Genetic Variations in Type 2 Diabetes and Cardiovascular Disease: A Focus on Gene-Lifestyle Interactions and Mendelian Randomization

Hindy, George

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Genetic Variations in Type 2 Diabetes and Cardiovascular Disease

A Focus on Gene-Lifestyle Interactions and Mendelian Randomization

George Hindy, MD

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Abstract

Type 2 diabetes (T2D) and cardiovascular disease (CVD) are highly prevalent complex diseases that result from lifestyle and genetic factors. Gene-lifestyle interactions are also believed to contribute to the etiology of these diseases. The aim of this thesis was to investigate gene-lifestyle interactions using the strongest T2D and CVD susceptibility genetic loci and to understand the causal nature of the associations of common cardio-metabolic biomarkers with T2D and coronary heart disease (CHD). The Malmö Diet and Cancer Study (MDCS), a population-based prospective study, was used and included around 30,000 individuals with comprehensive baseline dietary and lifestyle assessment between the years 1991-1996. The *TCF7L2* genotype modified the association between fiber intake and the risk of T2D. The lower risk of T2D by higher fiber intake was restricted to individuals carrying the CC non-risk genotype ($P_{interaction}$ = 0.049). Similar interaction was observed with baseline levels of HbA_{1C} ($P_{interaction}$ = 0.02). Other T2D associated genes were then investigated for links to WNT signaling pathway where TCF7L2 acts as a transcription factor. Of the 51 T2D associated gene loci 7 genes were annotated to the WNT pathway. Interaction analyses between single nucleotide polymorphisms (SNPs) in these loci and dietary fiber intake were significant for the *TCF7L2*, *NOTCH2* and *ZBED3* SNPs. Higher fiber intake associated with lower risk of T2D only among risk allele carriers of the *NOTCH2* SNP ($P_{interaction}$ = 0.01) and only among homozygotes for the risk allele of the *ZBED3* SNP ($P_{interaction}$ = 0.003). The interaction between *TCF7L2* and fiber intake was further explored in relation to the metabolic syndrome in 4,606 individuals. Higher fiber intake was observed to be associated with lower prevalence of the metabolic syndrome only among non-risk CC genotype carriers ($P_{interaction}$ = 0.02) and similar interactions were observed on baseline levels of several traits related to the metabolic syndrome. The chromosome 9p21 genotype modified the association of vegetable and wine intake with the risk of CVD. Lower risk of CVD by higher vegetable intake was restricted to non-carriers of the 9p21 risk G allele ($P_{interaction}$ = 0.043), while wine consumption appeared to lower the risk of CVD only among carriers of the risk allele ($P_{interaction}$ = 0.029). Instrumental variable analyses using cardio-metabolic genetic risk scores have indicated an inverse causal association between LDLC and T2D. Similar results were obtained in multivariable Mendelian randomization analyses using MDCS (P = 0.008) and genome-wide association studies data ($P = 5\times10^{-7}$). Using similar analyses, a direct causal association was observed between LDLC and CHD. In conclusion, this thesis provides important evidence for gene-lifestyle interactions in the development of T2D and CVD. It also provides evidence for an inverse causal relationship between LDLC and T2D indicating that LDLC has opposite roles in the causality of T2D and CHD.

Key words: Type 2 diabetes; cardiovascular disease; coronary heart disease; metabolic syndrome; genetics; gene-lifestyle interactions; diet; TCF7L2, WNT signaling; chromosome 9p21; Mendelian randomization

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Let food be thy medicine and medicine be thy food

–Hippocrates

To Roula

Contents

List of Papers

I. **Hindy G**, Sonestedt E, Ericson U, Jing XJ, Zhou Y, Hansson O, Renstrom E, Wirfalt E, Orho-Melander M. Role of *TCF7L2* risk variant and dietary fiber intake on incident type 2 diabetes. ***Diabetologia***. 2012; 202;55:2646-2654

II. **Hindy G**, Mollet I G, Rukh G, Ericson U, Orho-Melander M. Type 2 diabetes associated variants in genes annotated to WNT signaling and dietary fiber in relation to incidence of type 2 diabetes. ***Manuscript***

III. **Hindy G**, Ericson U, Sonestedt E, Nilsson PM, Melander O, Orho-Melander M. *TCF7L2* risk variant carriers lack the associated effect of dietary fiber intake on lower risk of metabolic syndrome. ***Manuscript***

IV. **Hindy G**, Ericson U, Hamrefors V, Drake I, Wirfält E, Melander O, Orho-Melander M. The chromosome 9p21 variant interacts with vegetable and wine intake to influence the risk of cardiovascular disease: a population based cohort study. ***BMC Med Genet***. 2014; 15:1220

V. **Hindy G**, Rukh G, Almgren P, Schulz CA, Ericson U, Fava C, Engström G, Melander O, Orho-Melander M. Low LDL-cholesterol and increased incidence of type 2 diabetes: a Mendelian randomization study. ***Submitted manuscript***

Papers not Included in the Thesis

I. Smith JG, Luk K, Schulz CA, Engert JC, Do R, **Hindy G**, Rukh G, Dufresne L, Almgren P, Owens DS, Harris TB, Peloso GM, Kerr KF, Wong Q, Smith AV, Budoff MJ, Rotter JI, Cupples LA, Rich S, Kathiresan S, Orho-Melander M, Gudnason V, O'Donnell CJ, Post WS, Thanassoulis G; Cohorts for Heart and Aging Research in Genetic Epidemiology (CHARGE) Extracoronary Calcium Working Group. Association of low-density lipoprotein cholesterol-related genetic variants with aortic valve calcium and incident aortic stenosis. ***JAMA***. 2014 Nov 5;312(17):1764-71.

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III. Crosby J, Peloso GM, Auer PL, Crosslin DR, Stitziel NO, Lange LA, Lu Y, Tang ZZ, Zhang H, **Hindy G**, Masca N, Stirrups K, Kanoni S, Do R, Jun G, Hu Y, Kang HM, Xue C, Goel A, Farrall M, Duga S, Merlini PA, Asselta R, Girelli D, Olivieri O, Martinelli N, Yin W, Reilly D, Speliotes E, Fox CS, Hveem K, Holmen OL, Nikpay M, Farlow DN, Assimes TL, Franceschini N, Robinson J, North KE, Martin LW, DePristo M, Gupta N, Escher SA, Jansson JH, Van Zuydam N, Palmer CN, Wareham N, Koch W, Meitinger T, Peters A, Lieb W, Erbel R, Konig IR, Kruppa J, Degenhardt F, Gottesman O, Bottinger EP, O'Donnell CJ, Psaty BM, Ballantyne CM, Abecasis G, Ordovas JM, Melander O, Watkins H, Orho-Melander M, Ardissino D, Loos RJ, McPherson R, Willer CJ, Erdmann J, Hall AS, Samani NJ, Deloukas P, Schunkert H, Wilson JG, Kooperberg C, Rich SS, Tracy RP, Lin DY, Altshuler D, Gabriel S, Nickerson DA, Jarvik GP, Cupples LA, Reiner AP, Boerwinkle E, Kathiresan S. Loss-of-function mutations in APOC3, triglycerides, and coronary disease. ***N Engl J Med***. 2014 Jul 3;371(1):22-31.

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sequence variants with blood lipids and coronary heart disease in 56,000 whites and blacks. ***Am J Hum Genet***. 2014 Feb 6;94(2):223-32.

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VII. Voight BF, Peloso GM, Orho-Melander M, Frikke-Schmidt R, Barbalic M, Jensen MK, **Hindy G**, Hólm H, Ding EL, Johnson T, Schunkert H, Samani NJ, Clarke R, Hopewell JC, Thompson JF, Li M, Thorleifsson G, Newton-Cheh C, Musunuru K, Pirruccello JP, Saleheen D, Chen L, Stewart A, Schillert A, Thorsteinsdottir U, Thorgeirsson G, Anand S, Engert JC, Morgan T, Spertus J, Stoll M, Berger K, Martinelli N, Girelli D, McKeown PP, Patterson CC, Epstein SE, Devaney J, Burnett MS, Mooser V, Ripatti S, Surakka I, Nieminen MS, Sinisalo J, Lokki ML, Perola M, Havulinna A, de Faire U, Gigante B, Ingelsson E, Zeller T, Wild P, de Bakker PI, Klungel OH, Maitland-van der Zee AH, Peters BJ, de Boer A, Grobbee DE, Kamphuisen PW, Deneer VH, Elbers CC, Onland-Moret NC, Hofker MH, Wijmenga C, Verschuren WM, Boer JM, van der Schouw YT, Rasheed A, Frossard P, Demissie S, Willer C, Do R, Ordovas JM, Abecasis GR, Boehnke M, Mohlke KL, Daly MJ, Guiducci C, Burtt NP, Surti A, Gonzalez E, Purcell S, Gabriel S, Marrugat J, Peden J, Erdmann J, Diemert P, Willenborg C, König IR, Fischer M, Hengstenberg C, Ziegler A, Buysschaert I, Lambrechts D, Van de Werf F, Fox KA, El Mokhtari NE, Rubin D, Schrezenmeir J, Schreiber S, Schäfer A, Danesh J, Blankenberg S, Roberts R, McPherson R, Watkins H, Hall AS, Overvad K, Rimm E, Boerwinkle E, Tybjaerg-Hansen A, Cupples LA, Reilly MP, Melander O, Mannucci PM, Ardissino D, Siscovick D, Elosua R, Stefansson K, O'Donnell CJ, Salomaa V, Rader DJ, Peltonen L, Schwartz SM, Altshuler D, Kathiresan S. Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. ***Lancet***. 2012 Aug 11;380(9841):572-80.

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Abbreviations

ADA	American Diabetes Association
AMPK	5' adenosine monophosphate-activated protein kinase
ANRIL	Antisense non-coding RNA in the INK4 locus
ApoA-I	Apolipoprotein A-I
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
ATP III	Adult Treatment Panel III
BMI	Body mass index
CHD	Coronary heart disease
CI	Confidence interval
CNV	Copy number variation
CRP	C-reactive protein
CVD	Cardiovascular disease
DAG	Diacylglycerol
DBP	Diastolic blood pressure
DIAGRAM	Diabetes and Genetics Replication and Meta-analysis
DPP	Diabetes Prevention Program
DPS	Diabetes Prevention Study
FBG	Fasting blood glucose
FFA	Free fatty acid
FPG	Fasting plasma glucose
FPI	Fasting plasma glucose
GLP-1	Glucagon-like peptide-1
GLUT4	Glucose transporter type 4
GRS	Genetic risk score
GSIS	Glucose stimulated insulin secretion
GWAS	Genome-wide association study
HbA_{1C}	Glycated hemoglobin
HDLC	High-density lipoprotein cholesterol
HOMA-IR	Homeostasis model assessment of insulin resistance

HR	Hazard ratio
IDF	International Diabetes Federation
IFCC	International Federation of Clinical Chemistry
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IL-6	Interleukin 6
IV	Instrumental variable
K^{+}_{ATP}	ATP sensitive potassium channel
Kb	Kilobase
LD	Linkage disequilibrium
LDLC	Low-density lipoprotein cholesterol
MAF	Minor allele frequency
MAGIC	Meta-Analyses of Glucose and Insulin-related traits Consortium
MCP-1	Monocyte chemotactic protein-1
MDC-CC	Malmö Diet and Cancer Cardiovascular Cohort
MDCS	Malmö Diet and Cancer Study
MI	Myocardial infarction
MODY	Maturity onset diabetes of the young
NAFLD	Non-alcoholic fatty liver disease
NCEP	National Cholesterol Education Program
NGSP	National Glycohemoglobin Standardization Program
NGT	Normal glucose tolerance
OGTT	Oral glucose tolerance test
OR	Odds ratio
PCOS	Polycystic ovarian syndrome
PKC	Protein kinase C
PPARG	Peroxisome proliferator-activated receptor gamma
PUFA	Polyunsaturated fatty acid
RCT	Randomized controlled trial
SBP	Systolic blood pressure
SCFA	Short chain fatty acid
SFA	Saturated fatty acid
SMC	Smooth muscle cell
SNP	Single nucleotide polymorphism
T2D	Type 2 diabetes
TC	Total cholesterol
TCF7L2	Transcription factor 7-like 2

TG	Triglycerides
TNF-α	Tumor necrosis factor α
TULIP	Tübingen Lifestyle Intervention Program
UKPDS	United Kingdom Prospective Diabetes Study
WC	Waist circumference
WHO	World Health Organization
WNT	Wingless-type MMTV integration site
ZBED3	Zinc finger BED domain-containing protein 3

Introduction

The prevalence of type 2 diabetes (T2D) and cardiovascular disease (CVD) is increasing in epidemic proportions around the globe. This epidemic accounts for huge health and economic burdens as a leading cause of morbidity and mortality. The etiology of these diseases is very complex and multifactorial. The shift to sedentary lifestyles and high caloric intake leading to obesity along with cigarette smoking are the main culprits behind this epidemic. The genetic component contributing to the etiology of these conditions is also evident due to familial aggregation and differences among ethnic groups. Gene-lifestyle interactions are also believed to be an important factor in the etiology of these diseases.

Several lifestyle risk factors for these diseases are well established and validated in different studies and meta-analyses. Genome-wide association studies have revolutionized the field of genetics of these diseases through the discovery of more than 50 genome loci for T2D and CVD separately. However, much more work is still to be done in this field, especially that these loci explain only around 10% of the heritability of these diseases. Efforts have also been directed into understanding gene-lifestyle interactions, however, most of the reported interactions have not been replicated highlighting the need for better well powered study designs and better assessment and harmonization of lifestyle factors across studies.

This thesis aims to investigate gene-lifestyle interactions in the development of T2D and CVD using the strongest genetic loci associated with these conditions to date. Understanding interactions is believed to contribute to developing better prevention strategies through identifying high risk groups of people. It may also help in understanding mechanisms through which lifestyle factors and genetic factors act.

This thesis also aims to use genetic variants to understand the causal nature of common cardio-metabolic biomarkers in the etiology of T2D and coronary heart disease (CHD). Understanding causality which is the ultimate goal in epidemiological studies would greatly improve treatment and prevention measures in these diseases.

Type 2 Diabetes

Diabetes mellitus is a disease characterized by chronic hyperglycemia leading to the development of vascular and neuropathic complications.[1] The development of diabetes results from absolute or relative insulin deficiency.

Diabetes can be classified clinically into four categories: 1) type 1 diabetes, resulting from autoimmune pancreatic β-cell destruction and accounting for 5-10% of all diabetes cases, 2) T2D, ranging from predominantly insulin resistance with relative insulin deficiency to predominantly secretory defect with insulin resistance and accounting for 90-95% of all diabetes cases, 3) rare specific types of diabetes including a) genetic defects of β-cell function (maturity-onset diabetes of the young (MODY) and mitochondrial DNA point mutations), b) genetic defects in insulin action (mutations in insulin receptor genes as in type A insulin resistance, leprechaunism, and Rabson-Mendenhall syndrome or mutations downstream of the insulin receptor as in lipoatrophic diabetes), c) diseases of the exocrine pancreas (pancreatitis, trauma, pancreatectomy, neoplasia, cystic fibrosis, hemochromatosis, fibrocalculous pancreatopathy), d) drug- or chemical-induced diabetes (vacor, pentamidine, nicotinic acid, glucocorticoids, thyroid hormone, diazoxide, β-adrenergic agonists, thiazides, phenytoin, interferon-α), e) infections (congenital rubella, coxsackievirus B, cytomegalovirus, adenovirus, mumps), f) uncommon forms of immune-mediated diabetes (stiff-man syndrome, anti-insulin receptor antibodies), and g) other genetic syndromes (Down syndrome, Klinefelter syndrome, Turner syndrome, Wolfram's syndrome), and 4) gestational diabetes mellitus affecting women with abnormal glucose tolerance first detected during pregnancy.[1,2]

T2D accounts for most (90-95%) of the diabetes cases encountered in clinical practice. Most patients with T2D are obese. Patients who do not fulfill the traditional criteria of obesity may have increased body fat in the abdominal region[1]. The international diabetes federation (IDF) estimates that around 382 million people have T2D accounting for 8.3% of the total global adult population and is projected to reach 592 million by the year 2035.[3]

Pathophysiology of T2D

T2D is a heterogeneous metabolic disease that results from core defects of insulin sensitivity mainly in muscle, liver, and adipose tissue in addition to progressive failure of sufficient insulin secretion from pancreatic β-cells leading to hyperglycemia.[4] However, the relative contributions of defects in insulin sensitivity and secretion differ inter-individually and among ethnic groups.[5]

Insulin Resistance

Changes in insulin sensitivity occur in a normal life cycle. Puberty,[6] pregnancy,[7] and aging[8] are associated with decreased insulin sensitivity. Insulin resistance is a critical factor in the etiology of T2D and it is associated with pathophysiological aspects of the metabolic syndrome (hypertension, dyslipidemia, and central obesity) and the polycystic ovarian syndrome (PCOS).[9] However, the mechanisms related to insulin resistance remain incompletely understood.

Role of the Adipose Tissue

The adipose tissue can modulate insulin sensitivity and metabolism through the release of free fatty acids (FFAs), glycerol, hormones, and proinflammatory cytokines.[10-12] These hormones and cytokines, collectively known as adipokines, include leptin, adiponectin, retinol-binding protein 4 (RBP-4), resistin, and proinflammatory cytokines as interleukin 6 (IL-6) and tumor necrosis factor α (TNFα).[13,14] Leptin and adiponectin have insulin sensitizing effects in both skeletal muscles and the liver which can be partially explained by their common ability to stimulate 5'AMP-activated protein kinase (AMPK) that activates glucose and fatty acid oxidation.[13] Adipose-specific glucose transporter type 4 (GLUT4) knockout mice exhibit both hepatic and muscle insulin resistance which highlights the important regulatory role of adipokines in other tissues.[15,16]

The important role played by the adipose tissue can be highlighted in both animals and humans with severe lipodystrophy, associated with severe muscle and hepatic insulin resistance.[17,18] Insulin sensitivity can be restored with transplantation of normal adipose tissue,[19] or leptin administration or overexpression in lipoatrophic mice models.[20,21] In humans, leptin administration has been shown to partially improve insulin sensitivity in patients with severe lipodystrophy.[17,22]

Observations of insulin resistance in septic patients,[23] the insulin sensitizing effects of high doses of salicylates,[24,25] and the chronic elevation of cytokines in obese and diabetic patients[26-28] support a role of inflammatory mediators in insulin resistance. The release of monocyte chemotactic protein-1 (MCP-1) from adipocytes and subsequent secretion of TNFα and IL-6 by adipose tissue

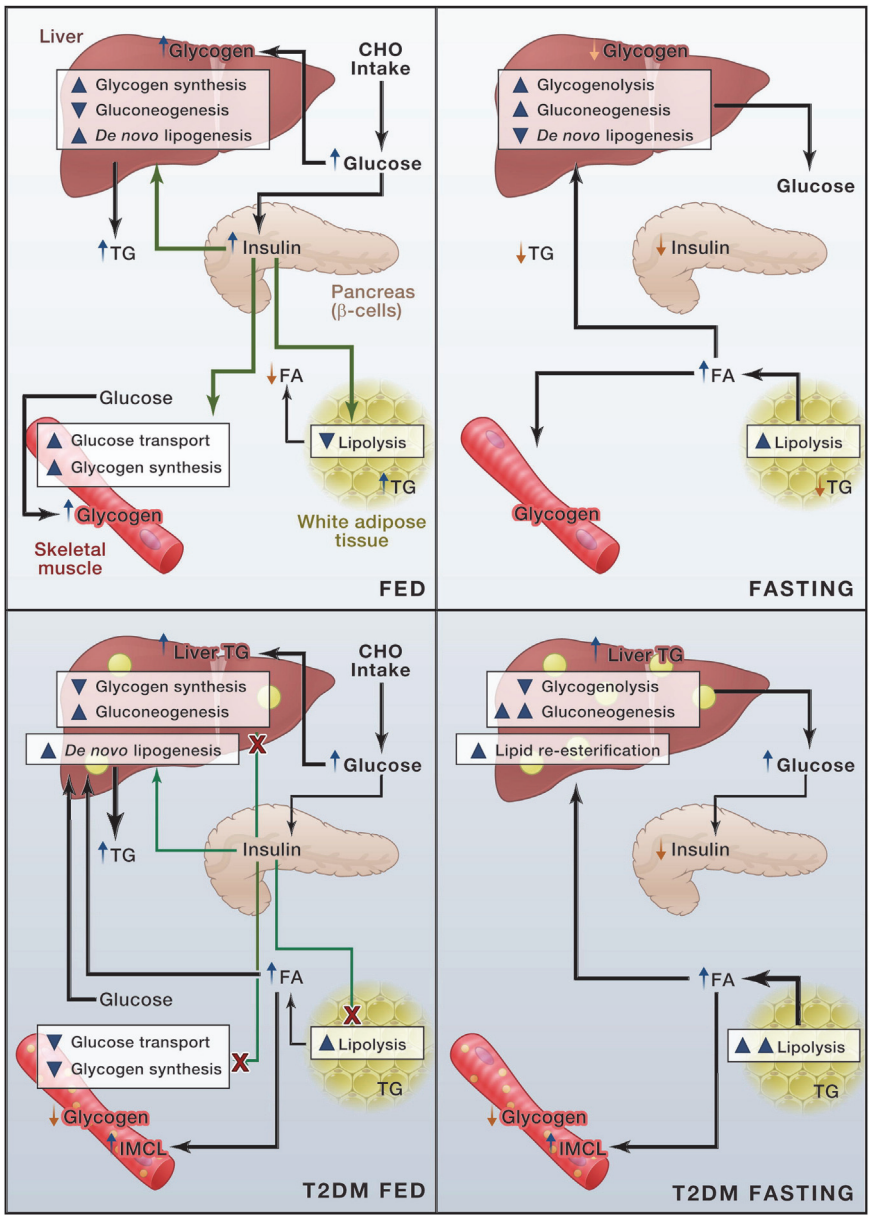

Figure 1. Mechanisms of insulin action in the normal and diabetic state.
CHO, Carbohydrate; TG, Triglycerides; FA, Fatty Acids; IMCL, Intramyocellular lipid. This figure was reproduced with permission from the publisher (Reference 42).

macrophages might play a role in the development of inflammation mediated insulin resistance.[12] These cytokines have been shown to impair insulin signaling in adipocytes through activation of certain transcription factors that stimulate production of more proinflammatory cytokines in a feed-forward mechanism.[29-31] When released into circulation these cytokines induce insulin resistance in other insulin sensitive tissues as the liver and skeletal muscles.

