devon and Exeter- Wonford Hospital	
Dr Hannah Cottis	Principal Investigator
Dr Vaughan Lewis	Previous Principal Investigator
Dr Christine McMillan	Clinician
Dr Nigel Osborne	Clinician
Caroline Harrill	Research Nurse
Suzanne Wilkins	Research Nurse
Royal Free London NHS Foundation Trust	
Royal Free Hospital (non-recruiting centre)	
Professor Robert Kleta	Laboratory
Royal United Hospitals Bath NHS Foundation Trust	
Royal United Hospital	
Dr Lynn Diskin	Principal Investigator
Dr Amanda Billson	Previous Principal Investigator
Alison Barratt	Research Nurse
Sheffield Children's NHS Foundation Trust	
Sheffield Children's Hospital	
Dr Gail Moss	Principal Investigator
Sherwood Forest Hospitals NHS Foundation Trust	
King's Mill Hospital	
Dr Simon Rhodes	Principal Investigator
Anna Coupe	Research Officer
Caroline Moulds	Research Nurse
Southern Health and Social Care Trust	
Craigavon Area Hospital	
Michael Smith	Principal Investigator
Sara Gilpin	Research Nurse
Judith Ratcliffe	Research Nurse
Southport and Ormskirk Hospital NHS Trust	
Ormskirk and District General Hospital	
Dr Sharryn Gardner	Principal Investigator
St Helens and Knowsley Teaching Hospitals NHS Trust	
Whiston Hospital	
Dr Lakshmi Chilukuri	Principal Investigator
Stockport NHS Foundation Trust	
Stepping Hill Hospital	
Dr Chris Cooper	Principal Investigator
Sara Bennett	Research Nurse
Tameside and Glossop Integrated Care NHS Foundation Trust	
Tameside Hospital	
Dr Anjali Petkar	Principal Investigator
Dr Ahsan Ul-Haq	Clinician
Gill Waring	Research Nurse
Taunton and Somerset NHS Foundation Trust	
Musgrove Park Hospital	
Dr Rebecca Mann	Principal Investigator
Nicola Thorne	Research Nurse
The Dudley Group NHS Foundation Trust	
Russells Hall Hospital	

Dr Zala Ibrahim	Principal Investigator
The Ipswich Hospital NHS Trust Ipswich Hospital	
Dr Jackie Buck	Principal Investigator
Deborah Beeby	Research Nurse
The Leeds Teaching Hospitals NHS Trust Leeds General Infirmary	
Dr Eric Finlay	Principal Investigator
Majorie Allen	Research Nurse
Rebecca Mottram	Research Nurse
The Mid Yorkshire Hospitals NHS Trust Dewsbury and District Hospital, Pinderfields Hospital	
Dr Kathryn Deakin	Principal Investigator
The Newcastle upon Tyne Hospitals NHS Foundation Trust Great North Children's Hospital	
Dr Sally Johnson	Principal Investigator
Dr Yincent Tse	Clinician
Kathryn Bell	Research Nurse
Denise Chisholm	Clinical Nurse Specialist
The Pennine Acute Hospitals NHS Trust North Manchester General Hospital, The Royal Oldham Hospital	
Dr Beena Padmakumar	Principal Investigator
Dr Talaivirichan Magadevan	**Clinician (North Manchester General Hospital)**
The Queen Elizabeth Hospital King's Lynn NHS Foundation Trust Queen Elizabeth Hospital	
Dr Mandy Hughes	Principal Investigator
Dr Susan Rubin	Previous Principal Investigator
Amy Frary	Research Nurse
The Rotherham NHS Foundation Trust Rotherham Hospital	
Dr Sanjay Suri	Principal Investigator
Dr Sherif El-Refee	Previous Principal Investigator
Dr Christine Harrison	Clinician
The Royal Wolverhampton NHS Trust Cannock Chase Hospital, New Cross Hospital	
Dr Karen Davies	Principal Investigator
Sharon Kempson	Research Nurse
The Shrewsbury and Telford Hospital NHS Trust Princess Royal Hospital, Royal Shrewsbury Hospital	
Dr Naeem Ayub	Principal Investigator
United Lincolnshire Hospitals NHS Trust Lincoln County Hospital, Pilgrim Hospital	
Dr Sourabh Mukhopadhyay	Principal Investigator (Lincoln County Hospital)
Dr Margaret Crawford	Principal Investigator (Pilgrim Hospital)
Dr Mujeeb Pervez	Previous Principal Investigator (Pilgrim Hospital)
Dr Douglas Thomas	Clinician (Lincoln County Hospital)