Ectopic Lipid Accumulation in the Skeletal Muscle

The skeletal muscle is the principal site of insulin mediated glucose uptake accounting for 75% of glucose disposal in the fed postprandial state.[32] A variety of rodent and human experiments demonstrated that lipid induced insulin resistance in skeletal muscles is related to impaired insulin signaling and decreased insulin stimulated glucose transport.[33-36] Other studies suggested that this impairment could be attributed to defects in GLUT4 translocation.[37,38]

It is not yet completely clear whether lipid induced insulin resistance is linked to circulating FFAs or intramyocellular lipid accumulation. In humans, studies have shown that the intramyocellular triglyceride (TG) content is a far stronger predictor of muscle insulin resistance than circulating FFAs.[39] In rodents, experiments have shown similar results; however, linking muscle insulin resistance and glucose uptake to intramyocellular levels of diacylglycerol (DAG) that are known to activate members of the protein kinase C (PKC) family.[34,40] In addition, human studies have indicated similar results highlighting the role of intramyocellular accumulation of DAG in the development of insulin resistance through activation of PKC.[41,42]

Ectopic Lipid Accumulation in the Liver

Ectopic lipid accumulation in the liver that is not a consequence of excessive alcohol consumption also termed non-alcoholic fatty liver disease (NAFLD) is considered the most common chronic liver disease in the United States.[43] Although, NAFLD patients have increased visceral adiposity, their hepatic insulin resistance has mainly been linked to hepatic fat deposition rather than visceral adiposity. This is evident in patients with severe lipodystrophy who have no visceral fat but manifest with severe hepatic insulin resistance and hepatic steatosis.[17] In addition, surgical removal of visceral fat does not alter glucose homeostasis or hepatic insulin sensitivity in patients with NAFLD, confirming that hepatic insulin resistance is primarily related to intrahepatic lipid content and not to visceral fat.[44,45] The accumulation of intrahepatic fat is thought to result from impaired fatty acid oxidation due to glucose induced elevations in malonyl-CoA which acts as a precursor for *de novo* lipogenesis and inhibitor of palmitoyltransferase-1 the rate limiting enzyme of fatty acid transport to the mitochondria.[16] This maintains insulin action on hepatic lipogenesis, while

cytosolic accumulation of lipid species as DAG, ceramides, and TGs impairs insulin induced suppression of gluconeogenesis.[16]

The Essential Role of β-cells

Plasma glucose concentrations are maintained in a narrow range through feedback loop between β-cells and insulin sensitive tissues.[46] In response to increasing insulin resistance the β-cells increase insulin secretion to maintain normal glucose tolerance. However, when β-cells fail to produce sufficient amount of insulin, glucose levels increase beyond the normal range.

The natural course of T2D pathogenesis is a continuum from normal glucose tolerance (NGT) to impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) (prediabetes) thereafter to T2D. In prediabetes insulin resistance is well established and elevations in glucose concentrations and progression to T2D are mainly attributed to continuous deteriorations in β-cell function.[47-49] β-cell dysfunction seems to occur very early in the disease pathogenesis and individuals in the upper tertile of IGT are almost maximally insulin resistant and have lost > 80% of their β-cell function.[50-53] In addition, individuals with elevated risk of T2D as those with family history,[54] gestational diabetes,[55] PCOS,[56] and elderly[57] have reduced β-cell function. β-cell function is a heritable trait.[58] Most of the gene loci associated with T2D have been associated with β-cell dysfunction, while only a few have been associated with obesity and insulin resistance.[59,60]

Adaptive Response of the β-cells

The β-cells are highly plastic with an ability to adapt to increasing insulin resistance by increasing their functional responsiveness and cell mass. The β-cells can enhance their insulin release up to 4- to 5-folds from an insulin sensitive to an insulin resistant state,[6,7,46,61,62] while the β-cell mass can only be increased by 50%.[63,64] Figure 2 shows the potential mechanisms by which β-cells adapt to insulin resistance.[65] The adaptive response could be mediated by elevations in glucose levels leading to activation of glucokinase (a) and increasing the ATP/ADP ratio which triggers the closure of the ATP sensitive potassium (K^+_{ATP}) channels, depolarization of the cell membrane and ultimately opening of voltage-gated calcium channels leading to calcium influx that stimulates insulin granule exocytosis.[66] Other glucose stimulated insulin secretion (GSIS) mechanisms include anaplerotic reactions (b) and replenishment of the tricarboxylic acid (TCA) cycle intermediates through pyruvate carboxylase.[67] Glucose metabolism also leads to elevations in citrate levels leading to malonyl CoA formation and elevations in DAG (c) which stimulates PKC and ultimately insulin release. Although increased β-cell glucose metabolism has been shown to maintain euglycemia in animal studies, human studies suggest that increased glucose levels

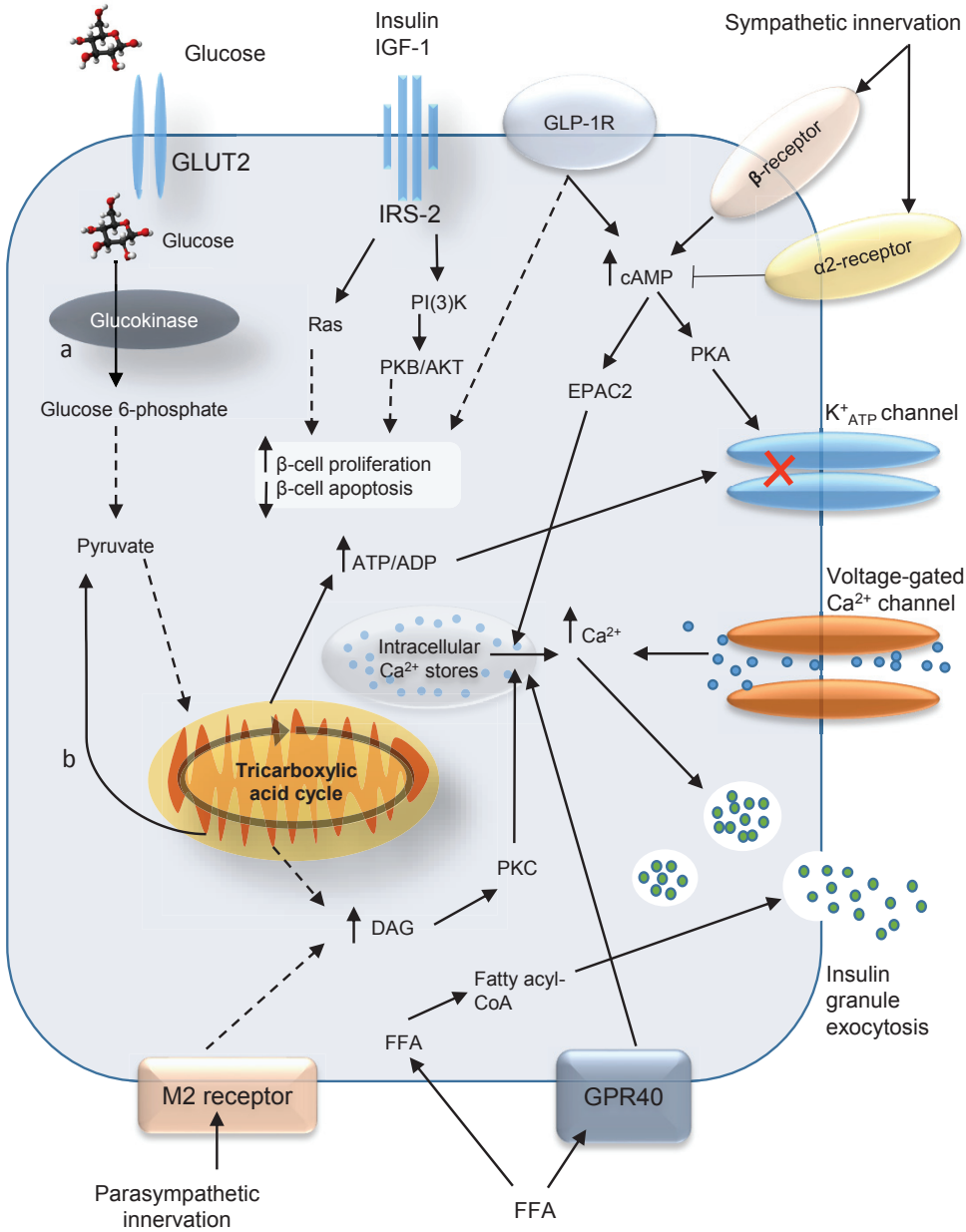

Figure 2. Possible mechanisms of adaptive insulin response to increased insulin resistance
This figure was adapted from reference 65.

are not responsible for the adaptive insulin release in response to insulin resistance.[68,69]

Other factors that could potentiate GSIS and adaptive insulin response include FFAs which can bind to the G-protein coupled receptor GPR40 (d) and subsequently stimulate intracellular signaling leading to exocytosis[70] or through generation of fatty acyl-CoA (e) that can both directly stimulate exocytosis and activate PKC.[71] Increased sensitivity to rather than increased levels of incretin hormones (f) secreted by the intestinal mucosa could be another adaptive mechanism to elevations in insulin resistance as evident in the lack of increased levels of glucagon-like peptide-1 (GLP-1) in obese individuals while insulin response to food intake is increased.[72] The central nervous system could also play an important role in the adaptive response of β-cells to decreased insulin sensitivity. Insulin secretion is stimulated through parasympathetic activation of M2 muscarinic receptors (g) and elevation of DAG, while sympathetic activation (h) of α2-adrenergic receptors inhibits insulin secretion and sympathetic activation of β-adrenergic receptors stimulates insulin secretion both acting through regulation of cyclic adenosine mono-phosphate (cAMP) levels.[73] Volume of β-cells also increases as an adaptive response to increasing insulin resistance. A 50% increased β-cell mass has been observed in healthy obese humans and this is more likely to be dependent on a hypertrophic rather than a hyperplastic response.[63,64] Animal studies have suggested that the increase in humans β-cell mass may more likely be mediated by FFAs rather than glucose.[74-76] In addition activation of the insulin/IGF-1 receptor (i) and the downstream signaling pathways may enhance β-cell survival.[77,78] Finally, GLP-1 (j) has been shown to increase β-cell proliferation and inhibit apoptosis in animal models.[65,79]

β-cell Dysfunction and Progression of Dysglycemia

The main defect in patients with T2D is the reduced capacity of β-cells to adequately respond to secretagogues as evident in the decline of insulin response to intravenous glucose and non-glucose secretagogues.[80] The dramatic loss of β-cell secretory function and to lower extent the loss of β-cell mass are characteristic of T2D patients.[63,64]

The progressive nature of T2D is mainly related to the continuous deterioration of β-cell function. This can be first attributed to the glucotoxic effects of chronicly elevated plasma glucose on β-cells. Insulin treatment has been shown to improve insulin sensitivity and β-cell function.[81] More recently the glucose lowering effects of sodium/glucose cotransporter 2 (SGLT2) inhibitors have been shown to improve β-cell function and insulin sensitivity despite the decrease in insulin secretion.[82] Furthermore, the lipotoxic effects of FFAs might also contribute to the progressive loss of β-cell function. *In vivo* lipid infusion and prolonged FFA elevation has been shown to increase insulin resistance and to decrease the compensatory β-cell response and insulin release in humans.[83] In addition to

glucolipotoxicity, chronic amyloid islet deposition leads to oxidative and endoplasmic-reticulum stress that results in β-cell apoptosis.[84] Dysfunction in the α-cell manifested as fasting hyperglucagonemia and its failure of suppression of postprandial glucagon release may also contribute to the progression of dysglycemia.[85]

Role of the Intestine

GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) are secreted by the entero-endocrine cells and are collectively known as incretins. These hormones act on pancreatic islet cells, with GLP-1 having a more important role as it can stimulate both insulin secretion and suppress glucagon secretion by acting on β- and α- cells respectively.[79] These hormones are responsible for the enhancement of insulin secretion after oral as compared to intravenous glucose which is termed as the incretin effect.[79] β-cell incretin resistance rather than decreased plasma levels of GLP-1 seems to be underlying the pathology in the entero-insular axis. First, plasma GLP-1 levels do not differ among individuals with NGT, IGT, or T2D.[86] Second, the β-cell response to GLP-1 seems to be deficient in patients with T2D as compared with controls.[87] In a recent study GLP-1 was shown to promote connectedness and coordination between β-cells through gap junctions and mobilization of intercellular calcium and these effects have been reversed through lipotoxic administration of FFAs.[88] In addition to the incretin system, the biliary system could play an important role in insulin secretion. Bile acids could play a beneficial role through stimulation of GLP-1 secretion by activating the G-protein bile acid receptor 1 on intestinal L-cells and through stimulation of farnesoid X receptor leading to the release of fibroblast growth factor 19 (FGF19) which has insulin-like effects.[89,90] Furthermore, the gut microbiota has been suggested to play an important role in the development in of T2D.[91,92]

Role of the Brain

The central nervous system can regulate insulin secretion through both sympathetic and parasympathetic effects.[73] Severing of the vagus nerve has been shown to impair insulin secretion.[93] The hypothalamus plays an important role in regulating hepatic glucose production through hypothalamic effects of insulin, glucose, and FFAs. In addition, hypothalamic insulin action can regulate body weight and impaired action results in obesity.[94-97] Finally, the clock genes, the circadian rhythm, and sleep patterns have been shown to regulate metabolic processes.[98,99]

Role of Inflammation

In addition to their effects on insulin sensitivity, systemic inflammatory cytokines associate with β-cell function. Interventions leading to a better systemic inflammatory profile have been shown to enhance β-cell function.[100,101]

Clinical Implications and Complications

Presentation

Patients with T2D may present with the classical signs and symptoms as polyuria, polydipsia, and/or weight loss. Hyperglycemia could also be suspected in individuals presenting with other signs as blurred vision, lower extremity paresthesias, or yeast infections. However, many patients with T2D are asymptomatic and at the time of diagnosis they would have had diabetes for 4-7 years.[102] The United Kingdom Prospective Diabetes Study (UKPDS) has shown that already at the time of diagnosis 25% of T2D patients had retinopathy; 9% had neuropathy; and 8% had nephropathy.[103]

Diagnosis

The diagnosis of diabetes can be based on fasting plasma glucose (FPG) levels, a 2-hour plasma glucose after a 75g oral glucose tolerance test (OGTT), glycated hemoglobin (HbA_{1C}) levels or random plasma glucose levels.[1,2] The diagnosis is established with a FPG $\geq$ 7.0 mmol/L (126 mg/dL) after a minimum of 8 hour fast, a 2-hr PG $\geq$ 11.1 mmol/L (200 mg/dL), a random plasma glucose $\geq$ 11.1 mmol/L (200 mg/dL) with classical hyperglycemic symptoms, or with an HbA_{1C} $\geq$ 6.5% using the United States National Glycohemoglobin Standardization Program (NGSP) which is concordant to 48 mmol/mol using the International Federation of Clinical Chemistry (IFCC) standardization.[2]

Prediabetes

Individuals are categorized as having prediabetes if they have IFG or IGT. IFG is defined as FPG levels between 5.6 and 6.9 mmol/L (100-125 mg/dL) by the American Diabetes Association (ADA) and as FPG levels between 6.1 and 6.9 mmol/L (110-125 mg/dL) by the World Health Organization (WHO). IGT is defined as 2-hour plasma glucose post OGTT between 7.8 and 11.0 mmol/L (140-199 mg/dL). In addition, individuals with HbA_{1C} levels between 5.7 and 6.4 % can be categorized as prediabetics.[2]

Prognosis

The degree of hyperglycemic control is a crucial factor in determining the prognosis of the disease. UKPDS has provided strong evidence for the association between chronic hyperglycemia and the increased risk for microvascular

complications.[104] In addition, reversion of prediabetes to NGT is associated with better prognosis and lower risk of future T2D.[105]

Diabetes complications are classified into microvascular and macrovascular complications. Microvascular complications lead to the development of retinopathy, nephropathy and neuropathy. Macrovascular complications are associated with atherosclerosis leading to CHD, stroke, and peripheral vascular disease. The mechanisms of diabetes complications are very complex and still far from being completely understood. Data from UKPDS has provided strong evidence that chronic hyperglycemia is the major determinant of microvascular but not macrovascular disease. An increase of HbA_{1C} from 5.5 to 9.5% was associated with a 10-fold increased risk of microvascular disease and but only a 2-fold increased risk of macrovascular disease.[104]

The glucotoxic effects on capillary endothelial cells of the retina, mesangial cells in the renal glomerulus, and on neurons and Schwann cells in the peripheral nervous system are thought to result mainly from four pathogenetic mechanisms. First, the increased influx in the polyol pathway, with aldose reductase reducing glucose to sorbitol which is then oxidized to fructose.[106] This process consumes nicotinamide adenine dinucleotide phosphate (NADPH) which is needed for the production of the antioxidant reduced glutathione leading to oxidative stress. Second, the production of advanced glycation end products (AGEs) and these agents damage the cells through regulation of certain genes, modifying the extracellular matrix and its signaling with the cell, and modifying circulating proteins as albumin leading to production of inflammatory cytokines and growth factors.[107-112] Third, activation of PKC by intracellular hyperglycemia through DAG which leads to lower levels of beneficial factors as nitric oxide synthase and higher levels of deleterious factors as transforming growth factor-β (TGF-β), endothelin-1, and plasminogen activator inhibitor-1 contributing to complications.[113-116] Finally, the fourth mechanism is via increased activity of the hexosamine pathway where fructose-6-phosphate is ultimately converted to N-acetyl glucosamine which is then attached to transcription factors leading to pathologic changes in gene expression.[117-119]

In 2004, Michael Brownlee proposed a unifying mechanism that could link together the four mechanisms of diabetic complications. He provided evidence for a common process, the hyperglycemic oxidative stress and increased production of superoxide by the mitochondria.[107] This overproduction of superoxide leads to activation of all four damaging pathways by inhibiting the glycolytic enzyme glyceraldehyde-3-phosphate dehydogenase.[107] Insulin resistance has been shown to be an independent risk factor for cardiovascular disease.[120] Since hyperglycemia is not a major determinant of macrovascular disease, Brownlee proposed that the increase in FFAs associated with insulin resistance may lead to overproduction of reactive oxygen species (as in hyperglycemia) that activate the four damaging pathways which could lead to macrovascular complications in diabetes.

Epidemiology, Risk Factors, and Prevention

The prevalence of T2D has been rapidly rising around the globe. This has been mainly attributed to aging and adoption of Westernized lifestyle with increased caloric intake and sedentary behavior.[121] In 2013, the IDF estimated around 382 million people (8.3% of the global adult population) to have diabetes and this number is estimated to reach 592 million by the year 2035. Although, the prevalence of the disease was relatively rare in developing countries a few decades ago, more than 80% of the current diabetes cases live in low- and middle-income countries. The mortality and financial burdens of diabetes are huge with the disease causing more than 5.1 million deaths worldwide and with more than 548 billion dollars in health spending in the year 2013.[3] Although T2D has traditionally been considered to be exclusive to the adult population, recent trends indicate increasing prevalence among adolescents and children.[122]

Overweight and Obesity

Overweight [body mass index (BMI) of 25-30 kg/m^2] or obesity (BMI of $\geq$ 30) are the most important risk factors for T2D.[123] The risk of T2D by obesity is stronger in younger adults, and weight gain in early adulthood is associated with higher risk and earlier onset of T2D than weight gain in late adulthood.[124,125] The association between obesity and T2D is mainly linked to the pathophysiological mechanisms of insulin resistance.[65] This association appears to be causal in nature as evident in randomized controlled trials (RCTs) that have shown reduced risk of T2D in relation to weight loss. This has been observed in lifestyle interventions as the Diabetes Prevention Program (DPP) and the Finnish Diabetes Prevention Study (DPS),[126,127] in pharmacologic interventions as lipase inhibitors,[128] and in bariatric interventions.[129] The direct causal association between BMI and T2D has also been reported in Mendelian randomization studies.[130,131]

Higher waist circumference (WC) has been previously shown to be a stronger risk factor for T2D than higher BMI.[132] In addition, the high risk of T2D in a group of normal weight individuals has led to the concept of metabolic obesity. Several studies have previously reported a higher risk of T2D in insulin resistant normal weight individuals as compared to insulin sensitive overweight individuals.[133-135] Metabolic obesity is particularly evident in Asian populations who tend to develop T2D at a lower BMI compared to Europeans.[136] At a given BMI, Asians tend to have higher body fat percentage and visceral adiposity than Europeans.[137,138] Although visceral adiposity is an independent risk factor for T2D, NAFLD has been shown to be a better predictor of insulin resistance and T2D showing that visceral adiposity could be a marker of intrahepatic fat accumulation and as such

not essentially causal.[139,140] In addition, surgical removal of visceral fat does not alter glucose homeostasis or insulin sensitivity.[44,45]

Physical Activity

Both lifestyle interventions of DPP and DPS were associated with reduced progression from IGT to T2D. Physical activity was an important part of these interventions (150 minutes per week and 30 minutes per day respectively).[126,127] Evidence from the Da Qing IGT and Diabetes Study have shown that diet alone, exercise alone, and diet plus exercise were equally effective in reducing the risk of IGT to T2D progression.[141] Furthermore, lower risk of T2D in association with moderate physical activity was observed in a meta-analysis of prospective trials even with BMI adjustment.[142] Experimentally, physical activity has been associated with increased insulin sensitivity and this is thought to be mediated by capillarization, increased oxidative capacity of mitochondria, and increased transport of the GLUT4.[143] In addition, physical activity has been associated with improved β-cell function and insulin secretion in patients with T2D.[144]

Dietary Factors

Dietary Fats

The risk of T2D by higher fat intake was earlier thought to be mediated directly through increasing insulin resistance or indirectly through increasing body weight.[145] However, previous studies do not support a direct role of the quantity of total fat intake in T2D, and rather highlight the importance of the quality of dietary fat. Several observational studies have shown that total fat intake is not associated with T2D.[146,147] In addition, an RCT of low dietary fat intake of postmenopausal women did not indicate a lower risk of T2D in the intervention group as compared to controls after 8.1 years.[148] Plant-derived fats and particularly omega-6 polyunsaturated fatty acids (PUFA) were associated with lower risk of T2D.[147,149] However, a systematic review found no association between seafood derived omega-3 PUFA (eicosapentaenoic acid, EPA; docosahexaenoic acid, DHA) and T2D, although it reported a modestly lower risk by plant derived omega-3 PUFA (alpha-linolenic acid, ALA).[150] Recent evidence from the EPIC-InterAct case-cohort study indicates that also saturated fatty acids (SFA) are not homogeneous in their association with T2D. Even-chained SFAs were associated with elevated risk of T2D, while odd-chained SFAs were associated with lower risk of T2D.[151]

Dietary Carbohydrates and Fibers

Since T2D is essentially a defect in glucose metabolism, dietary carbohydrate intake plays an important role in the pathogenesis of the disease. However, there is

variation in the absorption and effects on glucose metabolism between different food sources of carbohydrates.[152] Evidence from prospective studies does not indicate any change in the risk of T2D with changes in carbohydrate proportions in diet.[153] The glycemic index and glycemic load are used to assess the quality of carbohydrate intake. The glycemic index is the ratio of blood glucose elevation by a particular food to the glucose elevation by the same amount of a standard source usually white bread or glucose.[154] Since there is variation in the amount of carbohydrates in certain foods or diets, the glycemic load has been used to correct for this variation by multiplying the amount of carbohydrates in a certain food by its glycemic index.[152] Both higher glycemic index and glycemic load diets have been shown to associate with increased risk of T2D in a recent meta-analysis of three large cohorts.[155]

Dietary fiber can be defined as the edible parts of carbohydrates or plants that are resistant to enzymatic degradation in the small intestine. This definition traditionally included polysaccharides and lignin but more recently it has been expanded to include oligosaccharides and resistant starches.[156,157] Dietary fiber is commonly classified as either soluble or insoluble. Soluble fibers are viscous and fermentable and may exert their protective effects through delaying gastric emptying, and reducing macronutrient absorption and postprandial glucose responses.[158-160] Insoluble fibers have a bulking action and may be fermented for a limited extent in the colon.[156] Dietary fibers are fermented by colonic microbiota to produce several metabolites of which short chain fatty acids (SCFAs) as butyrate, acetate and propionate are the most common.[161] Animal studies have shown that SCFAs activate fatty acid oxidation through activation of AMPK, and inhibit adipose tissue lipolysis and *de novo* lipogenesis leading to reduced levels of circulating FFAs and decreased body weight.[162] In addition, SCFAs may lower fasting glucose levels which could be attributed to AMPK mediated lower expression of gluconeogenic enzymes. SCFAs may also increase plasma levels of GLP-1 and peptide YY (PYY).[162]

Both the ADA[163] and the European Association for the Study of Diabetes (EASD)[164] recommend higher fiber intake (14 g/1000 kcal) to prevent T2D. Higher dietary fiber intake has been shown to play a protective role in T2D based on evidence from several prospective studies.[123,165,166] In addition, evidence from DPS showed that individuals in the highest fiber intake quartile had a hazard ratio (HR) of 0.38 compared to individuals in the lowest quartile for progression from prediabetes to T2D.[167] Furthermore, a meta-analysis of prospective cohort studies demonstrated that cereal fiber might play a more important protective role in T2D compared to vegetable fiber.[168] In addition, the protective effect of cereal fiber appeared to be independent of the glycemic index and glycemic load in a recent meta-analysis.[155]

Higher fiber intake has also shown beneficial associations with the metabolic syndrome, lipid traits, insulin sensitivity, inflammatory markers, CHD, stroke, and

peripheral vascular disease.[169-172] In addition to its role in prevention of T2D, fiber intake has been associated with improvements in FPG, cholesterol and TGs in patients with diabetes.[173,174]

Food Groups and Beverages

A recent systematic review and meta-analysis of cohort studies reported that whole grain intake is associated with lower risk of T2D.[175] Another meta-analysis reported an increased risk of T2D with white rice consumption.[176] High intakes of red meat and processed meat have also been associated with elevated risk for T2D.[177,178] Total intakes of vegetables and fruits, when analyzed separately in large meta-analyses, did not show association with T2D. However, when studying subtypes of these food groups, only green leafy vegetables were associated with lower risk of T2D.[179,180] Higher consumption of dairy products and nuts have also been associated with lower risk of T2D.[181-183]

Sugar-sweetened beverages have been consistently associated with elevated risk of T2D.[184,185] In addition, coffee consumption has been demonstrated to have a protective association with T2D, and this association is likely to be mediated by compounds other than caffeine as decaffeinated coffee showed similar association.[186] The relationship between alcohol consumption and T2D fits a U-shaped curve with lower risk associated with moderate alcohol intake and elevated risk with both high alcohol consumption and zero or very low consumption.[187]

Minerals and Vitamins

Magnesium intake has been shown to have a protective association with T2D.[188] Blood levels of 25-hydroxy vitamin D have been associated with lower risk for T2D in a meta-analysis of prospective studies.[189] However, in a meta-analysis of RCTs vitamin D did not improve glycemic traits.[190] In addition, a recent Mendelian randomization study of circulating 25-hydroxy vitamin D failed to observe a causal association with T2D.[191] Finally, heme-iron intake and body iron stores have been associated with higher risk of T2D.[192]

Dietary Patterns

Mediterranean diets have been consistently associated with lower risk of T2D in prospective studies.[193] RCTs have also reported lower risk of T2D associated with Mediterranean diet.[194,195] Other dietary patterns that have been associated with lower risk of T2D include high quality diets assessed by the Alternate Healthy Eating Index (AHEI),[196] the Dietary Approaches to Stop Hypertension (DASH) diet,[197] vegetarian and vegan diets,[198] and prudent dietary patterns.[199,200]

Prediabetes and Prevention

In the United States, 35% of adults older than 20 years and 50% of those older than 50 years have prediabetes translating to a total of 79 million adults.[201] Each year around 5-10% of those with prediabetes become diabetic. However, the conversion rates are different in different populations and are dependent on which definition was used for prediabetes.[202,203] The annual conversion rate is 4-6% for isolated IGT, 6-9% for isolated IFG and 15-19% for combined IFG and IGT.[204] In DPP the annual progression rate from prediabetes to diabetes was 11%.[205] In the Da Qing Diabetes Prevention Study the 20-year cumulative conversion from IGT to diabetes was 93% in the control group.[206] Although T2D is currently incurable, studies have shown that it can be prevented or delayed through lifestyle and pharmacological interventions.[207-209]

The Metabolic Syndrome

The metabolic syndrome is a cluster of biochemical and anthropometric abnormalities that include glucose intolerance, dyslipidemia, hypertension and central obesity. This syndrome may be accompanied with abnormal findings as increased inflammatory markers, hyperuricemia, microalbuminuria, elevated alanine aminotransferase, and hemostatic derangements.[210,211]

The metabolic syndrome has several definitions and the most widely accepted are those by the WHO, the National Cholesterol Education Program (NCEP) and the IDF. In 1998, the WHO has stated insulin resistance to be the main cause of the metabolic syndrome and it was included as an essential criterion for the diagnosis of the metabolic syndrome.[212] Later on the NCEP and IDF replaced insulin resistance by abdominal obesity (as measured by the WC) as a criterion for the metabolic syndrome. The three definitions of the metabolic syndrome are summarized below.

WHO Criteria

The WHO report on the definition of the metabolic syndrome was finalized in 1999[213] and included insulin resistance (IGT, IFG, T2D, or insulin resistance as measured under hyperinsulinemic euglycemic conditions) as an essential criterion in addition to at least two of the following criteria:

- Central obesity: defined as a waist to hip ratio of > 0.90 in men and > 0.85 in women and/or a BMI > 30 kg/m^2
- Dyslipidemia: defined as TG levels ≥ 1.7 mmol/L (150 mg/dL) and/or high-density lipoprotein cholesterol (HDLC) levels < 0.9 mmol/L (35 mg/dL) in men or < 1 mmol/L (39 mg/dL) in women

- Hypertension: defined as blood pressure ≥ 140/90 mmHg and /or antihypertensive medication
- Microalbuminuria: defined as urinary albumin excretion rate ≥ 20 μg/min or albumin:creatinine ratio ≥ 30 mg/g

NCEP Criteria

The NCEP Adult Treatment Panel III (NCEP-ATP III)[214] definition of the metabolic syndrome published in 2002 includes individuals with 3 or more of the following criteria:

- Abdominal obesity: defined as a WC > 102 cm in men or > 88 cm in women
- Hypertriglyceridemia: defined as TG levels ≥ 1.7 mmol/L (150 mg/dL)
- Low HDLC: defined as levels < 1.03 mmol/L (40 mg/dL) in men or < 1.30 mmol/L (50 mg/dL) in women
- Elevated blood pressure: defined as blood pressure ≥130/≥85 mm Hg
- IFG: defined as FPG levels ≥ 5.6 mmol/L (100 mg/dL)

IDF Criteria

The IDF definition[215] for the metabolic syndrome published in 2006 includes central obesity as an essential criterion defined as an ethnicity specific elevated WC and/or a BMI > 30 kg/m^2 and at least two of the following criteria:

- Hypertriglyceridemia: defined as TG levels ≥ 1.7 mmol/L (150 mg/dL) or TG lowering medication
- Low HDLC: defined as HDLC < 1.03 mmol/L (40 mg/dL) in men or < 1.30 mmol/L (50 mg/dL) in women or HDLC raising medication
- Elevated blood pressure: defined as blood pressure ≥130/≥85 mm Hg or on antihypertensive medication
- IFG: defined as FPG levels ≥ 5.6 mmol/L (100 mg/dL)

Progression to Diabetes

Non-diabetic individuals with the metabolic syndrome are at a 5-fold increased risk of developing diabetes.[216] The metabolic syndrome accounted for approximately half of the new T2D after 8 years of follow-up in the Framingham Offspring Study.[217] Additionally, the metabolic syndrome is a strong risk factor for the progression to CVD.

Other Risk Factors

Early developmental factors during fetal embryogenesis can play an important role in the development of diabetes later in life. Studies have shown that low birth weight is a risk factor for T2D and IGT.[218] Furthermore, high birth weight has also been associated with greater risk of T2D supporting a U-shape curve relationship between birthweight and T2D.[219] In women, history of gestational diabetes or PCOS has been associated with increased risk of T2D.[55,56]

Non-Modifiable Risk Factors

Age

The risk of T2D increases dramatically with advancing age and most cases are diagnosed after the age of 45. The ADA recommends screening for both diabetes and prediabetes starting at the age of 45.[2]

Gender

T2D was more prevalent among women in the first half of the past century, but today the prevalence is higher among men. This has been mainly attributed to the increasing sedentary lifestyle among men.[220] Recent studies have shown that men develop T2D at a lower BMI than women.[221] This has been attributed to sex differences in body fat distribution, with men having more visceral and hepatic fat associated with insulin resistance and women with more subcutaneous fat associated with improved insulin sensitivity.[222]

Ethnicity

The differences in the prevalence of T2D among different ethnicities are well documented. In the United States the prevalence is higher for African Americans and Hispanics than for European Americans.[223] The age adjusted prevalence of diagnosed diabetes of adults above the age of 20 years was 7.6% for non-Hispanic whites, 9.0% for Asian Americans, 12.8% for Hispanics, 13.2% for non-Hispanic blacks, and 15.9% for American Indians or Alaska natives between 2010 and 2012.[224] The elevated risk among Asians, Hispanics, and African Americans have been previously shown to remain after controlling for BMI differences.[225] In Sweden, immigrants from the Middle East have shown a 4-fold higher risk of T2D as compared with native Swedes.[226]

Family History

T2D aggregates in families and studies have estimated that individuals with one or two affected parents are at a 2- to 6-fold increased risk and this risk has been found to be independent of other risk factors.[227-231] The lifetime risk for developing

T2D is approximately 40% if one of the parents is affected[232] and can reach 70% if both parents are affected.[233] Family history reflects shared genetic and environmental factors and possibly their interactions in the development of T2D.

Genetics of T2D

Heritability

The heritability of a certain phenotype measures how much of the total variation of the phenotype can be attributed to genetic effects. In family studies, broad sense heritability (H^2) can be measured as the ratio of the estimate of phenotypic variance attributable to all genetic effects (S^2_G) to the total phenotypic variance (S^2_T). In addition, the narrow sense heritability (h^2) can be obtained as the ratio of the estimate of phenotypic variance attributable to the additive genetic effects to the total phenotypic variance.[234] Both family and twin studies have provided evidence for a genetic component in the development of T2D. The concordance rates of T2D range between 35-58% in monozygotic twins and between 17-20% in dizygotic twins.[235,236] The heritability estimates from family and twin studies have been in the range of 26-70% with the highest estimates observed in the 35-60 age group.[237,238]

Genetic Variation in Complex Traits

Complex traits or diseases as T2D are phenotypes that do not exhibit Mendelian form of inheritance. These complexities arise when a single genotype can result in different phenotypes or when different genotypes can result in a similar phenotype.[239] No genetic markers show perfect co-segregation with complex traits. Complex traits are characterized by incomplete penetrance meaning that inheritance of the predisposing allele does not necessarily lead to disease; however, it may affect the probability of the disease. This probability may also depend on other non-genetic factors as age, sex and the environment. Complex traits also exhibit phenocopies, as individuals not carrying the risk allele may develop the disease.[239] Locus heterogeneity in complex traits is another problem where polymorphisms in several genes contribute to the same phenotype. In addition, complex traits exhibit polygenic inheritance, requiring variations in multiple loci to reach a certain threshold before developing the phenotype or disease. In addition, the alleles that associate with complex diseases typically have high population frequencies. All of these characteristics make it difficult to pinpoint and map genetic risk loci in complex traits.[239]

The most common type of variation in the human genome is known as the single nucleotide polymorphisms (SNP). SNPs are usually stable single base pair substitutions and more than 38 million common SNPs with a minor allele frequency (MAF) of more than 1% exist in the human genome.[240] Most of the SNPs are inherited as haplotype blocks that are defined by linkage disequilibrium (LD).[241-243] In addition to SNPs there are structural polymorphisms that include insertions, deletions, and larger copy number variations (CNVs).[244,245] One of the main tasks after finalization of the first draft of the human genome was to develop a map of genetic variations to investigate their role in complex diseases.[246-248] This has led to the creation of the International HapMap Project that has catalogued haplotypes of common genetic variations in three continental populations.[249-252] In addition, a catalog for uncommon genetic variations (1000 genomes project) has been recently developed from sequencing of more than 1000 genomes.[253]

Linkage Studies

Linkage studies which analyze the co-segregation of the disease or trait and genetic markers in families have been successful in evaluating the genetic basis of Mendelian traits.[254] However, genome-wide linkage scans that use microsatellites for discovering and mapping the genetic architecture complex traits was largely unsuccessful.[255] Therefore, it has been later argued that linkage analysis has limited resolution and power to detect genes with modest effects in complex traits and that large-scale association studies have far greater power even if every genetic marker needs to be tested.[256]

Linkage studies have been successful in identifying Mendelian or monogenic forms of non-autoimmune diabetes as MODY, neonatal diabetes, insulin resistance and Wolfram syndromes.[257] However, these conditions are usually rare and they account for 1-2% of all diabetes in the young.[258] Linkage studies for T2D were largely unsuccessful except for the discovery of the *TCF7L2* gene.[259]

Candidate Gene Approach

The lack of success in linkage studies led to the focus on association studies. Before the advent of large-scale genome-wide association studies (GWAS), researchers focused on certain candidate genes to test for association between variations in these genes and diseases or traits. Most of these studies were largely underpowered and only included variations in the protein coding regions of the candidate genes.[59,260] Candidate gene studies have further demonstrated the difficulties in pinpointing good candidate genes. However, few T2D candidate gene studies were successful in discovering the well-replicated associations of missense variants in the peroxisome proliferator-activated receptor gamma (*PPARG*) (Pro12Ala) and potassium inwardly-rectifying channel, subfamily J, member 11 (*KCNJ11*) (Glu23Lys) genes with T2D. *PPARG* encodes PPARγ which is a nuclear hormone receptor that regulates adipogenesis and a known

target for thiazolidinediones. The *KCNJ11* encodes the inwardly rectifying potassium channel (Kir6.2) of the β-cell K^{+}_{ATP} channel which is a target for sulfonylureas.[261,262] Monogenic forms of diabetes were studied as T2D candidate genes and association signals were discovered in variants in *WFS1* (Wolfram syndrome 1 gene) and *HNF1B* (MODY 5 gene).[263,264]

Genome-Wide Association Studies

GWASs have revolutionized the field of genetics of complex traits. These large scale association studies typically test the associations between ~ 1×10^6 SNPs with complex diseases and traits.[265] This became possible in 2006 with development of technologies that allow reliable genotyping of up to 1 million SNPs which can capture > 80% of common variants in a large number of individuals.[266] Due to the large number of tests performed in GWAS, the threshold for significance is set to $\alpha < 5 \times 10^{-08}$. In order to further rule out any false positive associations, SNPs that show significant association in the first GWAS discovery set are then tested in other replication studies.[265,267] After 2007, there has been an explosion in the number of GWAS publications and discovered SNP-trait associations. The National Human Genome Research Institute (NHGRI) GWAS catalogue included by the year 2013 more than 1,778 publications and 12,123 SNP-trait associations.[268] Even with discovery of a large number of SNP-trait associations, it is still very challenging to map the discovered SNPs into causal variants in genes and loci. Although exonic SNPs are overrepresented on genotyping arrays, only 12% of the discovered SNPs are located in protein coding genes and around 40% are located in intronic regions and another 40% in intergenic regions.[269]

Genome-Wide Association Studies and T2D

In 2007, the first GWASs for T2D were published and they successfully replicated the strong signal in the *TCF7L2* locus. These studies additionally identified several novel SNPs associated with T2D in different case-control studies of European ancestry.[270-276] Since all the discovered SNP associations were of modest effect sizes it was obvious that any additional common loci would probably have smaller effect sizes and a combined effort to form a large consortium was needed. This led to the creation of the Diabetes and Genetics Replication and Meta-analysis (DIAGRAM) consortium which have led to the discovery of most of the T2D associated loci in three large publications to date.[60,277,278] More than 50 loci have been identified so far to be associated with T2D in European populations.[60,277,278]

Most of the GWASs for T2D have been performed in populations of European ancestry. However, GWASs in populations with different ancestry can facilitate the discovery of additional genes. A good example is the association between the *KCNQ1* locus and T2D. This locus was first discovered in East-Asian populations and then replicated in European populations.[279,280] Differences in the allele

frequencies between European and Asian population for variants in this locus rendered Asian studies more powerful to detect this association.