University Hospital of South Manchester NHS Foundation Trust Wythenshawe Hospital	
Dr Gopikrishna Vemuri	Principal Investigator
Dr Radhika Puttha	Previous Principal Investigator
Dr Alice Setti	Previous Principal Investigator
Dr Faisal Al-Zidgali	Clinician
Claire Holliday	Research Nurse
Louise Woodhead	Research Nurse
University Hospital Southampton NHS Foundation Trust Southampton Children's Hospital	
Rodney Gilbert	Principal Investigator
University Hospitals Bristol NHS Foundation Trust Bristol Royal Hospital for Children	
Professor Moin Saleem	Principal Investigator
Dr Alison Kelly	Clinician
Michelle Ross	Research Nurse
Heather Smee	Research Nurse
University Hospitals Coventry and Warwickshire NHS Trust University Hospital	
Dr Nigel Coad	Principal Investigator
University Hospitals of Leicester NHS Trust Leicester Royal Infirmary	
Dr Angela Hall	Principal Investigator
Dr Peter Houtman	Previous Principal Investigator
University Hospitals of Morecambe Bay NHS Foundation Trust Furness General Hospital, Royal Lancaster Infirmary	
Dr Nayan Peepah Nardeosingh	Principal Investigator
Dr Mireille-Yvette Formosa	Previous Principal Investigator
Dr Patrick Ward	Previous Principal Investigator
University Hospitals of North Midlands NHS Trust County Hospital, Royal Stoke University Hospital	
Dr Yvonne Slater	Principal Investigator
Dr Saeeda Raja	Previous Principal Investigator (County Hospital)
Dr Kishor Tewary	Previous Principal Investigator (County Hospital)
Dr Shiva Shankar	Previous Principal Investigator (Royal Stoke University Hospital)
Warrington and Halton Hospitals NHS Foundation Trust Warrington Hospital	
Dr Rachel Delyth Webb	Principal Investigator
Natalie Rogers	Research Nurse
West Suffolk NHS Foundation Trust West Suffolk Hospital	
Dr Raman Lakshman	Principal Investigator
Dr Karine Cesar	Clinician
Western Health and Social Care Trust Altnagelvin Area Hospital	
Dr Damien Armstrong	Principal Investigator

Julie Brown	Research Nurse
Western Sussex Hospitals NHS Foundation Trust St Richard's Hospital, Worthing Hospital	
Dr Nicholas Brennan	Principal Investigator
Dr Stuart Nicholls	Previous Principal Investigator (Worthing Hospital)
Wirral University Teaching Hospital NHS Foundation Trust Arrowe Park Hospital	
Dr Elizabeth Breen	Principal Investigator
Sharon Hughes	Research Nurse
Lucy Lewis	Research Nurse
Worcestershire Acute Hospitals NHS Trust Alexandra Hospital, Worcestershire Royal Hospital	
Dr John Scanlon	Principal Investigator
Dr Munir Ahmed	Clinician
Dr Andrew Gallagher	Clinician
Wrightington, Wigan And Leigh NHS Foundation Trust Royal Albert Edward Infirmary	
Dr Vineeta Joshi	Principal Investigator
Dr Madhumita Mukherjee	Previous Principal Investigator
Dr Singara Velmurugan	Previous Principal Investigator
Wye Valley NHS Trust The County Hospital	
Dr Simon Meyrick	Principal Investigator
Yeovil District Hospital NHS Foundation Trust Yeovil District Hospital	
Dr Michael Fernando	Principal Investigator
Dr Christopher Zaborowski	Clinician
Alex Stannett	Research Nurse
York Teaching Hospital NHS Foundation Trust Scarborough Hospital, The York Hospital	
Dr Udupa Venkatesh	Principal Investigator (Scarborough Hospital)
Dr Guy Millman	Principal Investigator (The York Hospital)
Simon Dyer	Research Nurse (Scarborough Hospital)
Kerry Elliot	Research Nurse (Scarborough Hospital)

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Supplementary Tables

Supplementary Table 1: Effect estimates for the three lead genotyped SNPs at the associated loci

Locus	SNP	Minor allele	MAF cases	MAF controls	p-value
6p21.3	rs479536	T	0.22	0.07	1.87×10^{-47}
	rs4947342	A	0.45	0.24	4.90×10^{-42}
	rs9501626	A	0.27	0.11	3.70×10^{-39}
6q22.1	rs549262	A	0.30	0.40	4.30×10^{-9}
	rs648210	G	0.33	0.43	8.73×10^{-9}
	rs4142087	C	0.32	0.40	1.04×10^{-6}
4q13.3	rs9090	T	0.13	0.08	6.13×10^{-7}
	rs1043633	T	0.13	0.08	1.03×10^{-6}
	rs11098369	T	0.23	0.18	1.42×10^{-4}

Minor allele frequencies (MAF) and p-values of the three lead genotyped SNPs for each locus reaching significance in the imputed dataset.

Standard filtering steps with a call rate cut off of 97% for markers was used for quality control. Basic allele test was used for association testing.

Supplementary Table 2: Linkage equilibrium at the Chromosome 4q13.3 locus

	European		African	
	D'	r^2	D'	r^2
D'/r^2	rs59882675 (*BTC*)	rs59882675 (*BTC*)	rs59882675 (*BTC*)	rs59882675 (*BTC*)
rs10518133 (*PARM1*)	0.1983	0.0004	0.0414	0.0004

Shown are values for the normalized coefficient of linkage disequilibrium (D') and the coefficient of correlation (r^2) for the lead SNP at the chromosome 4q13.3 locus in our study (rs10518133) and the lead SNP identified at this locus by Debiec *et al*. (rs59882675). Note that linkage equilibrium is seen with reference data from European, as well as African ancestries, suggesting that these SNPs are independent. Reference data were pulled from the 1000 genome project (phase 3 version 5; https://www.genome.gov/27528684/1000-genomes-project/) using all European (CEU, TSI, FIN, GBR, IBS) or African (YRI, LWK, GWD, MSL, ESN, ASW, ACB) population data.