In addition, GWASs for quantitative glycemic traits have also led to the discovery of some of the T2D associated loci. This had been made possible with the creation of Meta-Analyses of Glucose and Insulin-related traits Consortium (MAGIC). Variations in the *MTNR1B*, *ADCY5*, *PROX1*, *GCK*, *GCKR* and *DGKB-TMEM195* gene were discovered to be associated with fasting glucose in MAGIC and also showed associations with T2D.[281-283]

Most of the T2D associated variants have been linked to β-cell dysfunction and few have been associated with insulin resistance. Early studies on the potential associated effect of the risk variants on insulin secretion and action have observed that most of the variants were associated with derangements in β-cell function in healthy individuals.[59,284] The few variants that have been associated with insulin resistance include variants with obesity-dependent mechanisms as *FTO* and obesity-independent mechanisms as *IRS-1* and *PPARG*.[59,285] The hypothesis-free approach of GWAS helped in highlighting certain pathways and mechanisms in the pathogenesis of T2D as the wingless-type MMTV integration site (WNT) pathway (*TCF7L2*), cell cycle signals (*CDKAL1*, *CDKN2A/B*), and the circadian rhythm (*MTNR1B*, *CRY2*).[59]

TCF7L2 and T2D

In 2006 Grant et al. discovered a microsatellite (DG10S478) in the *TCF7L2* gene region to be associated with T2D in an Icelandic case-control study.[259] Later this association was replicated in other case-control studies and the non-coding SNPs rs12255372 and rs7903146 showed the strongest LD with the DG10S478 microsatellite.[286] Up to this date these variants remain the strongest and most replicated in association with T2D. The locus shows an additive genetic effect with carriers of one copy of the risk allele having around 40% increased risk of T2D and the risk is almost doubled among carriers of two copies of the risk allele.[286-288] The *TCF7L2* risk allele has also been shown to be associated with elevated FPG and HbA_{1C} among non-diabetic individuals.[282,289]

The gene product, TCF7L2, also known as TCF4, belongs to a family of transcription factors. It acts as a principal transcription factor in the canonical WNT signaling pathway.[290] After activation of the WNT pathway, β-catenin is translocated into the nucleus and binds TCF7L2 and activates transcription of its target genes. WNT signaling plays an important role in cell proliferation and differentiation, organogenesis, and has been implicated in development and progression of some cancers.[291-294] TCF7L2 has been shown to play an important role in pancreatic development and protection and thus could affect β-cell mass.[295-298] In addition, TCF7L2 has been shown to affect insulin secretion, and this is based on evidence from animal studies showing that impairment of the canonical

WNT pathway results in glucose intolerance.[299] Furthermore, TCF7L2 depletion by small interfering RNA (siRNA) in human islets resulted in reduced β-cell survival and decreased GSIS.[300] TCF7L2 is involved in the induction of the transcription of the proglucagon gene in intestinal L-cells which encodes GLP-1 and that could affect insulin secretion and β-cell survival.[301] GLP-1 stimulated β-cell proliferation has been shown to be dependent on activation of the WNT signaling and TCF7L2.[302]

The role of the *TCF7L2* variation in T2D is still far from being completely understood. The rs7903146 SNP remains the strongest in association with T2D. As TCF7L2 is involved in the transcription of the proglucagon gene it was first thought that the variation could affect GLP-1 secretion and plasma levels. However, the risk T-allele has been shown to have no association with plasma GLP-1 levels.[303] On the other hand, several studies have observed an impaired incretin effect on insulin secretion among carriers of the risk T-allele.[304,305] Studies have reported increased, decreased, and no effect of the risk allele on the expression of *TCF7L2* in pancreatic β-cells.[304,306,307] These differences could be attributed to the extensive alternative splicing of the *TCF7L2* gene. In addition, the *TCF7L2* has a unique splice variant expression pattern in pancreatic islets as compared to other tissues.[308-310] Furthermore, the *TCF7L2* rs7903146 risk T-allele has been previously shown to be associated with an islet specific open chromatin state consistent with increased levels of expression.[311]

Genetic Risk Scores and Prediction

The clinical utility of the GWAS findings has been argued concerning contributions to better treatments by the uncovered new mechanisms, better risk prediction, and individualized treatment and prevention. For risk prediction purposes, genetic variants have been aggregated into genetic risk scores (GRSs). The GRS is usually created by summing the risk alleles and it can further be weighted based on the individual odds ratios (ORs) of each variant reported in the large meta-analyses.

The predictive capacity of the GRS for T2D have so far been quite modest. The areas under the receiver operating curve (AUC) which plots the sensitivity on the y-axis and the 1 minus specificity on the x-axis have been shown to be in the range of 0.54 to 0.63 in most of the genetic prediction studies done so far.[312] In addition, few studies have reported slight improvement in the AUC with addition of the GRS to the traditional clinical model of age, sex, family history, BMI, systolic blood pressure (SBP), FPG, HDLC and TG. However, the discriminatory and reclassification capacity of the GRS, although significant, has been marginal and of no clear clinical utility.[312,313] In addition, the predictive capacity of GRSs has been observed to increase with additional SNPs and then to plateau at a certain number where additional SNPs no more add to the prediction.[313] It is important to note that the prediction capacity of GRSs has been shown to be better in younger

individuals aged less than 50 years as compared to older individuals.[314] This shows that genetic prediction may fair better if done at a younger age.

Missing Heritability

One of the major challenges after the discovery of a large number of T2D associated variants is the low proportion of the total genetic influence explained by these variants. It is estimated that these variants explain only 10-15% of the heritability of T2D.[277] This has led to the concept of missing heritability in T2D and other complex traits. One of the possible explanations is that GWASs so far have only included common variants (MAF > 5%) and that a large number of low frequency variants (MAF 0.5%-5%) and rare variants (MAF < 0.5%) could contribute. It is argued that low frequency variants with intermediate effect sizes and rare variants with large effect sizes could explain part of the missing heritability.[315] This will be facilitated with the comprehensive catalogue of low frequency variants of the 1000 Genomes project.[240] Detection of rare variants is now possible with recent development of next-generation sequencing technologies that allow cheaper sequencing of exomes or genomes. In addition, structural variations as CNVs, inversions, or translocations have been argued to account for part of the missing heritability and more efforts are needed to integrate CNVs in GWAS.[315] Other explanations for the missing heritability include gene-gene interactions, gene-lifestyle interactions, and epigenetic effects.

Gene-Lifestyle Interactions

Both genetic and lifestyle factors play an important role in the pathogenesis of T2D. In addition, interactions between lifestyle and genetic factors are believed to play an important role in T2D. The evidence for gene-lifestyle interactions is based on several observations: the prevalence of T2D differs dramatically between ethnic groups, with certain ethnicities at a higher risk with adoption of a Westernized lifestyle. One of the best examples is the case of the Pima Indians of Arizona and Northern Mexico who are closely related and share a very similar gene pool. The Arizona Pima Indians abandoned their traditional lifestyle a century ago. They became obese and have a prevalence of 38% of T2D which is 5-fold of the Mexican Pima Indians (6.9%) who kept their traditional lifestyle. This example shows that certain environmental changes can induce certain genetic predisposition.[316] In 1962, the thrifty gene hypothesis was introduced by James Neel, proposing that the elevated prevalence of T2D and obesity in certain ethnicities could be related to the positive selection of certain genotypes that allow more efficient energy storage and metabolism.[317] The heterogeneity in the response to certain interventions between related and unrelated individuals in family based studies support the role of gene-lifestyle interactions in the pathogenesis of complex traits as T2D.[318]

Departure from additivity of genetic and environmental effects is the most common statistical definition of gene-lifestyle interactions which may not necessarily imply any biological inference.[319] If a statistical interaction exists, the effects of the genotype and the environment are not independent: the effect of the genotype is dependent on the environment and/or the effect of the environment is dependent on the genotype.[320]

Understanding gene-lifestyle interactions in the pathogenesis of T2D can have profound implications in both prevention and treatment of the disease. Prevention may be targeted to individuals carrying certain genotypes that render them more susceptible to the deleterious effects of certain environmental exposures. In addition, identifying differences in treatment response among different genotype carriers would allow for better tailoring of treatments and interventions especially that T2D is very heterogeneous disease and there is a growing need for characterization of diabetic subgroups.[321] Furthermore, understanding gene-lifestyle interactions can contribute in understanding the biological mechanisms by which certain lifestyle factors and genetic variants affect the risk of T2D.

Study Designs and Challenges

Population-based studies are the most commonly used for studying gene-lifestyle interactions.[322] These include case-control, nested case-control, and cohort studies. Case-control studies allow the study of gene-lifestyle interactions on the prevalence of T2D. Although this study design allows for efficient recruitment of subjects it is prone to exposure recall bias. Cohort studies allow the study of interactions on incidence of T2D, and since exposures are assessed at baseline they are not subject to recall bias. However, it suffers from lower efficiency in subject utilization and decreased power if the number of individuals who develop the disease is too low. The nested case-control is most commonly used design as it allows efficient use of subjects and does not suffer from exposure recall bias.[323-325] Cross-sectional gene-lifestyle interaction are usually performed with glycemic traits. These studies can provide valuable insight into the possible biological pathway through which interactions are acting which cannot be captured by studying T2D as the outcome variable.[326] The RCT approach in studying interactions has great advantages in eliminating confounding bias and permitting causal inference. However, most of RCTs suffer from low power in detecting interactions due to their small size and short duration of follow up.[326]

Gene-Lifestyle Interactions and T2D

Gene-lifestyle interaction studies focused on T2D candidate genes before the GWASs. These studies were hypothesis driven and focused on shared biological pathways between certain lifestyle factors and candidate genes. Dietary PUFAs have been shown to bind and upregulate PPARγ.[327] This led to the hypothesis of potential interactions between *PPARG* and dietary fat intake. In fact some

observational studies have reported that carriers of the Ala12 may have more favorable effects of PUFAs on glycemic and anthropometric traits compared to the Pro12 carriers, and the Ala12 were observed to be less sensitive to the deleterious effects of SFAs on these traits.[328-332]

The strongest T2D locus, *TCF7L2*, which has been mainly associated to defects in the incretin system has been extensively investigated for gene-lifestyle interaction as dietary intakes can modulate the entero-insular axis. In the DPP the associated effect of the risk T-allele on the progression from IGT to T2D was stronger in the placebo as compared to the metformin and lifestyle intervention groups, however, the test for interaction was not statistically significant.[333] The *TCF7L2* risk allele has been associated with a more pronounced risk of T2D among individuals consuming high glycemic load diets as compared to those on low glycemic load diets.[334] In the European Prospective Investigation into Cancer and Nutrition (EPIC) Potsdam cohort higher intake levels of whole grains were associated with lower risk of T2D only among homozygotes for the non-risk allele.[335] A similar observation was reported in the Stockholm Diabetes Prevention Program in which the protective associated effect of both whole grains and cereal fibers on T2D was restricted to non-risk genotype carriers.[336] In the Tübingen Lifestyle Intervention Program (TULIP) study, a higher fiber intake was associated greater weight loss among homozygotes for the *TCF7L2* non-risk allele as compared to risk allele carriers.[337] These findings suggest a common mechanism by which *TCF7L2* and cereal fiber or whole grain intake may modulate the risk of T2D.

Several T2D GWAS loci have been found to interact with lifestyle factors on the risk of T2D and related traits. The *FTO* variant has been consistently associated with T2D through its effects on adiposity.[271] This variant has been previously reported to interact with physical activity on the risk of obesity in a large meta-analysis.[338] Higher levels of physical activity attenuated the elevated risk of obesity by the *FTO* risk allele. Risk variants in the *FTO* and another obesity associated locus (*MC4R*) have been reported to be associated with increased risk of T2D only among individuals with low adherence to Mediterranean diet.[339] A variant in the *SLC30A8* gene associates with T2D and FPG. *SLC30A8* encodes a zinc transporter which provides zinc for insulin hexamer formation.[340] Interaction between variants in *SLC30A8* and zinc intake was tested in a large meta-analysis in the Cohorts for Heart and Aging Research in Genetic Epidemiology (CHARGE) on FPG levels. A stronger inverse association between zinc intake and FPG was observed among carriers of the *SLC30A8* risk allele as compared to non-carriers.[341] Sex-specific interactions between a variant in the *IRS1* locus and carbohydrate and fat intake on the risk of T2D was previously reported. Variants in the *GIPR* locus have been previously associated with 2-hour post OGTT glucose levels, but not with T2D.[342] However, the minor allele of this variant has been previously reported to increase the risk of T2D among individuals with low fat intake and decrease the risk among those with high fat intake.[343] Most of the studies using

T2D GRS have failed to observe interactions with lifestyle factors on the risk of T2D.[331] However, an interaction between a GRS of 10 T2D SNPs and a Western dietary pattern has been observed on the risk of T2D.[344]

Most of the gene-lifestyle interaction studies suffer from power issues and measurement inaccuracies of lifestyle factors as diet and physical activity. As a general rule, gene-lifestyle interactions in case-control studies require four times the cases of what is required to detect the main effects of genotypes and environment seperately.[325,345] In addition, moderate deteriorations in the measurement accuracy have been estimated in simulation studies to decrease the power by 20-fold for detecting interactions.[346] To overcome these restrictions efforts are now directed into improving the accuracy of measurements of lifestyle factors. More objective measures of dietary factors are focused on the discovery of biomarkers for nutrient intakes. Since interactions studies require large sample sizes and in most cases large meta-analyses of several studies may be needed, efforts are now also focused on harmonizing measurements of lifestyle exposures across different studies.[322]

Mendelian Randomization

Validated T2D risk prediction models incorporating traditional risk factors as age, sex, family history, BMI, SBP, FPG, HDLC and TG have been developed in observational epidemiology.[347,348] Even though these cardio-metabolic biomarkers have independent associations and tend to improve risk discrimination, these associations are not necessarily causal in nature and could be confounders for other real causal associations.[347-350] To address the issue of causality RCTs have been traditionally used as means of balancing known and unknown confounders between two comparison groups. Many behavioral, physiological, and pharmacological measures that show robust associations in observational studies fail to show these associations in RCTs.[351] Reverse causation, confounding, and other biases can be behind observational associations, and incorrect causal inference may happen even with careful study design and statistical adjustments.[351,352]

Mendelian randomization can mimic RCTs in the random allocation of individuals (Figure 3). Instead of actively randomly assigning participants into treatment and control groups to balance confounders and establish causal inference, Mendelian randomization benefits from the random allocation of alleles at conception which can be exploited in observational studies.[353,354] Therefore, those who inherit the trait raising allele are randomly assigned to the group with higher levels of the trait of interest and those that do not inherit the trait raising allele are randomly assigned to the group with lower levels of the trait of interest.[353] This form of

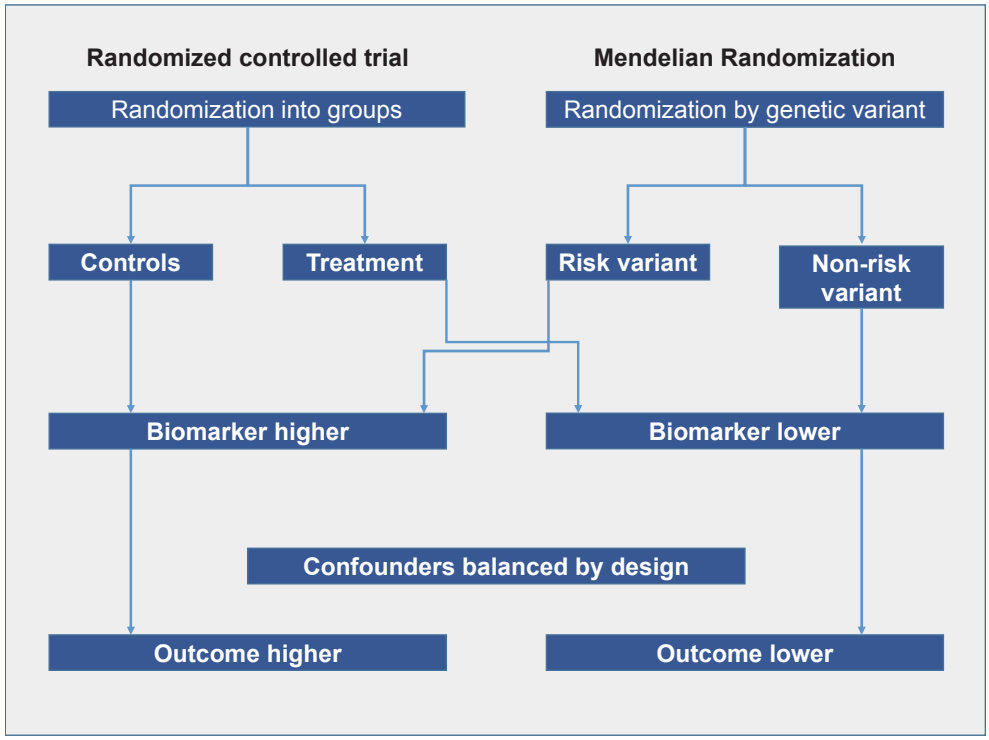

Figure 3. Schematic analogy between randomized controlled trials and Mendelian randomization
This figure was adapted from reference 354.

randomization generally prevents confounding bias, although it is important to take appropriate measures to prevent confounding by population stratification.

To infer causal association between a trait and an outcome, genetic variants can be used as instrumental variables. Genetic variations are excellent instruments for several reasons. The genetic variant associates with the trait of interest in one direction only and this can remove bias by reverse causation. Genetic variants can be used as unconfounded indicators of the trait of interest. Measurements of genetic variation are usually low in errors and biases. In addition, genetic variants in high LD with the causal variant may be used as instruments.[353,355] However, genetic variants should fulfill certain assumptions to be used as instrumental variables in Mendelian randomization as shown in Figure 4. (a) The genetic variant should be reliably associated with the intermediate phenotype or trait of interest. (b) The genetic variant should not have independent associations with the outcome and should only associate with the outcome through the intermediate

phenotype or trait. (c) The genetic variant should not be associated with measured or unmeasured confounders.

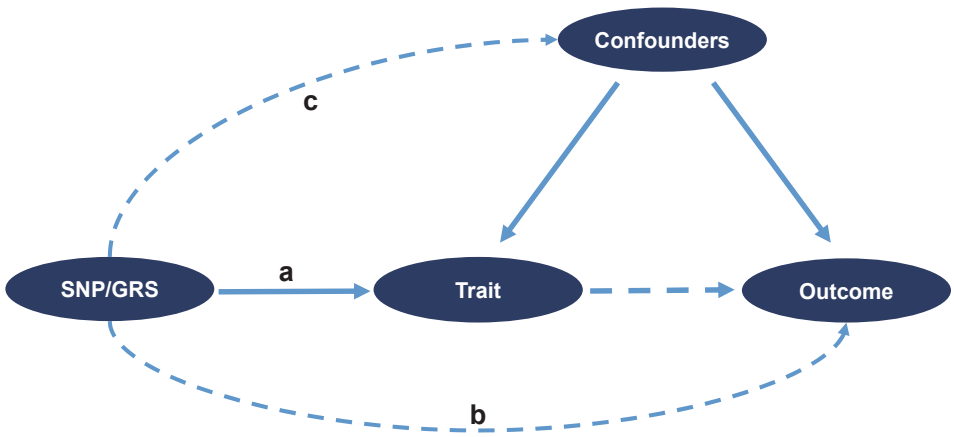

Figure 4. A schematic representation of instrumental variable analysis.

Genetic Risk Scores as Instrumental Variables

A single genetic variant (most commonly SNP) usually explains very little of the variance of the trait of interest leading to very low power to detect true causal associations. The variance explained can be increased by increasing the number of associated variants. SNPs can be combined into a GRS to create more powerful instrumental variables for inference of causality. A previous simulation study indicated that a weighted GRS is a more powerful instrumental variable than an un-weighted GRS.[356] However, including more SNPs can increase the chance of pleiotropic effects and bias.

Cardiovascular Disease

CVD is the leading cause of the global morbidity and mortality. Around 17.3 million deaths occurred in 2008 due to CVD accounting for 30% of the total global deaths (56 million).[357] CVD includes heart diseases, vascular diseases of the brain, and diseases of blood vessels. CVD can occur due to atherosclerotic disease as CHD, cerebrovascular disease, diseases of the aorta and peripheral vascular disease. Non-atherosclerotic causes of CVD include congenital heart disease, rheumatic heart disease, cardiomyopathies and cardiac arrhythmias. Of the 17.3 global CVD deaths, 7.3 million were attributed to CHD and 6.2 million were attributed to strokes.[357] Therefore, the majority of CVD morbidity and mortality is linked to atherosclerotic events in the coronary, carotid or cerebral arteries. We have therefore, focused on atherosclerotic forms of CVD affecting the heart and brain.

Pathophysiology of Atherosclerosis

Atherosclerosis is a disease of gradual thickening of the walls of medium- and large-sized arteries from chronic inflammatory and fibroproliferative processes exacerbated by lipids leading to the formation of atherosclerotic plaques.[358] This narrowing will consequently lower the amount of blood supply to target organs leading to ischemic injuries most commonly in the heart and brain. However, atherosclerosis by itself is seldom fatal and mortality usually results from plaque rupture leading to thrombosis and obstruction of blood vessels manifesting usually as myocardial infarction (MI) or stroke.[359] Atherosclerotic plaques usually develop in areas with disturbed laminar blood flow and turbulence such as bifurcations and arterial branches.

Fatty streaks are considered precursors of atherosclerotic plaques and usually develop as early as childhood and adolescence and consist of foam cells that are macrophages laden with cholesterol.[360] These fatty streaks may later develop into more complex fibrous lesions consisting of lipid rich necrotic cores covered by fibrous caps of smooth muscle cells (SMCs) and extracellular matrix. These plaques may be further complicated with hemorrhages from rupture of angiogenic vessels from the media, calcification, or ulceration.[360]

Early endothelial dysfunction in lesion prone areas leads to subendothelial accumulation of apolipoprotein B (ApoB) lipoproteins.[358,361,362] Changes in the permeability of the endothelial layer accompanied with modifications in the extracellular matrix composition facilitate the entry and retention of these atherogenic lipoproteins, mainly low-density lipoprotein cholesterol (LDLC).[363] LDLC undergoes oxidation acquiring pro-inflammatory and cytotoxic properties, leading to the activation of endothelial cells and expression of adhesion molecules, mainly the vascular adhesion molecule-1 (VCAM-1) that facilitates the adhesion of primarily circulating monocytes and T-cell lymphocytes to a lower extent.[364,365] Transmigration of these leukocytes across the intimal endothelial layer is stimulated by chemokines that include oxidized LDLC and MCP-1 produced by endothelial cells, SMCs and macrophages.[358,364]

A critical step in the development of atherosclerosis is the differentiation of monocytes into macrophages induced by the macrophage colony-stimulating factor,[364] which also upregulates pattern recognition receptors of the innate immunity as scavenger receptors and toll-like receptors. Type A scavenger receptor (SRA) and a member of the type B scavenger receptors, CD36, mediate the uptake of oxidized LDLC.[366] The cholesterol derived from LDLC accumulates as cytosolic lipid droplets forming foam cells. Fatty streaks are formed by accumulation of foam cells.

A fibrous plaque is formed by proliferation of SMCs recruited from the media into the intima and secreting large amounts of a collagen rich matrix which increases the retention and accumulation of atherogenic lipoproteins.[360] On the other hand, the connective tissue produced by SMCs and formation of the fibrous cap is considered beneficial as it protects against rupture and thrombosis.[367] T-cell

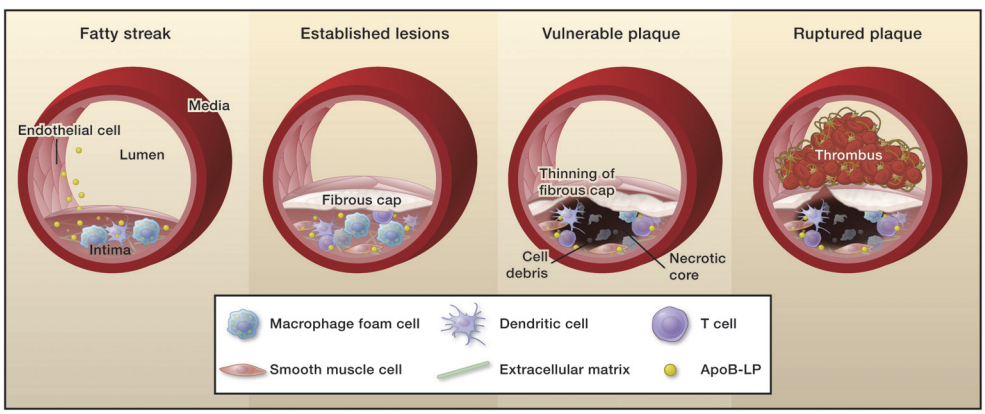

Figure 5. Atherosclerotic lesion progression
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lymphocytes play an important role in the progression of the atherosclerotic lesion. Antigens presented on macrophages and dendritic cells trigger the activation of T-cells mostly into type 1 helper T-cells that in turn secrete cytokines as interferon-γ which activates macrophages to augment the production of inflammatory cytokines as tumor necrosis factor and interleukin-1 leading to a perpetual state of chronic inflammation. To a lesser extent T-cells are activated into type 2 helper T-cells that secrete anti-inflammatory cytokines as interleukin-10 and transforming growth factor β and have anti-atherosclerotic properties.[364]

With the progression of atherosclerosis the uptake of lipids by macrophages continues until these cells die by apoptosis or necrosis. This leads to the release of cholesterol and cellular debris and formation of a lipid-rich necrotic core that increases the risk of rupture of the fibrous cap. Plaque rupture exposes thromobogenic material resulting in thrombus formation and occlusion of the lumen.[358,360] These high risk plaques are usually termed vulnerable plaques.

Atherosclerotic plaques can be further complicated by calcification, neovascularization, and remodeling of the artery. Calcification of plaques is common, increases with age, and acts as a marker for the severity of atherosclerotic disease.[368] Another common process that can complicate atherosclerotic plaques is angiogenesis of new vessels originating in the vasa vasorum of the adventitia and reaching the base of the plaque through the media. These new vessels are usually fragile which can lead to intraplaque hemorrhage. Neovascularization is a marker of disease progression into high risk vulnerable plaques.[358,369-371]

Changes in the vascular wall also occur during plaque development. Two types of vascular remodeling usually occur: expansive remodeling as a compensatory mechanism to preserve the lumen by outward expansion of the arterial wall, or constrictive remodeling when fibroproliferative processes predominate and inward growth of the arterial wall leading to narrowing of the lumen.[372,373] Coronary artery segments with high shear stress have been shown to have expansive remodeling with progression of necrotic core formation, calcification, and regression of the fibrous tissue. In contrast, segments with low shear stress have been shown to have constrictive remodeling with progression of the plaque and its necrotic core.[374]

Vulnerable plaques leading to acute coronary syndromes are usually associated with expansive remodeling while stable plaques leading to ischemia and stable angina are associated with constrictive remodeling.[358,375] Vulnerable plaques are also usually characterized by an elevated inflammatory activity with high concentration of macrophages and T lymphocytes leading to enlargement of the lipid core and thinning of the fibrous cap.[376,377] Increased levels of matrix metalloproteinases involved in collagen degradation and decreased concentration of SMCs are also common characteristics of vulnerable plaques.[378-380]

Clinical Consequences

Atherosclerosis affects mainly large- and medium-sized arteries and thus its ischemic effects may affect target organs as the heart, brain, kidneys and lower extremities. MI, stroke, aortic aneurysms, and peripheral vascular disease are the major manifestations of atherosclerosis.[381] Progressively stenotic atherosclerotic plaques usually lead to critical stenosis when they occlude more than 70 % of the artery. Critical stenosis leads to the development of cardiac ischemia manifesting as stable angina on modest exertion, chronic ischemic heart disease, ischemic encephalopathy, lower extremity ischemia manifesting as intermittent claudication and mesenteric ischemia.[381]

Acute Coronary Syndrome

Plaque rupture or erosion exposes thrombogenic material and ultimately leads to thrombus formation that may occlude the lumen either partially or completely. Acute changes in coronary plaques with absence of collateral perfusion results in the acute coronary syndrome (ACS) manifesting clinically as unstable angina, non-ST elevation MI (NSTEMI), ST elevation MI (STEMI), or sudden cardiac death.[382] Individuals with unstable angina usually present with angina at rest, new-onset severe angina, and/or angina with a crescendo pattern with increasing frequency, intensity and duration. Unstable angina and NSTEMI share similar pathophysiologic origins and clinical presentation. However, NSTEMI is generally more severe causing myocardial necrosis and elevation of cardiac-specific troponins and creatine kinase (CK-MB).[382,383] In the United States, around 60% of hospitalizations due to ACS are because of MI and around two thirds of MI cases have NSTEMI.[383]

Stroke

Strokes or cerebrovascular accidents are classified as either ischemic or hemorrhagic. In the United States, it is estimated that around 87% of strokes are due to ischemia, 10% are due to intracerebral hemorrhage and 3% are due to subarachnoid hemorrhage.[384] Acute ischemic strokes are generally caused by thrombotic or embolic occlusion of the carotid or cerebral arteries. Progressive and stable atherosclerotic disease may allow the development of collateral blood supply which could prevent stroke in the cases of complete occlusion. In the case of acute plaque change and thrombosis the extent of the damage is dependent on the collateral perfusion. If collateral perfusion allows marginal blood supply to the ischemic areas the neurologic damage becomes dependent on blood pressure and any drop in blood pressure may lead to stroke or transient ischemic attacks. In some cases detachment of thrombi from the plaque leads to *artery-to-artery embolization*. Emboli are more likely to reach distal ends with no collateral supply and are more likely to cause cerebral infarction. Around 20% of emboli are of

cardiac origin mainly from mural thrombi associated with infarcted myocardium. Other cardiogenic emboli result from vulvular heart disease, atrial fibrillation, and paradoxical emboli of venous origin in case of cardiac defects as patent foramen ovale and atrial septal defect.[385]

Epidemiology, Risk factors, and Prevention

CVD is the leading cause of death around the globe and it has accounted for more than 30% of deaths in the year 2008. Over 80% of these deaths occur in in low- and middle-income countries. Unhealthy lifestyle and behavioral factors as cigarette smoking, sedentary lifestyle, and increased caloric intake leading to obesity, dyslipidemia, hypertension, and diabetes are the major risk factors of CVD.[357]

Obesity and Lifestyle Factors

The obesity epidemic associated with a sedentary lifestyle and increased caloric intake has been shown to worsen most of CVD major risk factors including plasma lipids, blood pressure, glucose and inflammation.[386] Although obesity has been associated with CVD in most of observational studies, evidence from weight loss lifestyle intervention trials have been inconclusive. Weight loss intervention with sibutramine among individuals with CVD, diabetes or both associated with increased risk of non-fatal MI and stroke.[387] Another multicenter weight loss trial did not indicate any reduction in CVD event rate in the intervention group after 9.6 years of follow-up.[388] However, a recent report from the Da Qing Prevention Study among individuals with IGT indicated that individuals in the weight loss intervention group experienced lower cardiovascular mortality compared to controls.[389] In the past decades several studies have observed an "obesity paradox" where obese individuals with CVD have had better prognosis than lean individuals.[386] Higher levels of physical activity have been previously associated with lower CVD risk in large meta-analyses of prospective studies.[390] Mendelian randomization studies have also provided conflicting results for the causal nature of the association between obesity and CVD.[130,131,391]

Dietary factors

Several dietary factors have been reported to associate with CVD. High glycemic index and glycemic load diets have been previously associated several CVD risk factors including BMI, blood pressure, dyslipidemia, glycemic traits and diabetes.[392] High Glycemic index and glycemic load diets have also been associated with increased risk of CHD in systematic reviews and meta-analyses of

published studies.[392,393] Higher total fiber intake has been associated with lower risk of CHD and total CVD with around 9% risk reduction with each additional 7g/day of total fiber.[393,394]

The current dietary recommendations that support a high intake of omega-3 PUFA and low intake of SFA have been challenged by a recent systematic review and meta-analysis.[395] In the meta-analysis of observational studies neither SFAs, mono-unsaturated fatty acids (MUFA) nor omega-6 PUFA showed association with the risk of CHD. Higher consumption of seafood-derived long-chain PUFAs was associated with lower risk of CHD. Furthermore, the meta-analysis of RCTs did not support an association between omega-3 PUFA (both plant and seafood derived) and omega-6 PUFA supplementations and the risk of CHD.[395] Higher intakes of *trans*-fatty acids have been consistently associated with elevated risk of CHD.[393,395]

Higher consumption of vegetables and fruits have been associated with lower risk of CHD, stroke and CVD mortality with green leafy vegetable showing the strongest association.[393,396-399] Although this was initially attributed to vitamins and antioxidants in vegetables and fruits, a recent systematic review and meta-analysis of RCTs reported no evidence that supports the use of vitamin and antioxidant supplements for the prevention of CVD.[400] In addition, higher intakes of foods rich in whole grains, fish and nuts have been associated with lower risk of CHD.[393] Moderate consumption of alcohol and wine has been linked to favorable effects on CVD. A large systematic review and meta-analysis reported lower risk of multiple CVD outcomes with moderate consumption of alcohol, while high consumption was associated with increased stroke incidence and CVD related mortality, consistent with U- or J-shaped curves.[401] Strong evidence also exists for a more pronounced cardio-protective effect of wine intake compared with other alcoholic beverages. [402,403] In addition, lower salt intake has been linked to reduced blood pressure and lower risk of CVD.[404]

Strong evidence from RCTs supports the association between a Mediterranean dietary pattern and a lower risk of CVD.[405,406] In addition, previous evidence indicates elevated CVD risk with a Western dietary pattern and decreased risk with high-quality and prudent dietary patterns.[393,406]

Cigarette smoking

Cigarette smoking is highly prevalent around the globe with around 1 billion smokers and it has been consistently associated with elevated risk of CVD and CVD related mortality.[357] Even passive smoking has been linked with approximately 30% increased risk of CVD compared with 80% increased risk among active smokers.[407,408] The exact mechanisms by which smoking increases the risk of CVD remain unclear. However, studies have implicated smoking in

vasomotor dysfunction, free radical-mediated oxidative stress, atherogenesis, and thrombosis.[409]

Dyslipidemia

Dyslipidemia is defined as elevations in total cholesterol (TC), LDLC, TG or decrease in HDLC and it is associated with higher risk of atherosclerotic disease. Evidence from prospective observational studies have consistently shown higher risk of CVD and CVD mortality associated with elevated levels of LDLC.[410] Monogenic diseases associated with elevated plasma levels of LDLC increase the risk of CVD. Familial hypercholesterolemia is a monogenic disorder that results from mutations in the hepatic LDLC receptor (*LDLR*) gene. Individuals heterozygous for mutations in the *LDLR* who have 2 to 3 times higher LDLC levels as compared to control subjects develop CVD early in their 30s and 40s while rare homozygotes with 6 to 10 times higher levels develop CVD in their adolescence and early adulthood.[411,412] Meta-analyses of RCTs have consistently shown lower risk of CVD endpoints with statin therapy.[413,414] In addition, recent evidence from the IMPROVE-IT trial support additional benefits of LDLC lowering on CVD outcomes by ezetimibe on top of statin therapy.[415] Mendelian randomization studies have indicated that genetically lower LDLC levels lead to a 3-fold greater reduction in CHD risk as compared to the same level of LDLC reduction by statins.[416] This suggests that LDLC reduction beginning early in life is associated with much lower CHD risk as compared with reduction beginning later in life.

Early observational studies reporting inverse association between HDLC and CHD have led to the concept of reverse cholesterol transport through which high HDLC might protect against CHD.[417-419] This hypothesis was supported by results from animal studies which demonstrated protection against atherosclerosis through infusion of HDLC or overexpression of apolipoprotein A-I (ApoA-I).[419-421] The strong inverse association between plasma levels of HDLC and several CVD outcomes support the predictive value of HDLC.[422,423] However, the causal nature of this association has been challenged by RCTs and genetic studies. Monogenic disorders associated with low HDLC as those caused by rare mutations in the *APOA-I*, ATP Binding Cassette A1 (*ABCA1*) and lecithin cholesteryl acyl transferase (*LCAT*) genes have shown inconsistent associations with CVD.[419] In addition, recent Mendelian randomization studies reported no causal association between HDLC and CHD.[424-426] The lack of causal association has also been supported by data from RCTs that showed no effect of HDLC raising drugs as fibrates, niacin, and cholesteryl ester transfer protein (CETP) inhibitors on CVD outcomes.[427,428]

Elevated TG levels have been consistently associated with higher risk of CVD.[429,430] This association has been reported to be independent of HDLC which levels are highly inversely correlated with TG levels.[431] The cholesterol component of TG rich lipoproteins (remnant cholesterol) have been suggested to be the main cause of association between TG and atherosclerotic CVD.[432] This has been based on observations from monogenic forms of hypertriglyceridemia as many individuals with familial chylomicronemia syndrome do not develop CVD.[432] This has also been supported by a large meta-analysis of prospective studies where no association between TG and CVD was observed after adjustment for HDLC and non-HDLC.[432,433] Mendelian randomization studies have suggested causal associations between remnant cholesterol and CHD.[434] Moreover, a recent Mendelian randomization study using a total of 185 lipid and lipoprotein SNPs from GWASs reported a causal association between TG and CHD after correcting for pleiotropic associations of these SNPs on LDLC and HDLC.[425]

Hypertension

Hypertension is one of the most prevalent conditions worldwide with prevalence of 30-50%.[407,435] The American Heart Association estimates that around 75 million individuals suffer from hypertension in the United States.[436] The Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC 7) classifies blood pressure into four categories: Normal (SBP < 120 mmHg and/or DBP < 80 mmHg), prehypertension (SBP 120-139 and/or DBP 80-90), stage 1 hypertension (SBP 140-159 and/or DBP 90-99) and stage 2 hypertension (SBP $\geq$ 160 and/or DBP $\geq$ 100).[437] Prospective studies have consistently shown increased risk of CVD and CVD related mortality associated with elevated blood pressure.[438] Each 20 mmHg increase in SBP or 10 mmHg increase in DBP was associated with a 2-fold increased risk of CVD. In a recent meta-analysis, prehypertension was associated with increased risk of stroke reaching 95% among individuals in the high range of prehypertension (SBP 130-139 and/or DBP 85-90).[439] Evidence for the causal nature of this association comes from RCTs of blood pressure lowering treatments.[440,441]

Diabetes Mellitus and the Metabolic Syndrome

Diabetes mellitus has been recognized as a major risk factor for CVD. Individuals with type 1 or type 2 diabetes are at a 2- to 3-fold increased risk of CVD events and around 60% of individuals with diabetes die from CVD related causes.[357,442,443] In addition, prediabetic states of IFG and IGT have shown increased risk of CVD.[357,444] Glycemic abnormalities tend to cluster with other CVD risk factors as obesity, dyslipidemia, and hypertension leading to the metabolic syndrome.[445,446]

In a meta-analysis of prospective studies the metabolic syndrome was associated with a 2-fold increased risk of CVD.[447] Moreover, whether hyperglycemia is the causal factor in developing CVD is still under discussion. The causality of hyperglycemia and CVD has been challenged in the Diabetes Control and Complications Trial (DCCT) which failed to observe reduction of CVD events after intensive treatment in patients with type 1 diabetes.[448] On the other hand, intensive glucose control has been associated with reduced risk of CVD but not with CVD related mortality in a meta-analysis of 5 RCTs.[449]

Inflammation

Inflammation plays an important role in the pathogenesis of atherosclerotic CVD. Several markers of inflammation have been associated with CVD.[450] Earlier studies have reported increased CVD risk with elevated WBC count and fibrinogen.[451-453] Additionally, newer inflammatory markers as C-reactive protein (CRP), IL-6 and serum amyloid A (SAA) have been consistently associated with increased risk of CVD outcomes independent of other risk factors.[450] A Mendelian randomization study using *CRP* gene variants did not provide evidence for a causal role of CRP in CHD.[454] In contrast, another Mendelian randomization study using genetic variants in the IL-6 receptor gene (*IL6R*), an upstream regulator of CRP, indicated a causal association of IL-6 receptor with CHD. This study suggests that blocking the IL-6 receptor could be a novel therapeutic approach to prevent CHD.[455]