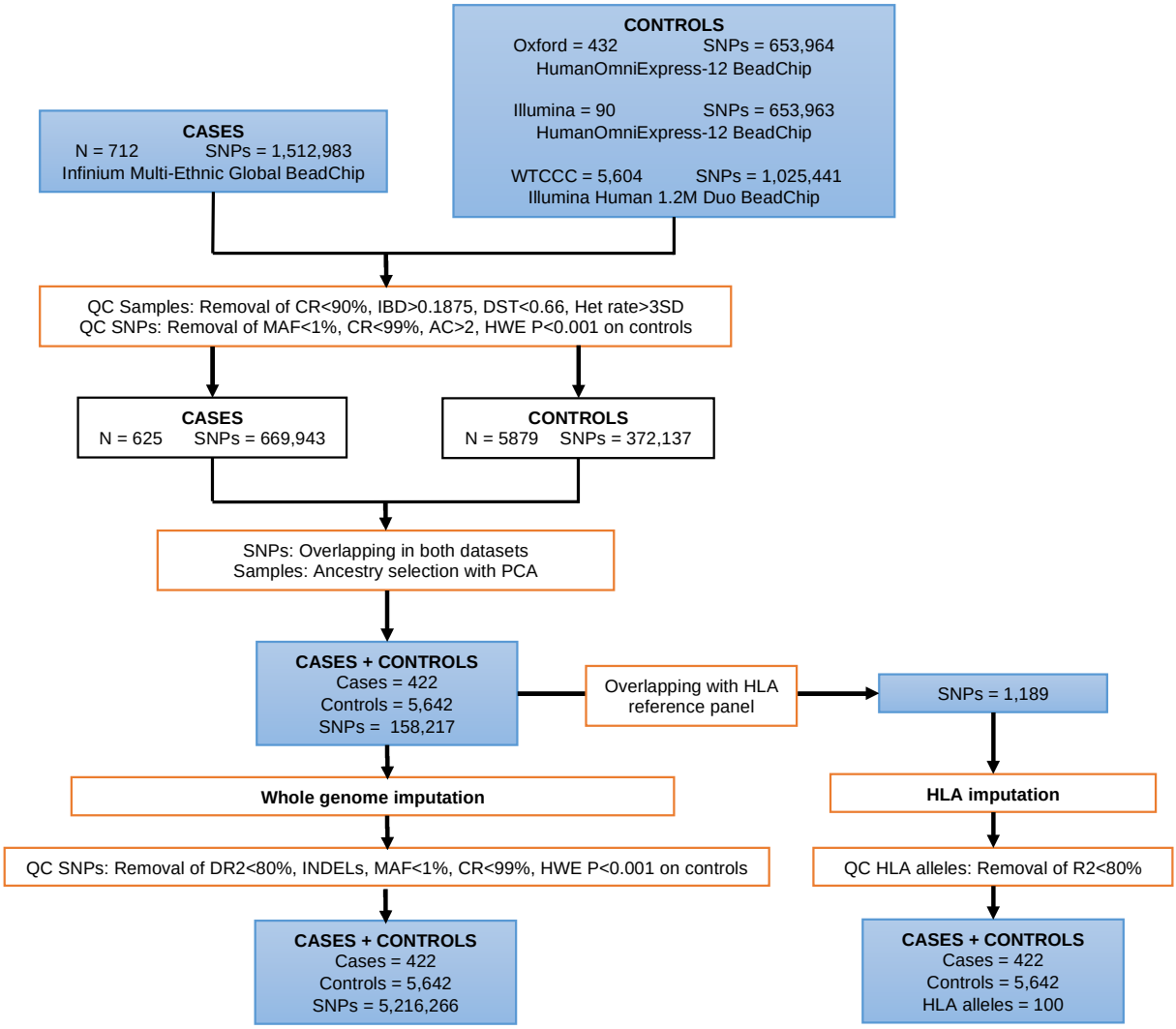
Supplementary Table 3: List of SNPs with a suggestive association

Locus	SNP	Minor allele	MAF cases	MAF controls	OR	95% CI	p-value
1q32.1	rs113752715	G	0.04	0.08	0.47	0.33-0.66	1.62e-06
3q21.1	rs530462	T	0.15	0.21	0.63	0.52-0.77	2.63e-06
3q25.1	rs6763024	C	0.34	0.27	1.41	1.21-1.63	8.89e-06
3q27.2	rs487575	T	0.15	0.22	0.63	0.52-0.77	1.27e-06
4p14	rs6531527	G	0.46	0.38	1.38	1.20-1.58	6.74e-06
6q16.1	rs2674382	T	0.33	0.07	0.46	0.32-0.68	9.15e-06
6q23.2	rs2746419	A	0.55	0.46	1.42	1.23-1.63	1.08e-06
7p14.1	rs111796602	C	0.16	0.10	1.63	1.34-1.99	3.31e-06
9p23	rs201899638	C	0.004	0.02	0.11	0.03-0.40	4.96e-07
10p11.22	rs1891621	G	0.55	0.47	1.39	1.21-1.60	3.56e-06
19q13.12	rs34217742	A	0.18	0.12	1.56	1.29-1.88	7.64e-06

Listed are lead SNPs from loci achieving suggestive genome-wide significance with a p-value between $5x10^{-8}$ and $1x10^{-5}$. Minor allele frequency (MAF) and odds ratio (OR) with 95% confidence intervals (95% CI).