Genetics of CVD

Family history is an important risk factor for CVD. The Framingham Study cohort has reported that parental history of death due to CHD increases the risk of CHD in the offspring independent of other risk factors and this risk factor is more important in individuals with premature CHD (age < 60 years).[456] Premature CVD in at least one parent was associated with a 2.6-fold increased risk of CVD in men and 2.2 fold increased risk in women.[457,458] The risk increase associated with family history was attenuated but remained significant after adjusting for traditional risk factors indicating that part of the heritability of CVD can be attributed to the heritability of its risk factors as dyslipidemia, hypertension and T2D.[458] In a Swedish twin study, premature CHD death in one of the twins was associated with a 3.8-fold increased risk of CHD mortality among dizygotic male twins and an 8.1-fold increased risk among monozygotic male twins; while in females there was a 2.5-fold increased risk among dizygotic twins and a 15-fold increased risk among monozygotic twins.[459] These findings highlight the

importance of the genetic component in CHD. The heritability of CHD is estimated to be in the range of 40-60%.[460,461] The heritability estimates for different lipid traits range from 28% to 78% and for blood pressure from 50 to 70%.[462,463] Studies of family history in stroke have been more challenging due to the heterogeneity of this disease even in its ischemic forms. Hypertension is the most important risk factor for stroke and it has been estimated that around a quarter to a third of stroke heritability can be attributed to the heritability of hypertension.[463]

Linkage Studies

Early successes in identifying genes that influence the risk of CVD was mainly attributed to monogenic rare forms of dyslipidemia identified in linkage studies of families. Familial hypercholesterolemia represents one of the earliest forms of monogenic inheritance of elevated LDLC levels associated with increased risk of premature CHD. Autosomal dominant forms of this condition include mutations in the hepatic LDLC receptor gene (*LDLR*),[464] ApoB gene (*APOB*),[465] and the proprotein convertase subtilisin/kexin type 9 gene (*PCSK9*).[466] Other mutations in the *PCSK9*, *APOB*, and angiopoietin-like 3 (*ANGPTL3*) genes have been linked to familial hypobetalipoproteinemia with low LDLC levels and low risk of CHD.[465,467,468] An autosomal recessive form of familial hypercholesterolemia has been identified and linked to mutations in the low-density lipoprotein receptor adapter protein 1 (*LDLRAP1*).[469] Another monogenic form of dyslipidemia is sitosterolemia associated with elevated levels of circulating plant sterols and cholesterol and higher risk of CHD. This disorder results from mutations in the adenosine triphosphate (ATP)-binding cassette (ABC) transporter genes (*ABCG5* and *ABCG8*).[470] Other monogenic forms of dyslipidemia exist but their link to CVD is not well established due to rarity of these mutations.[457] Like most other complex traits and diseases linkage studies were largely unsuccessful in identifying common variants for CVD. Although few loci have been found to associate with CHD and stroke in genome-wide linkage scans, these signals were not confirmed in replication studies nor GWASs.[457,471]

Candidate Gene Studies

The first study to successfully discover a CVD susceptibility gene using the candidate gene approach reported an association between a deletion in the angiotensin converting enzyme (*ACE*) gene, previously associated with elevated circulating levels of ACE, and MI.[472] However, a meta-analysis of later studies showed no association between this deletion and the risk of MI. Another example was the discovery of the association between variants in the apolipoprotein E (*APOE*) gene and CHD.[473] Already earlier *APOE* had been discovered to be polymorphic with three genetically determined isoforms (ε2, ε3, and ε4).[474] Most of the subsequent candidate gene studies findings were inconsistent and not reliably replicated in large independent samples.

Genome-Wide Association Studies

In 2007 the first GWASs for CHD were published. Three independent studies in European populations identified a signal in the same locus on chromosome 9p21 which remains the strongest in association with CVD.[475-477] Thereafter, the Coronary Artery Disease Genome-Wide Replication and Meta-Analysis (CARDIoGRAM) consortium was formed and later on the CARDIoGRAMplusC4D.[478-480] These large meta-analyses have identified more than 50 variants that are associated with CHD explaining approximately 10% of the heritability of the disease. Most of these variants exert their risk independent of traditional risk factors of CHD.[481]

Separate GWASs have been conducted on CVD risk factors which are themselves complex in nature and heritable. Compared with CHD, there have been few GWASs for stroke mainly due to the heterogeneity of the disease and the need for subtyping of ischemic stroke endpoints. Two loci that have been previously linked to atrial fibrillation have been also linked to cardio-embolic stroke but not with other subtypes of ischemic stroke.[482-484] The chromosome 9p21 variant has been associated with atherosclerotic ischemic stroke but not with other subtypes.[485] The Global Lipids Genetics Consortium (GLGC) has so far identified 185 variants in 157 loci to be associated with plasma levels of lipid traits.[486] A total of 54 variants were associated with TC levels, 37 with LDLC levels, 55 with HDLC levels and 24 with TG levels. Additionally, other consortia have identified more than 30 blood pressure associated loci.[487-489]

Chromosome 9p21

Variants on chromosome 9p21were the first to be discovered to be associated with CHD in GWASs.[475-477] These variants have also been associated with atherosclerotic stoke, abdominal and intracranial aneurysms, and periodontitis.[485,490,491] Approximately 25% of the European population carry both risk alleles and have around 50% increased risk of CHD and a 2-fold increased risk of premature CHD.[475-477] However, the risk increase by this locus has been found to be independent of all conventional risk factors such as dyslipidemia, diabetes, hypertension, or inflammation.[476,492] The mechanisms by which this locus confers its risk remains poorly understood.

Fine mapping have demonstrated that the risk locus is confined to a 58 kilo base pair (kb) LD block that lacks any protein-coding genes and can be tagged by the rs4977574 SNP.[493] The antisense noncoding RNA in the INK locus (*ANRIL*) (also known as *CDKN2BAS*) has been mapped to the risk interval.[494,495] ANRIL has been shown to induce the expression of three cyclin-dependent kinase inhibitors which include CDKN2A and CDKN2B that inhibit cell cycle.[495] The association of the 9p21 variant with down-regulation of ANRIL expression and subsequent down-regulation of cyclin-dependent kinase inhibitors has been hypothesized to lead to SMC proliferation and apoptosis and thus weakening of vessel walls and

development of atherosclerosis and aneurysms. However, studies testing this hypothesis have so far reported conflicting results.[481] A previous study reported that two SNPs in the 58-kb LD block are located in an enhancer region and that the risk alleles disrupt the binding of STAT1 to the enhancer sequence.[496] This enhancer region was observed to physically interact with the *CDKN2A/B* locus and the *IFNA21* gene which encodes interferon α21.[496] Moreover, the same study reported chromatin structure alteration by interferon-γ on the 9p21 locus including the STAT1 binding site and alteration in the expression of *ANRIL* and *CDKN2B* in vascular endothelial cells. This study led to the hypothesis that the 9p21 variant could exert its effects through modulation of interferons. However, later studies have shown that the effects of these interferons either on the expression of *CDKN2A/B* or on the risk of CHD are independent of the 9p21 variant.[497,498]

Gene-Lifestyle Interactions

Before the advent of GWAS, gene-lifestyle interactions in CVD have focused on candidate genes and lifestyle factors that possibly share a common biological pathway with these genes. One of the early findings was the reported interaction between *APOE* polymorphisms and smoking on the risk of CVD among middle-aged men.[499,500] The magnitude of risk elevation of CHD by smoking was largest among individuals carrying ε4 genotype and the risk by the ε4 genotype was restricted to smokers. However, a recent meta-analysis of 13 studies did not indicate a significant interaction between smoking and *APOE* polymorphisms on the risk CHD.[501]

Of the GWAS findings, the chromosome 9p21 locus is the strongest and most validated CVD susceptibility locus to date. Variation in this locus has been previously tested for interactions with several lifestyle factors including dietary patterns, physical activity and smoking in case-control and cohort studies.[502] The risk of CHD by the 9p21 risk allele was attenuated among individuals with a high prudent diet score. This interaction was mainly driven by the raw vegetable component of the prudent dietary pattern. This study, however, reported no interactions with physical activity and smoking. In a more recent study, we examined interactions between a variant in the 9p21 locus and smoking on the risk of CHD.[503] In this study, we observe that the risk of CHD by the 9p21 variant was attenuated among smokers.

Aims

General Aims

This thesis generally aims to understand how the strongest genetic variants for T2D and CVD may modify the association of lifestyle factors and particularly dietary factors with T2D and CVD. It also aims to understand the causal nature of traditional cardio-metabolic traits in T2D and CHD using Mendelian randomization.

Specific Aims

Study I: To test if dietary intakes of carbohydrates, fats, proteins, or fibers modify the risk of T2D associated with *TCF7L2* rs7903146 genetic variant

Study II: To annotate T2D loci into the WNT signaling pathway and test if known genetic variants in annotated loci interact with dietary fiber intake on the risk of T2D

Study III: To test if *TCF7L2* rs7903146 genetic variant modifies the association between dietary fiber intake and the metabolic syndrome and its associated traits

Study IV: To test if dietary vegetable, fruit, wine, or alcohol intake modify the associated effect of the chromosome 9p21 rs4977574 genetic variant and the risk for CVD

Study V: To understand the causal nature of BMI, SBP, LDLC, HDLC, TG and FPG associations with T2D and CHD using trait-specific GRSs

Methods

Malmö Diet and Cancer Study

The Malmö Diet and Cancer Study (MDCS) is a population-based prospective cohort study in the city of Malmö, Sweden. The main aim of this study is to assess the impact of diet on cancer incidence and mortality.[504] The study recruited individuals living in Malmö born between 1926 and 1945 from March 1991 until May 1995. The recruitment was extended to October 1996 to include men born between 1923 and 1945 and women born between 1923 and 1950. The reason for including more women of younger age than men was to be able to study breast cancer in premenopausal women.[505] Eligibility was determined on the basis of adequate Swedish reading and writing skills and no history of mental disability. A source population of 74,138 individuals were invited through public advertisements or personal letters. The study included at total of 30,447 individuals with a participation rate of 41%. The difference between participants and non-participants was previously assessed and participants had lower mortality compared to non-participants pointing to a possible selection bias for individuals with more conscious health behavior.[505] All participants provided informed consent. The study was approved by the Ethical Committee at Lund University (LU 51-90).

At baseline the study participants were invited to visit the screening center twice. During the first visit, nurses carried out anthropometric assessment, measured blood pressure and withdrew non-fasting blood samples for storage. Participant were also instructed on how to fill a diet questionnaire and an extensive questionnaire covering socioeconomic and lifestyle factors. Approximately 10 days later participants returned for the second visit and underwent a diet history interview and the questionnaires completed at home were reviewed. By the end of the baseline examination period, 28,098 individuals (11,063 men and 17,035 women) had complete dietary, anthropometric, and lifestyle data.

Malmö Diet and Cancer Cardiovascular Cohort

A random 50% of MDCS were invited to participate in a sub-cohort study for the epidemiology of the carotid artery disease between October 1991 and February

1994. A total of 6,103 participants were included and formed the Malmö Diet and Cancer-Cardiovascular Cohort (MDC-CC). These individuals underwent additional fasting blood measurements including fasting glucose and fasting lipids and lipoproteins.[506] From the MDC-CC cohort, 4,924 individuals, who were still alive and had not emigrated, were invited for re-investigation between the years 2007and 2012 of whom 3,734 individuals attended.

Dietary Assessment

A modified diet history methodology was specifically developed for the MDCS. This assessment method consisted of a 7-day menubook, a 168-item diet questionnaire and a 45-60 minute diet history interview. Participants were asked to record cooked meals, cold beverages, dietary supplements, natural remedies and medicinal drugs. Meal patterns, frequency and portion sizes of regularly consumed foods in the preceding year were recorded in a diet history questionnaire with the aid of a 48-page booklet of pictures. This methodology choice was mainly directed by the need to assess total diet in a middle-aged urban population on a typical Western diet patterns which is rich in fats and low in vegetable and fiber intake. Trained interviewers asked participants to describe their meal patterns and food choices. They were also asked to describe in detail the preparation procedures and ingredients of the foods recoded in the menubook. The interviewers used an extensive book of photographs to help participants estimate the usual portion sizes of foods and dishes in the menubook. The interviewers also checked that the reported food intake did not overlap between the questionnaire and menubook according to predefined rules. All information from the menubook, questionnaire, and interview was coded and entered into a data system. The average daily consumption of food groups (grams per day) was summarized for each individual and then converted into energy and nutrient intakes using the Malmö Diet and Cancer Food and Nutrient Database which was designed specifically for MDCS and was derived from PC KOST2-93 of the Swedish National Food Administration.[507,508]

The dietary data collection routines were slightly modified in September 1994. These modifications aimed at simplifying data collection and making interviews faster without substantial impact on the ranking of individuals or mean levels of nutrient intakes. Although reported energy intakes were lower and energy-adjusted fat intakes were higher after these modifications, the impact on the ranking of individuals was small.[508]

To identify individuals potentially reporting inaccurate energy intakes, the physical activity level was calculated from self-reported information on leisure time physical activity, physical activity at work and household work, and from estimates of sleeping duration. Individuals with potentially inaccurate reporting of

energy were defined as those with a total energy intake to basal metabolic rate ratio outside the 95% confidence interval limits of the calculated physical activity level. Around 12% of men and 18% of women were classified as under-reporters of energy intake and 3.5% of men and 2.8% of women were classified as over-reporters of energy intake.[509] Additionally, one questionnaire item was used to identify individuals with a change in their dietary habits in the past due to illness or other factors.

Dietary Variables

In Study I, macronutrient and fiber intakes were used. Macronutrient intakes included carbohydrate, fat, and protein intakes and were studied as percentages of non-alcohol total energy intake. The conversion factors used for carbohydrates, fats, and proteins were 4 kcal/g, 9 kcal/g, and 4 kcal/g respectively. Carbohydrates included monosaccharides, disaccharides, and starch but not fiber. Fats included saturated-, monounsaturated- and polyunsaturated fats, and different fatty acids and cholesterol. Fiber intake (Study I, II, and III) included all types of fiber, however data on specific fiber subtypes was not available.

In study IV, vegetables, fruit, wine, and total alcohol intake were used as the dietary exposure variables. The vegetable intake variable included all raw, dried, and cooked vegetables. The fruit intake variable included citrus and non-citrus fruits, berries, and dried fruits. The weights of dried fruits were corrected for their lower water content. The wine variable included red-, white-, and fortified wines. Vegetable, fruit, wine, and total alcohol intakes were estimated as grams per day.

Validation

The validity of the dietary assessment method of MDCS was previously studied in 241 individuals aged 50-69 years. The correlation coefficients of energy and nutrients were very high as compared to studies in other populations. Comparisons were done between the MDCS modified diet history method and 18-day weighed food records collected during one year as a reference. Pearson's correlation coefficients of energy adjusted nutrient and food group intakes were in men and women separately as follows: carbohydrates (0.66; 0.70), fats (0.64; 0.69), proteins (0.54; 0.53), fiber (0.74; 0.69); vegetables (0.65; 0.53); fruits (0.60; 0.77), wine (0.53; 0.63) and total alcohol (0.80; 0.78).

Clinical Measurements

Blood pressure measurement was performed using a mercury-column sphygmomanometer in the supine position after ten minutes of rest. Body weight (kilograms) was measured using a balance-beam scale with subjects wearing light clothing and no shoes. Height (centimeters) was measured using a fixed

stadiometer. BMI was calculated as the ratio of weight in kilograms to height in meters squared. WC (centimeters) was measured midway between the lowest rib margin and iliac crest. A bioelectric impedance analyzer (BIA 103; JRL Systems, Mt. Clemens, MI) was used to estimate body composition. Body fat percentage was calculated using an algorithm provided by the manufacturer.

Fasting blood measurements were only available in MDC-CC. Samples were analyzed by routine standard methods at the Department of Clinical Chemistry, Malmö University Hospital. Fasting blood glucose (FBG) was measured using a routine hexokinase method and was converted to FPG by multiplying the values by 1.13. Fasting plasma insulin (FPI) was determined by a non-specific radioimmunoassay method. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the formula: (FPG × FPI)/22.5. TG and TC measurements were done using a DAX 48 automatic analyzer (Bayer AB, Göteborg, Sweden). Similarly, HDLC was measured after precipitation of LDLC and very low-density lipoprotein cholesterol (VLDL) with dextran sulphate. LDLC was calculated using the Friedewald formula.[510] The levels of ApoA-I ApoB were measured in non-fasted plasma samples of the entire MDCS by Quest Diagnostics (San Juan Capistrano, CA, USA), blinded to case-control status, using an immunonephelometric assay run on the Siemens BNII (Siemens, Newark, DE, USA). The inter-assay variability was < 4.0% for both ApoA-I and ApoB.

Genetic Variants and Genotyping

Non-fasting blood collected at the baseline was used for DNA extraction in MDCS. DNA extraction from frozen granulocyte or buffy coat samples from blood was done using the QIAamp96 spin blood kits (QIAGEN, VWR, Gaithersburg, MD, USA).

TCF7L2 rs7903146 and Chromosome 9p21 rs4977574

Genotyping of the *TCF7L2* rs7903146 SNP was performed using the TaqMan PCR method (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Post-PCR allelic discrimination was determined by the ABI Prism Sequence Detection System ABI 7900HT (Applied Biosystems) by measuring allele-specific fluorescence. As a quality control, the genotyping success rate was more than 96%, the concordance rate in 325 randomly repeated samples was more than 99%, and the genotypes were in Hardy–Weinberg equilibrium ($P = 0.16$). The chromosome 9p21 rs4977574 was genotyped using the same method. The concordance rate in a randomly selected sample (20% of MDCS) was more than 99.9% and the genotypes were in Hardy–Weinberg equilibrium ($P = 0.18$).

T2D and Cardio-Metabolic Genetic Variants

Previously reported T2D and cardio-metabolic traits associated SNPs were genotyped in the entire MDCS. The main method used for genotyping was the Sequenom iPLEX using a MALDI-TOF mass spectrometer (Sequenom MassArray, Sequenom, San Diego, CA, USA) and Sequenom reagents and protocols. When the commercial primers were not available, proxy SNPs were identified using SNAP version 2.2.2 (http://www.broadinstitute.org/mpg/snap/). TaqMan or KASPar allelic discrimination on an ABI 7900HT (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) were used to genotype SNPs individually when they have failed to be genotyped successfully using Sequenom. We excluded individuals with < 60% successfully genotyped SNPs as marker of bad DNA quality. SNPs were excluded when they had a genotype success rate of < 90% or when they deviated from Bonferroni-corrected Hardy-Weinberg Equilibrium in each set of SNPs of the specific traits. The concordance rate was > 99% in 5,500 samples which were additionally genotyped using Human Omni Express Exome Bead Chip Kit (Illumina, San Diego, CA, USA). A total of 58 T2D (Study II), 31 BMI, 29 SBP, 32 LDLC, 41 HDLC, 26 TG. And 15 FPG SNPs (Study V) were included.[59,60,282,511-515]

Ascertainment of Diabetes Cases

Follow up of the MDCS was regularly performed by record linkage with national registries. Both prevalent and incident diabetes cases were identified using different national and regional diabetes registers: 1) the nationwide Swedish National Diabetes Register;[516] 2) the regional Diabetes 2000 register of the Scania region, of which Malmö is the largest city;[517] 3) the Swedish National Patient Register, which is a principal source of data for numerous research projects and which covers more than 99% of all somatic and psychiatric hospital discharges and Swedish hospital-based outpatient care.[518] In addition, individuals were classified as diabetic if: 1) the cause of death was registered as diabetes in the Swedish Cause-of-Death Register, which includes all deaths in Swedish residents occurring in Sweden or abroad;[519] 2) anti-diabetic medication was prescribed according to the Swedish Prescribed Drug Register;[520] 3) they had at least two HbA_{1C} recordings ≥ 6.0% using the Swedish Mono-S standardization system (corresponding to 7.0% according to the US NGSP) in the Malmö HbA_{1C} register, which analyzed and catalogued all HbA_{1C} samples at the Department of Clinical Chemistry taken in institutional and non-institutional care in the greater Malmö area from 1988 onwards.

Self-reports of a physician diagnosis or the use of diabetes medication in the baseline questionnaire were used to identify diabetes cases at the baseline examination. In addition, in MDC-CC, an FBG concentration of ≥ 6.1 mmol/L

(corresponding to FPG concentration of ≥ 7.0 mmol/L) was used to identify diabetes cases. Furthermore, self-reports of a physician diagnosis, the use of diabetes medication based on a questionnaire, FPG of ≥ 7.0 mmol/L or a 2-hour plasma glucose post-OGTT of > 11.0 mmol/L were used to identify cases of diabetes among the 3,734 individuals from MDC-CC who underwent reinvestigation.

Ascertainment of Cardiovascular Disease Cases

Individuals with CVD were classified as those with CHD (defined as fatal or nonfatal MI or death due to ischemic heart disease) or individuals with fatal or non-fatal stroke using three registers: the Swedish Hospital Discharge Register, the Swedish Cause of Death Register, and the Stroke Register of Malmö. The identification of these register end-points has been described and validated elsewhere.[521-523] MI cases were defined using codes 410 and I21 of the 9th and 10th revisions of the *International Classification of Diseases* (ICD9 and ICD10), death due to ischemic heart disease was defined based on the codes 412 and 414 of the ICD9 or codes I22-I23 and I25 of the ICD10, and the stroke cases were defined using codes 430, 431, 434, and 436 of the ICD9, and codes I60, I61, I63, and I64 of the ICD10.