Supplementary Figures

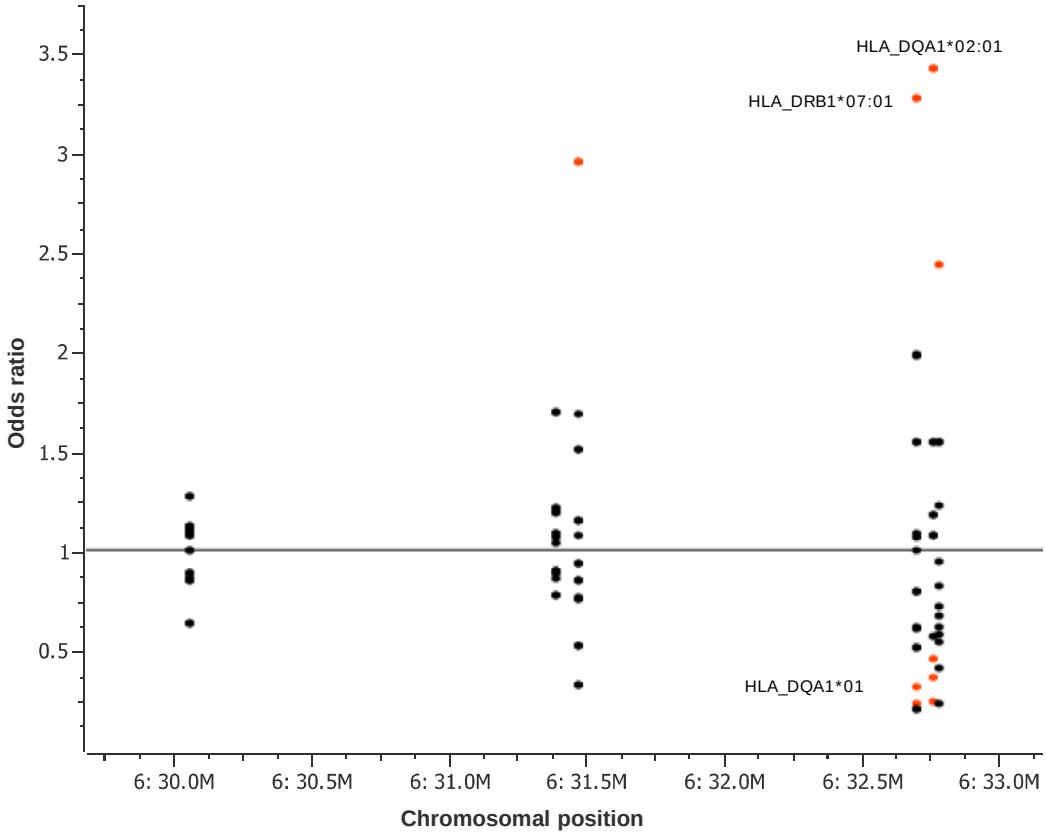
Supplementary Figure 1. Flowchart of quality control steps and GWAS



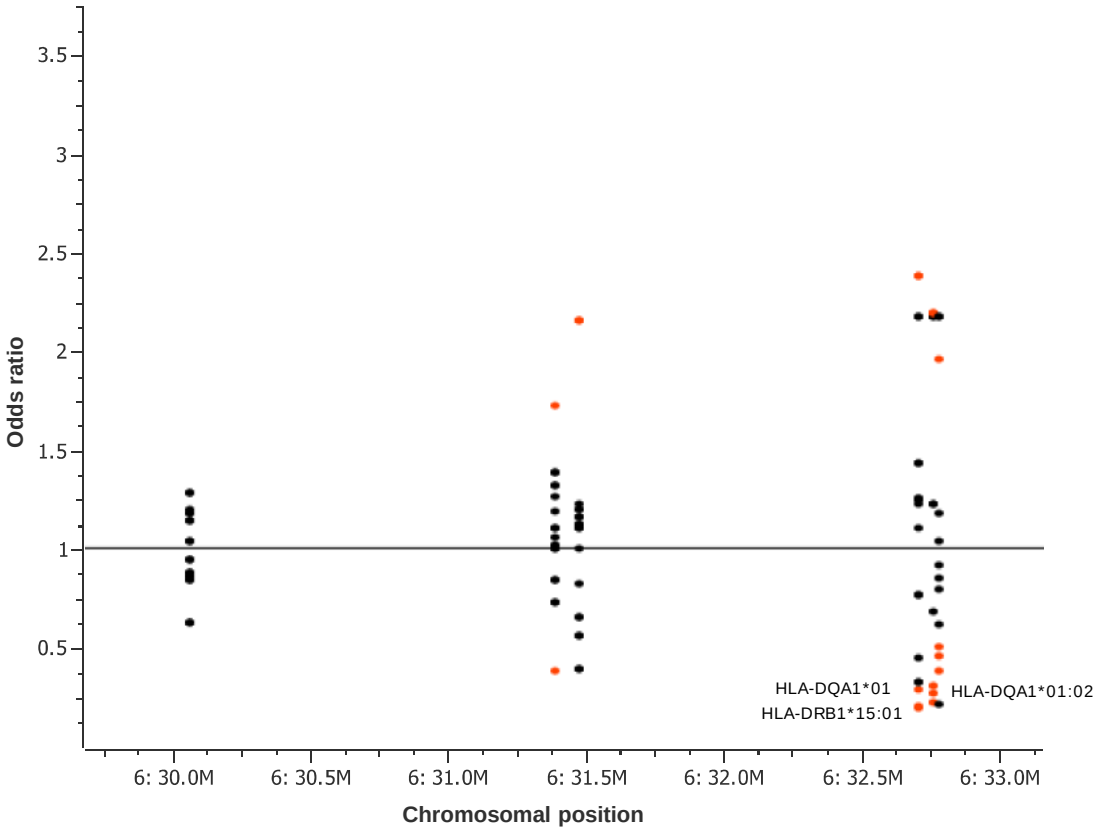
Shown is a flowchart providing information on data input and processing. QC: quality control; CR: call rate; IBD: identity by descent; DST: identity by state distance; Het rate: heterozygosity rate; MAF: minor allele frequency; HWE: Hardy-Weinberg equilibrium.