Other Variables

An extensive lifestyle questionnaire adapted from the Minnesota Leisure Time Physical Activity Questionnaire was used to assess leisure-time physical activity in the MDCS. The number of minutes spent on performing each of 17 different types of physical activity each week was estimated by each participant. A leisure-time physical activity score was created by multiplying the duration of each physical activity by an intensity factor. A smoking variable was created by classifying individuals as current smokers, former-smokers and never-smokers. A variable was created classifying individuals into four categories based on sex-specific grams of total alcohol consumed per day: abstainers defined as those reporting zero consumption of alcohol in the menu book, and indicating no consumption of alcohol in the previous year in the questionnaire , low consumption (< 15 g/day in women or < 20 g/day in men), medium consumption (15–30 g/day in women or 20–40 g/day in men) or high consumption (> 30 g/day in women or > 40 g/day in men). Categorization of alcohol intake was based on an assumption of biological risk.[524] An education variable was created by classifying participants into four categories based on their highest educational level: elementary education, primary and secondary education, upper secondary education, and further education with or without university degree. To correct for

changes in dietary data collection over time dietary assessment method and season variables were created.

Study Specific Methods and Statistics

Macronutrients, *TCF7L2*, and T2D (Study I)

In study I we included the whole MDCS cohort (*n* =28,098) with complete dietary data. After excluding individuals with prevalent diabetes and those with missing DNA or genotype data we ended with a study population of 24,799 (9,789 men and 15,010 women). Over a mean follow-up period of 11.8 years (until December 2006), 1,649 individuals were ascertained as incident T2D cases. In addition, we performed cross-sectional analyses in the MDC-CC (*n* =5,216) with FPG and HbA_{1C} as outcome variables.

The main exposure variables were the *TCF7L2* rs7903146 (C/T) SNP, which was coded as 0,1 and 2 based on the number of risk T-alleles, and dietary intakes of carbohydrates, fats, and proteins expressed as percentages of total non-alcohol energy intake, and fiber intake expressed as grams per 1000 kcal. Logistic regression was first used to study the additive per T-allele odds ratio (OR) of incident T2D adjusting for age, sex and BMI. The additional adjustment for BMI was motivated by the association observed between the T-allele and lower BMI in some studies.[286] The association analyses between the T-allele and risk of T2D was done in quintiles of the dietary exposures. Interaction analyses were performed by including the *TCF7L2* genotype variable, the dietary quintiles variable and their multiplicative term in the same model. To correct for potential confounding bias we performed adjustments by including age, sex, BMI, total energy intake, dietary assessment method, and season of dietary data collection in the model. In sensitivity analyses, we first excluded individuals potentially reporting inaccurate energy intake and in the second analyses we further excluded those reporting changes in the dietary habits in the past.

After observing interaction between fiber intake and the *TCF7L2* variant on T2D we performed additional analyses. We first used logistic regression to obtain OR of incident T2D in each quintile of fiber intake in strata of the different *TCF7L2* genotypes (CC, *n* = 13,571; CT, *n* =9,488; TT, *n* = 1,740) using the first quintile as a reference (OR = 1) with similar adjustments as described above for interaction analyses. In MDC-CC, we performed linear regression to obtain the effect sizes per T-allele on baseline levels of FPG and HbA_{1C} adjusted for age, sex, and BMI. Interaction analyses between *TCF7L2* genotype and fiber intake quintiles on

baseline levels of FPG and HbA_{1C} were done using linear regression using the same model described for T2D.

Further adjustments for leisure-time physical activity, total alcohol intake, smoking habits and level of education were performed.

Dietary Fiber, WNT Signaling, and T2D (Study II)

Gene annotation

In Study I we observed interaction between fiber intake and the *TCF7L2* variant on the risk of T2D. The major role played by TCF7L2 in the WNT signaling pathway led us to the hypothesis that other T2D genes involved in this pathway may interact with fiber intake potentially highlighting a role of dietary fibers in this pathway.

A total of 58 T2D associated SNPs in 51 unique gene loci were genotyped in the entire MDCS. Using a combination of annotation from Gene Ontology,[525] the Kioto Encyclopedia of Genes and Genomes (www.kegg.jp/kegg/), Biocarta pathways (www.biocarta.com/genes/index.asp), Panther pathways[526,527] and Literature integrated within the DAVID Annotation Bioinformatics Resource (david.abcc.ncifcrf.gov) we analyzed links to the WNT pathway for the 51 genes.

Design and analysis

In study II we included participants from MDCS (n = 26,930) and MDC-CC (n = 5,507) after excluding prevalent cases of diabetes. Over a mean follow up period of 14.3 years (until December 2009) a total of 2,860 incident cases of T2D were registered. Cox regression was used to obtain the hazard ratio (HR) per quintile of fiber intake for the risk of T2D in MDCS. Linear regression analyses were performed to obtain cross-sectional per fiber quintile effect sizes on the levels of BMI, FPG, FPI, HOMA-IR and HbA_{1C} in MDC-CC adjusting for age, sex, BMI, total energy intake, dietary assessment method, and season of dietary data collection.

The per risk-allele HR of T2D of SNPs in genes linked to the WNT signaling pathway were obtained in Cox regression models adjusted for age and sex. The per risk-allele effect size of the same SNPs on glycemic traits (FPG, FPI, and HbA_{1C}) were obtained using linear regression models adjusting for age and sex. Interaction analyses between the annotated SNPs and quintiles of fiber intake were performed in Cox regression models by including these variables and the their multiplicative terms in the same model adjusted for age, sex, BMI, total energy intake, dietary assessment method, and season of dietary data collection. In a secondary model, leisure-time physical activity, total alcohol intake, smoking habits and level of education were added to the interaction analyses as covariates.

Dietary Fiber, *TCF7L2*, and the Metabolic Syndrome (Study III)

Previous studies have shown that higher dietary fiber intake is also associated with favorable effects on insulin sensitivity, lipid profile, inflammatory markers and the metabolic syndome.[169-172] We included participants in the MDC-CC to test if *TCF7L2* variant modifies the associated effect of fiber on the metabolic syndrome and its components. We included individuals free from diabetes and CVD and with complete data on glycemic and lipid traits and *TCF7L2* rs7903146 genotypes. In this population (n = 4,606) we identified individuals with baseline metabolic syndrome using the NCEP-ATPIII criteria defined earlier (n = 1,269). To test if the *TCF7L2* risk T-allele associates with metabolic syndrome at baseline we used a logistic regression model adjusting for age and sex. The per fiber tertile OR of baseline metabolic syndrome was obtained using a logistic regression model adjusting for age, sex, total energy intake, dietary assessment method, season of dietary data collection, leisure-time physical activity, total alcohol intake, smoking habits and level of education. Linear regression was used to perform similar association analyses with baseline levels of WC, HDLC, TG, FPG, FPI, SBP, DBP, body fat percentage, BMI, TC, LDLC, and HOMA-IR. Tests for interactions were performed adjusting for age, sex, total energy intake, dietary assessment method, and season of dietary data collection, leisure-time physical activity, total alcohol intake, smoking habits and level of education. Finally, we performed similar analyses with incident metabolic syndrome (n = 755) in the MDC-CC reinvestigation sample of 2,337 individuals after excluding baseline individuals with diabetes, CVD and metabolic syndrome.

Vegetable and Wine Intakes, 9p21 and CVD (Study IV)

In study IV we included 23,949 individuals from the MDCS after excluding baseline cases of diabetes, CVD and those with missing DNA or genotype data. Over a mean follow-up period of 15 years (until December 2010) a total of 3,164 individuals developed CVD. The HR of incident CVD per risk G-allele of chromosome 9p21 rs4977574 was obtained using Cox regression adjusted for age and sex. Linear regression analyses were performed to obtain the cross-sectional per G-allele effect sizes on different cardio-metabolic traits with similar adjustments.

Vegetable and fruit intakes were ranked into tertiles. Wine non-consumers were defined as those reporting no consumption of wine in the menu book, and indicating no consumption of wine during the previous month in the questionnaire and categorized as a separate group and consumers were stratified into two groups using the median split. Cox regression and linear regression models were used to obtain per category associations of vegetables, fruits, wine and total alcohol intake with incident CVD and baseline levels of cardio-metabolic traits, respectively,

adjusting for age, sex, total energy intake, dietary assessment method, season of dietary data collection, leisure-time physical activity, total alcohol intake, smoking habits, level of education, BMI, SBP, use of lipid-lowering medication and use of anti-hypertensive medication. Using the same multivariable model interactions between rs4977574 and categories of vegetable, fruit, wine, and total alcohol intakes on incidence of CVD were tested.

Significant interactions on the risk of CVD were further explored in cross-sectional interaction analyses in MDC-CC (n = 4,828) on the baseline levels of glycemic, lipid and inflammatory traits with the same adjustments as described above. Additional interaction analyses were performed excluding BMI, SBP, and use of lipid-lowering or antihypertensive medication as covariates.

Cardio-Metabolic Traits, T2D and CHD (Study V)

In study V a total of 28,589 individuals from MDCS and 5,432 individuals from MDC-CC were included. In MDCS, 4,427 cases of T2D and 2,997 cases of CHD were registered; 3,257 and 2,428 of these were incident cases of T2DM and CHD, respectively, occurring during the follow-up period of 15.4 years. Cardio-metabolic traits (LDLC, HDLC, TG, BMI, SBP, and FPG) were natural log-transformed and converted to z-scores to achieve normality of distribution and comparability between the studied traits.

Using the PLINK software (version 1.07) we created weighted GRSs based on published SNP effect estimates for BMI, SBP, LDLC, HDLC, TG, and FPG. The association analyses between the weighted GRSs z-score and their respective traits were performed using linear regression adjusting for age and sex. When the outcome variable was LDLC, HDLC, or TG, cholesterol-lowering therapy was added as a covariate to the model. When the outcome variable was SBP, antihypertensive therapy was added as a covariate. When the outcome variable was FPG, individuals with diabetes at baseline were excluded.

Associations between cardio-metabolic traits and incident T2D or CHD were analyzed using Cox regression models adjusting for age and sex. When lipid traits or blood pressure were used as exposure variables the analyses were adjusted for cholesterol-lowering therapy or antihypertensive therapy, respectively. When the exposure variable was FPG, individuals with diabetes at baseline were excluded.

Instrumental variable analyses

We first performed Mendelian randomization analyses employing a 2-stage regression approach to estimate the un-confounded causal effect of each cardio-metabolic trait on the risk of incident and overall (incident and prevalent combined) disease. Predicted values from the first stage linear regression of the traits by their respective GRSs were used as the predictor variables for disease in

the second stage regression. Cox regression was used in the analysis of incident endpoints and logistic regression was used in the analysis of overall disease risk. Age and sex were included as covariates in all regression analyses.

Multivariable Mendelian randomization

Although using GRSs instead of single SNPs improve power in instrumental variable analysis they increase the chance of including SNPs with pleiotropic associations. The GRSs used in our study included pleiotropic SNPs, which may have biased our results. To correct for potential pleiotropic bias without removing SNPs from GRSs and weakening these instrumental variables we used a multivariable Mendelian randomization approach, previously described and applied.[425] In this approach, the β coefficients obtained from the logistic regression of each of the 153 SNPs on the outcomes were regressed on the β coefficients obtained from the linear regression of the same SNPs on each cardio-metabolic trait. The β coefficients obtained with the cardio-metabolic traits were included in a single multivariable model as predictor variables to correct for potential pleiotropic effects.

We performed additional multivariable analyses[425] using the β coefficients of 185 LDLC, HDLC, and TG SNPs obtained from the latest GLGC (188,577 individuals) GWAS,[486] the β coefficients of these 185 SNPs on T2D obtained from the DIAGRAM (34,840 cases and 114,981 controls)[60] GWAS, and the β coefficients of these 185 SNPs on homeostasis model assessment of beta cell function (HOMA-B) and insulin resistance (HOMA-IR) obtained from the MAGIC (46,186 non-diabetic individuals) GWAS.[282]

IBM SPSS Statistics (SPSS Inc., Chicago, IL, USA), *R* (version 3.1.1, The *R* Foundation for Statistical Computing) and PLINK (version 1.07) were used for the statistical analyses. To achieve normality of distribution, natural log transformation was performed for skewed continuous variables. All *P* values reported are two-sided.

Results

Study I

Dietary Fiber, TCF7L2, and T2D

Table 1 shows the characteristics of the MDCS participants (n = 24,799) among different *TCF7L2* rs7903146 genotype carriers. Each risk T-allele was associated with 44% increased risk of T2D (95% Confidence Interval [CI]: 33-56; $P = 4.6 \times 10^{-19}$) in MDCS. Each *TCF7L2* T-allele was additionally associated with elevated baseline levels of FPG (β = 0.059 mmol/L, P = 0.004) and HbA_{1C} (β = 0.27 mmol/mol, P = 0.02). The mean intakes of carbohydrates, fats, proteins and fibers did not differ among different genotype carriers.

Table 1. Characteristics of the MDCS participants by the *TCF7L2* rs7903146 genotype

Characteristic	*TCF7L2* genotype			OR (95% CI)[a] or β (SE)[b,c]	P_{rend}[c]
	CC	CT	TT		
n	13,571	9,488	1,740		
Age (years)	58.1 ± 7.6	58.0 ± 7.6	58.0 ± 7.6	–0.08 (0.07)	0.30
BMI (kg/m^2)	25.7 ± 3.9	25.7 ± 3.9	25.5 ± 3.8	–0.08 (0.04)	0.06
Incident T2D (%)	741 (5.5)	757 (8.0)	151 (8.7)	1.44 (1.33, 1.56)[a]	4.6×10^{-19}
FPG (mmol/L)[d]	5.6 ± 0.9	5.7 ± 0.9	5.7 ± 0.8	0.06 (0.02)	0.004
FPI (pmol/L)[d]	46.8 ± 43.6	47.6 ± 52.6	44.1 ± 27.2	0.22 (1.00)	0.83
HbA_{1C} (%)[d]	4.8 ± 0.5	4.8 ± 0.5	4.9 ± 0.5	0.03 (0.01)	0.02
HbA_{1C} (mmol/mol)[d]	39.7 ± 5.2	40.0 ± 5.3	40.1 ± 4.8	0.27 (0.12)	0.02
Energy (kcal)	2283 ± 652	2281 ± 648	2288 ± 687	0.30 (1.38)	0.28
Carbohydrate (%E)	45.2 ± 6.0	45.2 ± 6.1	45.2 ± 5.9	0.01 (0.06)	0.99
Fat (%E)	39.1 ± 6.1	39.0 ± 6.2	39.1 ± 6.0	–0.02 (0.06)	0.75
Protein (%E)	15.7 ± 2.6	15.8 ± 2.5	15.8 ± 2.5	0.01 (0.03)	0.79
Fiber (g/1,000 kcal)	9.0 ± 2.7	9.0 ± 2.7	9.1 ± 2.8	0.003 (0.025)	0.91

Data are means ± SD unless otherwise stated

Number of individuals included in MDCS cohort, n = 24,799

[a] Logistic regression assuming an additive genetic model adjusting for age, sex, and BMI

[b] β represents the difference generated by each additional T allele

[c] Linear regression assuming an additive genetic model adjusting for age, sex, BMI, total energy intake, dietary assessment method and season of dietary data collection

[d] Data available only for the MDC-CC, n = 5,216

After testing for interactions between quintiles of carbohydrate, fat, protein, and fiber intake and *TCF7L2* rs7903146 variant on the risk of T2D, only fiber intake showed significant interaction ($P_{interaction}$ = 0.049). The magnitude of the elevated risk by each risk T-allele increased from 24% in the lowest quintile of fiber intake to 56% in the highest quintile (Figure 6). The interaction between rs7903146 and fiber intake was more evident ($P_{interaction}$ = 0.006) after excluding potential inaccurate reporters of energy intake (18.3% of the study sample). Further exclusion of individuals who reported change in dietary habits in the past (35.9% of the study sample) did not affect the observed result ($P_{interaction}$ = 0.046).

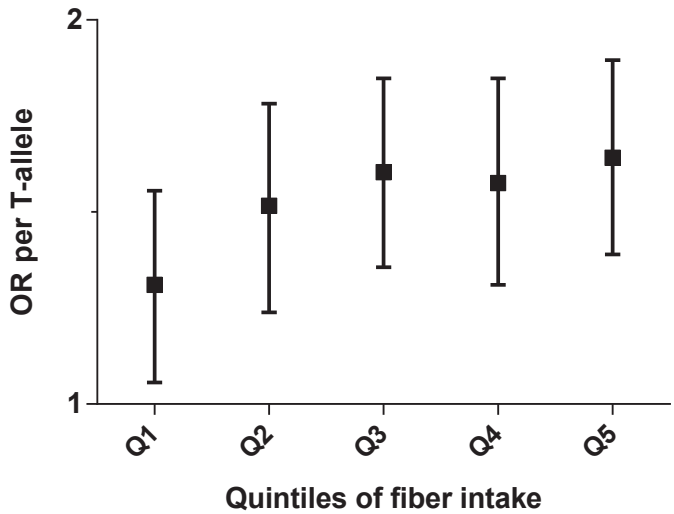

Figure 6. OR per *TCF7L2* rs7903146 risk T-allele in quintiles of fiber intake
The magnitude of the risk elevation of T2D by each risk T-allele increased from the lowest quintile (Q1) of fiber intake to the highest quintile (Q5). In the lowest quintile the OR per T-allele was 1.24 (95% CI: 1.04-1.47, P = 0.014) compared to an OR of 1.56 (95% CI: 1.31-1.86, $P = 8 \times 10^{-7}$) in the highest quintile. This effect modification was statistically significant ($P_{interaction}$ = 0.049) after adjusting for age, sex, BMI, total energy intake, dietary assessment method and season of dietary data collection.

We next analyzed this interaction from the perspective of how fiber intake was associated with incidence of T2D among the different *TCF7L2* genotype carriers. Individuals in the highest quintile of fiber intake were at a significantly lower risk of T2D (OR = 0.74, 95% CI: 0.58-0.94) compared to the lowest quintile among CC genotype carriers, but not among CT (OR = 1.03, 95% CI: 0.80-1.32) or TT (OR = 1.13, 95% CI: 0.62-2.07) genotype carriers (Figure 7).

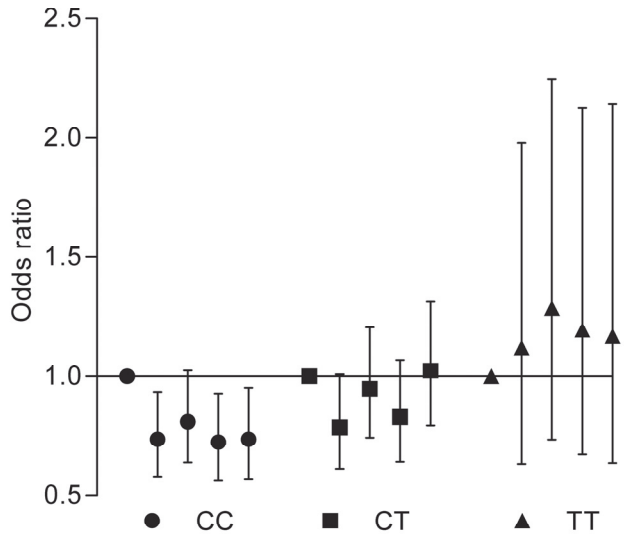

Figure 7. Association between fiber intake and T2D in strata of *TCF7L2* genotype
The lowest quintile of fiber intake in each genotype group was used as a reference. Higher fiber intake was associated with lower risk of T2D among carriers of non-risk CC genotype (P = 0.025) when comparing the highest quintile to the lowest. No associations were observed between fiber intake and risk of T2D among CT (P = 0.77) or TT (P = 0.60) genotype carriers. These analyses were adjusted for age, sex, BMI, total energy intake, dietary assessment method, and season of dietary data collection

We next performed cross-sectional interaction analyses between quintiles of fiber intake and *TCF7L2* genotype on baseline levels of FPG and HbA_{1C}. We did not find any interaction on the levels of FPG (P = 0.20). However, the magnitude the associated effect on HbA_{1C} levels by each risk T-allele increased from the lowest quintile (β = −0.021% [-0.21 mmol/mol]) to highest quintile of fiber intake (β = 0.079% [0.80 mmol/mol]) (Figure 8). The test for interaction was statistically significant ($P_{interaction}$ = 0.02) and remained significant in the sensitivity analysis after excluding inaccurate reporters of energy intake ($P_{interaction}$ = 0.03). When analyzed in strata of diffeternt *TCF7L2* genotypes, higher fiber intake showed the strongest association with lower baseline levels of HbA_{1C} among CC genotype carriers (−0.036% [−0.37 mmol/mol] per quintile, $P = 6.5 \times 10^{-7}$). A weaker association was observed among CT genotype carriers (−0.023% [−0.24 mmol/mol] per quintile, $P = 0.009$) and no association was observed among TT genotype carriers (0.012% [0.13 mmol/mol] per quintile, $P = 0.52$).