Supplementary Figure 2. Results of classical HLA type association analysis

A)



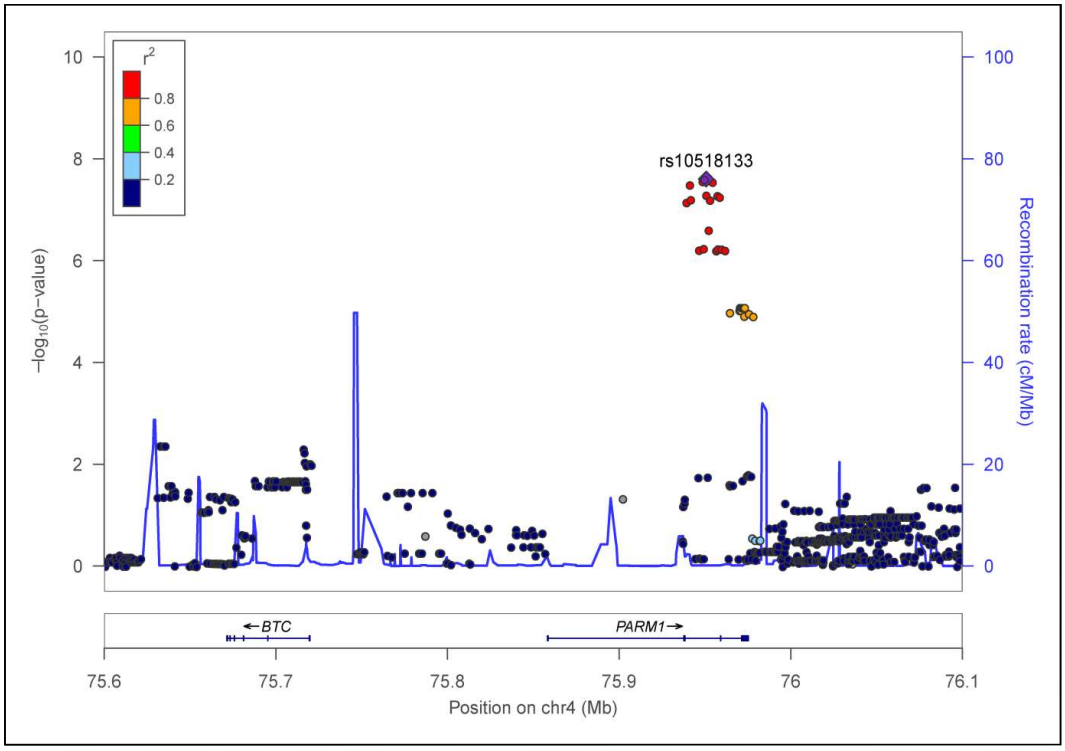
B)



A) Results for HLA allele imputation for HLA class I (HLA-A, -C and -B) and class II (HLA-DRB1, -DQB1) genes. Shown are the odds ratios (OR) for classical HLA alleles on Y-axis and chromosomal position on X-axis. HLA alleles with an OR > 1 are deemed to be disease causing versus HLA alleles with an OR < 1 deemed to be protective. Significantly associated HLA alleles are colored in red (p-value $<5x10^{-8}$). Indicated are the three HLA alleles with the lowest p-value for the independent association with risk for SSNS.

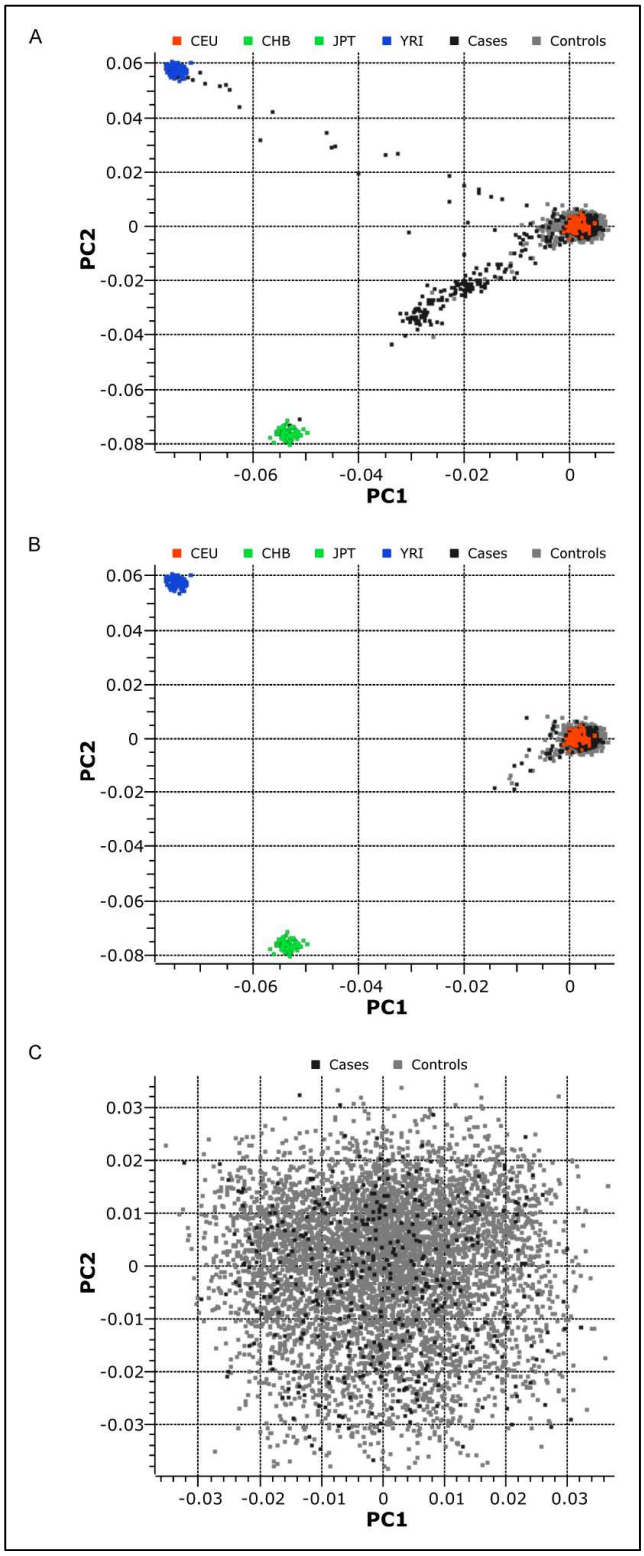
B) Same as A), but after conditioning on HLA-DQA1*02:01. Note that the protective allele HLA-DQA1*01 is essentially unchanged, indicating that its effect is independent of HLA-DQA1*02:01.

Supplementary figure 3. Locus Zoom plot of the association on chromosome 4



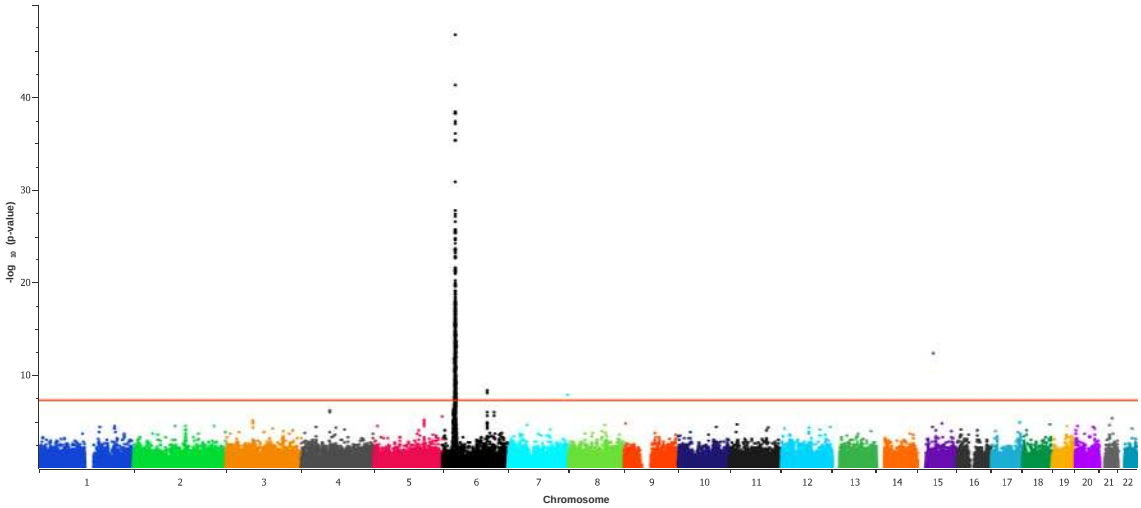
X-axis indicates chromosomal position, the left Y-axis the log-transformed p-value and the right Y-axis the recombination rate. The purple dot indicates the top SNP for each region. Recombination hotspots indicated by blue lines. The purple diamond indicates SNP with the smallest p-value within each region. SNPs are colored based on their pairwise LD to the lead SNP as per 1000 Genomes European reference data, according to the key. Recombination hotspots are indicated by blue lines. Data are shown for 200 kb either side of the lead SNP.
Note that *PARM1* and *BTC* are approximately 150 kb apart and separated by a strong recombination hotspot (> 50cM/Mb).

Supplementary Figure 4. Principal component analysis of cases and controls



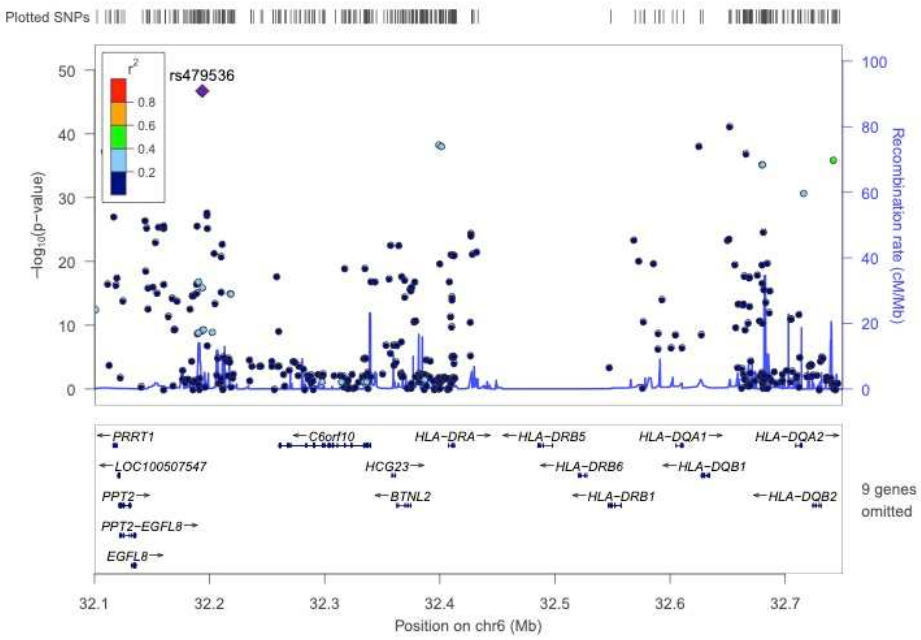
Shown is the principal component analysis.
A) Prior to the exclusion of non-European individuals. Distribution of cases samples along the top two principal components (PC1 and PC2) identified by principal component analysis of 687 cases and 5,879 controls. The results are visualized in comparison to the Illumina ethnicity controls (CEU, YRI and CHB-JPT) for panels A and B.
B) and C) Distribution of remaining European 422 cases and 5,642 controls after removal of cases and controls with principal components of more than 3 standard deviations from the mean. Panel C is a zoomed in image of panel B without the Illumina ethnicity controls. Inflation factor lambda = 1.027.

Supplementary Figure 5. Manhattan plot with genotyped markers only



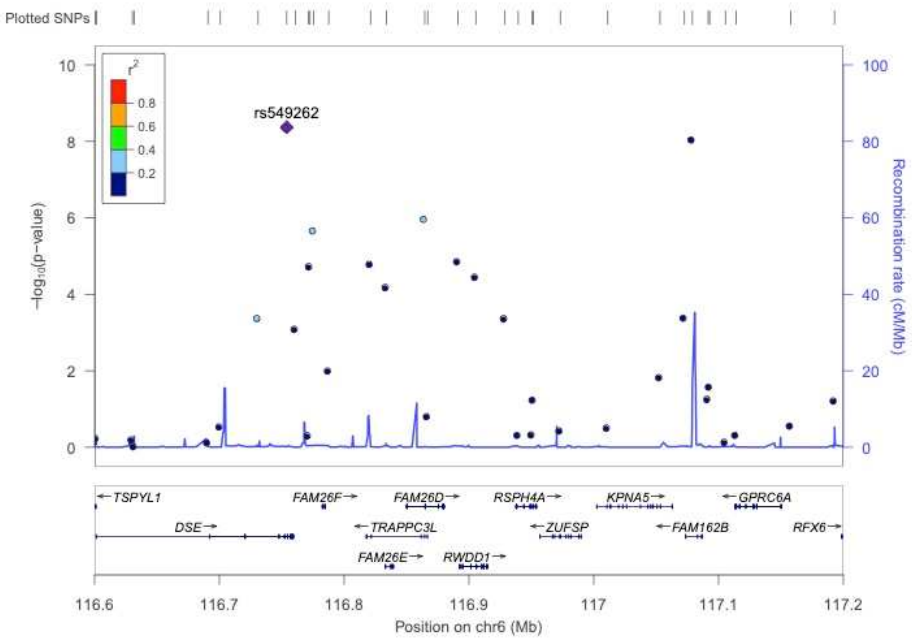
Shown is the Manhattan plot for the analysis with the 158,217 directly genotyped SNPs. Note that the same three peaks are seen, as in the analysis with the imputed dataset (Figure 1), although the peak on chromosome 4 is not significant with genotyped markers only. Same QC criteria were used as in the full analysis, except for a cut-off for the CR of <0.97.

Supplementary Figure 6. Locus zoom plot with genotyped markers only for the HLA locus



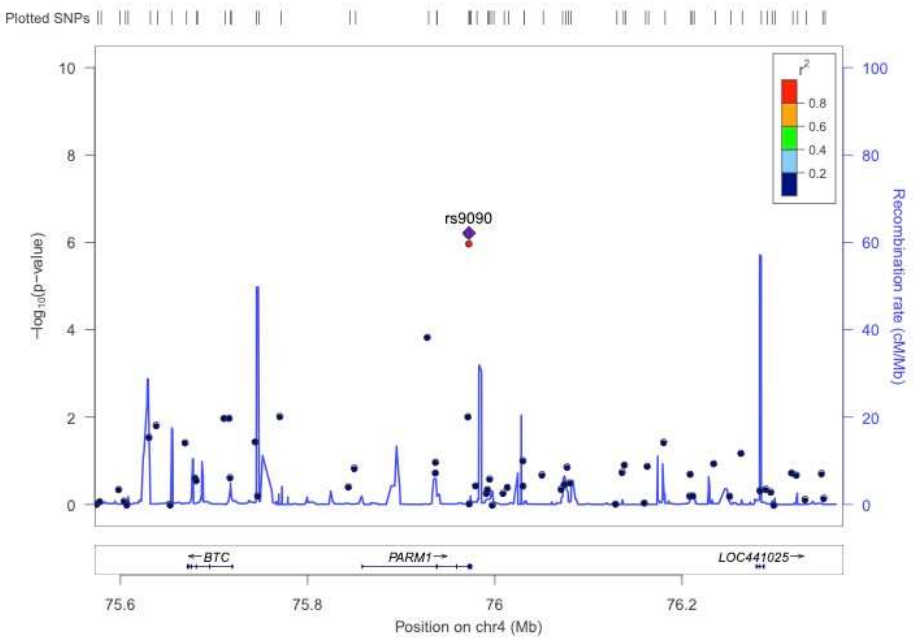
Shown is the locus zoom plot for the HLA locus with genotyped markers only. Note that the SNP with the lowest p-value (rs479536) is approximately 0.4 Mb telomeric to the peak over *HLA-DQB1*, seen with the imputed analysis. However, the SNP with the second lowest p-Value (rs4947342, upstream of *HLA-DQB1*, not annotated) is within the peak identified in the imputed analysis.

Supplementary Figure 7. Locus zoom plot with genotyped markers only for the *CALHM6* locus



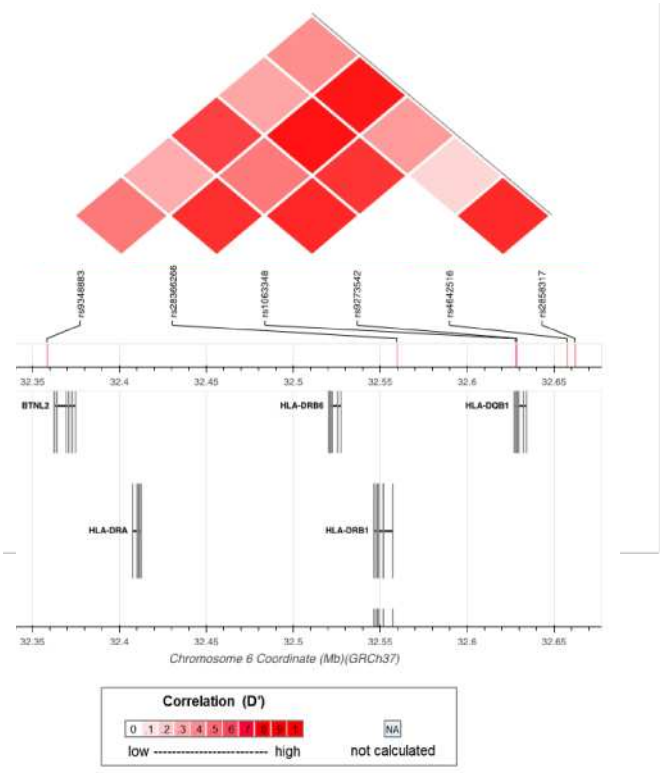
Shown is the locus zoom plot for the peak on chromosome 6q22.1 with genotyped markers only. Note that the SNP with the lowest p-Value (rs549262) is in the same peak over *CALHM6* (here annotated with the old name *FAM26F*), identified in the imputed analysis.

Supplementary Figure 8. Locus zoom plot with genotyped markers only for the chr. 4q13.3 locus



Shown is the locus zoom plot for the peak on chromosome 4q13.3 with genotyped markers only. Note that the SNP with the lowest p-Value (rs9090) is in the same peak over *PARM1*, identified in the imputed analysis.

Supplementary Figure 9: Linkage Disequilibrium of SNP in the HLA region associated with SSNS



Shown is the LD data for the lead HLA SNPs identified in previous GWAS of SSNS and this analysis, based on European reference data from the 1000 genome dataset. Note that the SNPs rs9273542 and rs2858317, identified in this analysis are in strong LD with rs4642516, identified by Jia *et al*.[23] and with two markers, rs1063348 and rs28366266, identified by Debiec *et al*..[24] In contrast, the third, most telomeric marker identified by Debiec *et al*. rs9348883 is only in weak LD with the other markers.

Summary Statistics

Due to the large file size (spreadsheet with >5.000.000 rows), this is provided as a separate document (SSNSSummaryStatistics.csv). Each row contains information for one of the 5,216,266 SNP analysed. The column headers indicate the information provided. Columns 1-6 contain general information about the respective SNP from dbSNP (www.ncbi.nlm.nih.gov/snp), whereas columns 7-10 contain information derived from our analysis.

Column 1 “rsid”: SNP identifier
Column 2 “chromosome”: name of chromosome on which the SNP is located
Column 3: “position”: base pair position on the chromosome
Column 4 “minor_test_allele”: the base that constitutes the minor allele
Column 5 “major_allele”: the base that constitutes the major allele
Column 6 “maf”: the frequency of the minor allele, indicated as a fraction of 1
Column 7 “allele_freq_cases”: the minor allele frequency in cases
Column 8 “allele_freq_controls”: the minor allele frequency in controls
Column 9 “regression_pvalue”: the p-value for the difference in allele frequency between cases and controls
Column 10 “odds_ratio”: the odds ratio, as calculated using logistic regression under an additive model with adjustment for the first ten principal components of ancestry

This data will be deposited with the European Genome-phenome Archive (EGA) (https://ega-archive.org/) and is available through collaboration / corresponding authors.