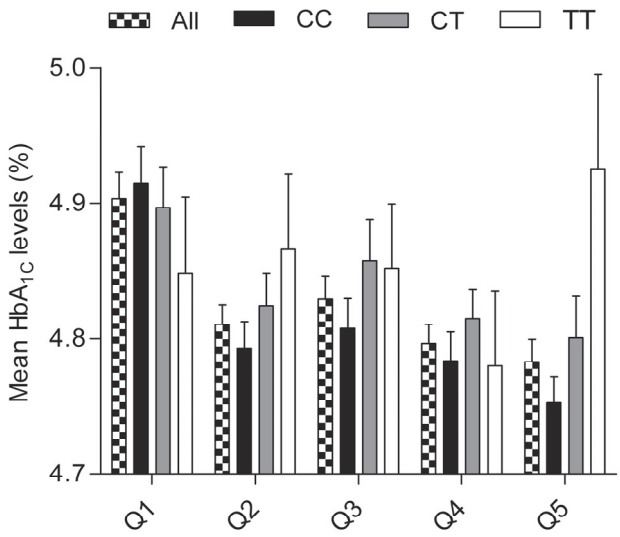

Figure 8. Mean HbA_{1C} levels by *TCF7L2* genotype in quintiles of fiber intake.
The *TCF7L2* T-allele was associated with elevated baseline HbA_{1C} levels in MDC-CC (n = 5,216) (β = 0.03%, P = 0.02). After stratification by quintiles of fiber intake the significant association was restricted to the highest quintile of fiber intake (β = 0.08%, P = 0.002). Higher fiber intake was associated with lower baseline HbA_{1C} levels in MDC-CC ($P = 1.7 \times 10^{-4}$) and this appeared to be driven by the strong association among the CC genotype carriers (β = −0.036%, $P = 6.5 \times 10^{-7}$). No such association was observed among TT genotype carriers (β = 0.012, P = 0.52) while carriers of the CT genotype appeared as an intermediate group (β = −0.023%, P = 0.009). Genotype carriers: hatched bar, all; black bar, CC; grey bar, CT; white bar, TT. The error bars denote the SEM.

As several studies have previously shown that the risk of T2D by the *TCF7L2* variant may be stronger among lean individuals. We were concerned about any potential residual confounding despite adjustment for BMI. Therefore, we first tested for interaction between the *TCF7L2* rs7903146 and BMI quintiles on the risk of T2D. The risk of T2D by the T-allele decreased from 86% to 31% from the lowest to the highest BMI quintile ($P_{interaction}$ = 0.009). However, no correlation was observed between fiber intake and BMI in MDCS (r^2 = −0.006, P = 0.88) and stratification by tertiles of BMI did not indicate changes in the results of interaction analyses. In addition, quintiles of leisure time physical activity did not show interaction with *TCF7L2* variant on the risk of T2D ($P_{interaction}$ = 0.46) indicating no potential residual confounding by physical activity.

Study II

Dietary Fiber and Incidence of T2D

In Study II a total of 2,860 cases of T2DM were recorded over a mean follow-up period of 14.3 years. Higher fiber intake was associated with lower risk of T2D (HR = 0.96; 95% CI: 0.94-0.99 per quintile). Higher fiber intake was also strongly associated with lower baseline levels of FPG, FPI, HOMA-IR, and HbA_{1C} ($P < 0.0001$) (Table 2). In addition, both age and sex were associated with fiber intake with men and younger individuals reporting lower levels of fiber consumption ($P < 0.0001$).

Table 2. Characteristics of the MDCS by quintiles of fiber intake (Study II)

	Quintiles of fiber intake						
	Q1 *n* = 5,386	Q2 *n* = 5,386	Q3 *n* = 5,386	Q4 *n* = 5,386	Q5 *n* = 5,386	β (SE) or HR (CI)	*P*[b]
T2D (%)	11.3	11.0	11.4	9.56	10.9	0.96 (0.94-0.99)[a]	0.01
Men (%)	56	45	38	30	24	–	5×10^{-83}
Age (years)	57.4 ± 7.5	57.7 ±7.7	58.2 ± 7.7	58.3 ± 7.7	58.3 ± 7.5	–	4×10^{-88}
BMI (kg/m^2)	25.5 ± 3.95	25.7 ± 3.81	25.8 ± 3.94	25.7 ± 3.87	25.5 ± 3.98	0.02 (0.02)	0.20
FPG (mmol/L)[c]	5.82 ± 1.05	5.69 ± 0.81	5.63 ± 0.79	5.58 ± 0.74	5.56 ± 0.81	−0.04 (0.008)	3×10^{-7}
FPI(pmol/L)[c]	50.8 ± 43.4	46.3 ± 33.7	47.5 ± 50.5	45.1 ± 59.3	43.9 ± 38.9	−1.09 (0.46)	2×10^{-7}
HOMA-IR[c]	1.75 ± 1.32	1.64 ± 1.06	1.65 ± 1.62	1.53 ± 1.04	1.49 ± 1.00	−0.06 (0.01)	9×10^{-9}
HbA_{1C} (%)[c]	4.90 ± 0.59	4.82 ± 0.45	4.82 ± 0.53	4.80 ± 0.43	4.77 ± 0.49	−0.03 (0.005)	3×10^{-10}

Data are means ± SD unless otherwise stated

Number of individuals included in MDCS cohort, *n* = 26,930

[a] HR of incident T2D per quintile of fiber intake, adjusted for age and sex.

[b] Linear regression assuming an additive genetic model adjusting for age, sex, BMI, season and method where appropriate

[c] Data available only for the MDC-CC, *n* = 5,507

Annotation of T2D Associated Genes to WNT Pathway

Annotation of the 51 T2D associated genes including 58 SNPs was performed in study II. A total of 7 genes including 9 SNPs were found to have links to the WNT signaling pathway where TCF7L2 is a principal transcription factor. These genes (rs numbers of SNPs) include *TCF7L2* (rs7903146 and rs12255372), *HHEX* (rs1111875), *HNF1A* (rs7957197), *NOTCH2* (rs10923931), *TLE4* (rs13292136), *ZBED3* (rs4457053) and *PPARG* (rs1801282 and rs13081389).

Functional positioning of these genes to the WNT pathway is represented in Figure 9. When WNT receptor ligands are absent, transcriptional cofactor β-catenin is bound to a destruction complex established around the Axin/APC scaffolding complex (Figure 9a), (only major components shown: GSK3β, Glycogen synthase kinase 3; CK, Casein kinases; PP2A, Protein phosphatase 2A;

APC, Adenomatous polyposis coli). β-catenin is regulated not only through changes to its cytoplasmic concentration, but also through its cellular localization and extensive protein modification. The Axin degradation complex marks β-catenin for proteolysis (Figure 9b) via phosphorylation and ubiquitination. When

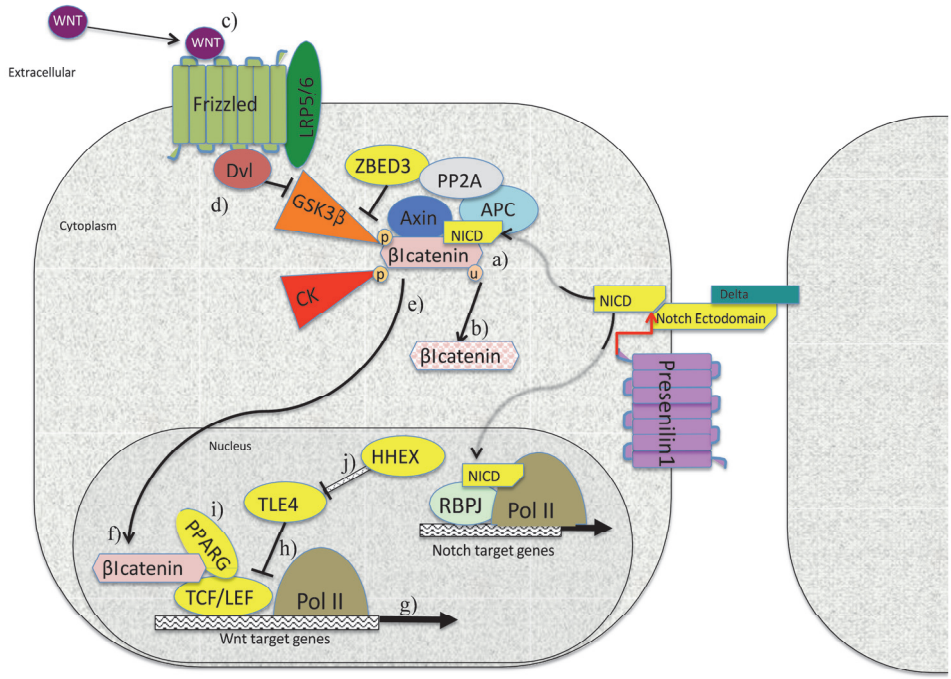

Figure 9. Functional positioning of ZBED3, TLE4, TCF7L2, HNF1A, HHEX, PPARG and NOTCH2 within the WNT canonical pathway

ligands bind to the Frizzled-LRP5/6 receptor complex (Figure 9c), it activates Dishevelled (Dvl) in the cytoplasm of that cell (Figure 9d). Dvl in turn promotes the attachment of the Axin/APC destruction complex to the Frizzled-LRP5/6 (LRP, Low density lipoprotein receptor-related protein) receptor complex followed by LRP5/6-mediated breakdown of Axin and concomitant release of β-catenin (Figure 9e).[528,529] Zinc-finger BED domain-containing 3 (ZBED3)[530] binds to Axin leading to inhibition of GSK3β-mediated β-catenin phosphorylation and resulting in cytoplasmic accumulation of free β-catenin, that subsequently translocates to the nucleus where it binds to T-cell factor/lymphoid enhancer factor (TCF/LEF, e.g. TCF7L2 a.k.a. TCF4, HNF1A a.k.a TCF1)[531] family of transcription modulator complexes on WNT target gene promoters (Figure 9f) to

activate their transcription (Figure 9g). Factors such as TLE1, TLE2, TLE3 and TLE4 repress the transactivation mediated by TCF/LEF complexes and β-catenin (Figure 9h). Transcription factor PPARG interacts with β-catenin and TCF7L2 (Figure 9i), but also appears to be a target of the WNT pathway in cancer cells.[532,533] HHEX, on the other hand wields its enhancing action on WNT signaling by repression of *TLE4* expression (Figure 9j).[534] The direct NOTCH-signaling pathway involves interaction of NOTCH with its ligand Delta, then NOTCH undergoes proteolytic cleavage by Presenilin releasing the NOTCH Intra-Cellular Domain (NICD),[535] which enters the nucleus and interacts with RBPJ to regulate transcription of specific NOTCH target genes. Convergence between NOTCH signaling and WNT signaling appears to mostly mediate cell fate.[535,536] However, there are several pieces of evidence supporting interaction between WNT and NOTCH signaling pathways upstream of gene transcription through which NOTCH downregulates WNT signaling.[537-541] β-catenin interacts directly with, and is modulated by the NOTCH receptor in a ligand independent manner,[537,542] there is also evidence for a functional interaction between Axin and APC in fine-tuning the intracellular traffic of NOTCH.[541,543] Dvl also interacts with NOTCH[544] and GSK3 can also phosphorylate NOTCH.[545,546] In addition to proteolytic processing of NOTCH there is evidence that presenilin 1 also associates with β-catenin.[547] This supports the notion that WNT and NOTCH signaling are an integrated functional module along with the adherens junctions/cadherin pathway (not shown) regulating β-catenin activity and localization and consequently influencing cell fate of physically adjacent cells.

WNT genes, T2D, and Glycemic Traits

Figure 10 and Table 3 show the associations between SNPs in the genes that were annotated to the WNT pathway and T2D and baseline levels of glycemic traits. Both of the *TCF7L2* variants (r^2 = 0.70), rs7903146 (HR = 1.32, 95% CI: 1.24-1.39 per risk allele) and rs12255372 (HR = 1.23, 95% CI: 1.16-1.30 per risk allele) associated with higher risk of T2D. The *HHEX* rs1111875 and *HNF1A* rs7957197 risk alleles also showed association with elevated risk of T2D (HR = 1.07, 95% CI: 1.01-1.12 and HR = 1.14, 95% CI: 1.07-1.22). Both of the *TCF7L2* variants showed elevated baseline levels of FPG and HbA_{1C} by their risk alleles. The *HHEX* risk allele associated with lower baseline levels of FPI. The *ZBED3* risk allele associated with elevated levels of HbA_{1C} at baseline.

Gene-Fiber Interactions and T2D

We observed nominally significant interactions between 4 SNPs in 3 loci and fiber intake on incidence of T2D. The associated effect of *TCF7L2* rs7903146 T-allele on the risk of T2D was significantly modified in quintiles of fiber intake ($P_{interaction}$ = 0.04). This observation is similar to our results in study I but with a longer period of follow-up. Study II included 2,860 incident T2D cases compared to 1,649 incident cases in study I.

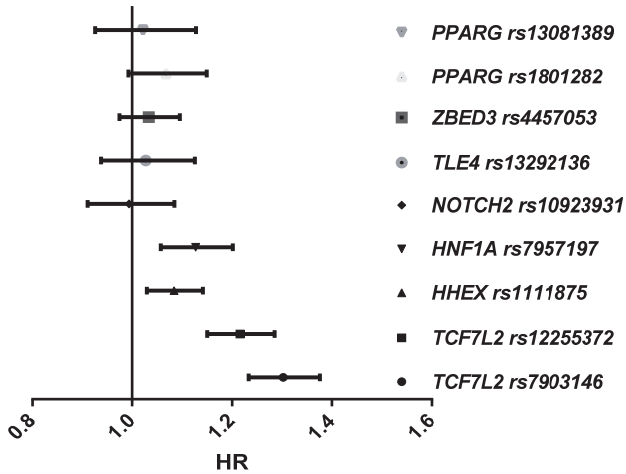

Figure 10. Association between SNPs in genes annotated within the WNT signaling pathway with incident T2D (*n* = 2,860) over 14.3 years of follow-up in MDCS (*n* = 26,930)

Table 3. Association between SNPs in genes annotated within the WNT signaling pathway and glycemic traits in MDC-CC (*n* = 5,507)

	FPG (mmol/L)		FPI (pmol/L)		HbA_{1C}(%)	
	β (SE)	*P*	β (SE)	*P*	β (SE)	*P*
TCF7L2 rs7903146	0.05 (0.02)	0.01	0.04 (0.18)	0.73	0.03 (0.01)	0.03
TCF7L2 rs12255372	0.04 (0.02)	0.04	−0.11 (0.18)	0.69	0.02 (0.01)	0.03
HHEX rs1111875	−0.02 (0.02)	0.22	−0.51 (0.17)	0.015	0.01 (0.01)	0.27
HNF1A rs7957197	0.05 (0.02)	0.02	0.19 (0.20)	0.52	0.02 (0.01)	0.22
NOTCH2 rs10923931	0.03 (0.03)	0.30	−0.03 (0.29)	0.72	0.01 (0.02)	0.43
TLE4 rs13292136	0.004 (0.03)	0.88	0.28 (0.29)	0.41	0.02 (0.02)	0.26
ZBED3 rs4457053	0.02 (0.02)	0.22	−0.23 (0.19)	0.18	0.03 (0.01)	0.03
PPARG rs1801282	0.03 (0.02)	0.16	0.20 (0.23)	0.39	0.00 (0.01)	1.00
PPARG rs13081389	0.04 (0.03)	0.26	0.28 (0.30)	0.32	0.01 (0.02)	0.60

Adjusted for age and sex; data available only for the MDC-CC, *n* = 5,507

In contrast to study I, in study II we additionally tested interactions between quintiles of fiber intake and another *TCF7L2* variant (rs12255372) which indicated somewhat stronger interaction ($P_{interaction}$ = 0.007). The protective association between higher fiber intake and T2D was restricted to individuals carrying the non-risk GG genotype (HR = 0.94, 95% CI: 0.90-0.98, per quintile) and this association was absent among individuals carrying the GT (HR =0.97, 95% CI: 0.92-1.01) and TT genotypes (HR =1.02, 95% CI: 0.93-1.13) (Figure 11). Among

individuals in the lowest quintile of fiber intake the *TCF7L2* rs12255372 risk T-allele was not associated with T2D.

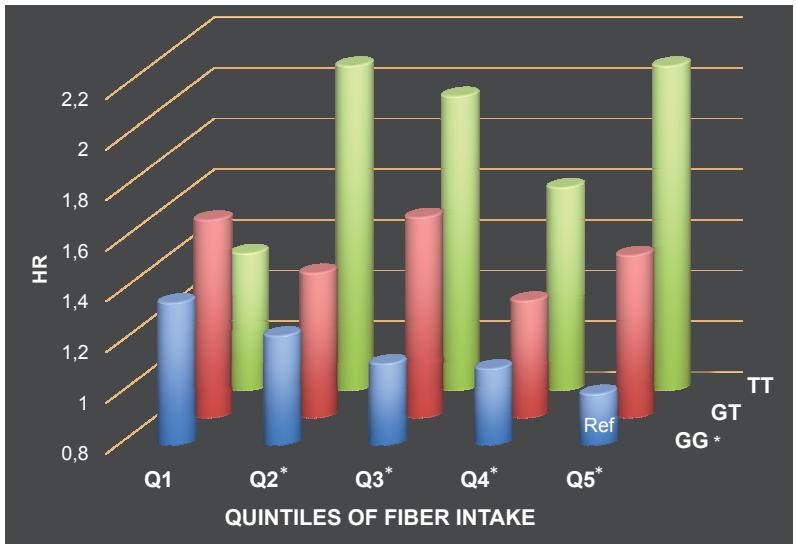

Figure 11. HR of T2D in quintiles of fiber intake by the *TCF7L2* rs12255372 genotype
* indicates a significant association between *TCF7L2* genotype and T2D in fiber intake quintiles ($P < 0.05$) or a significant association between fiber intake and T2D among different *TCF7L2* genotypes ($P < 0.05$)

The *NOTCH2* rs10923931 also showed nominal interaction with quintiles of fiber intake on the risk of T2D ($P_{interaction} = 0.01$). Higher fiber intake was associated with lower risk of T2D only among carriers of the risk T-allele; GT (HR = 0.90; 95% CI: 0.84-0.97) and TT (HR = 0.70; 95% CI: 0.50-0.99) genotypes. (Figure 12). A third nominally significant interaction was observed between the *ZBED3* rs4457053 and quintiles of fiber intake on the risk of T2D ($P_{interaction} = 0.003$). The protective associated effect of higher fiber intake was restricted to individuals carrying the GG risk genotype (HR = 0.84; 95% CI: 0.74-0.94) (Figure 13). Both variants in *ZBED3* and *NOTCH2* showed no associations with T2D incidence in MDCS (Figure 10).

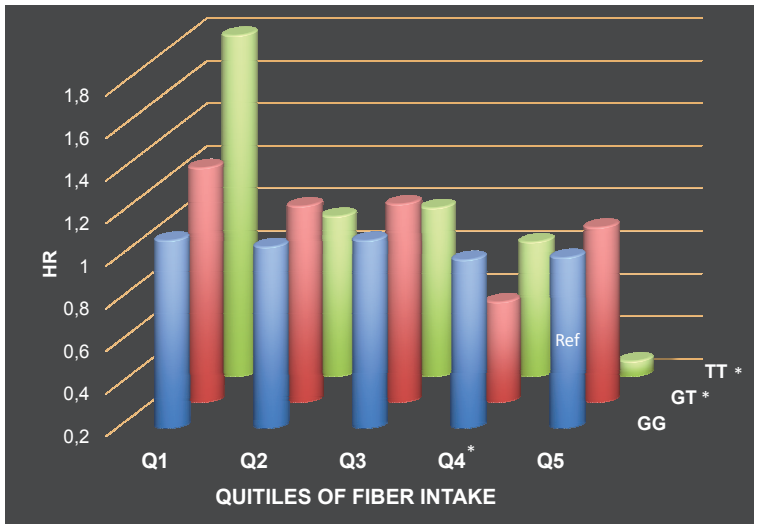

Figure 12. HR of T2D in quintiles of fiber intake by the *NOTCH2* rs10923931 genotype
* indicates a significant association between *NOTCH2* genotype and T2D in fiber intake quintiles ($P < 0.05$) or a significant association between fiber intake and T2D among different *NOTCH2* genotypes ($P < 0.05$)

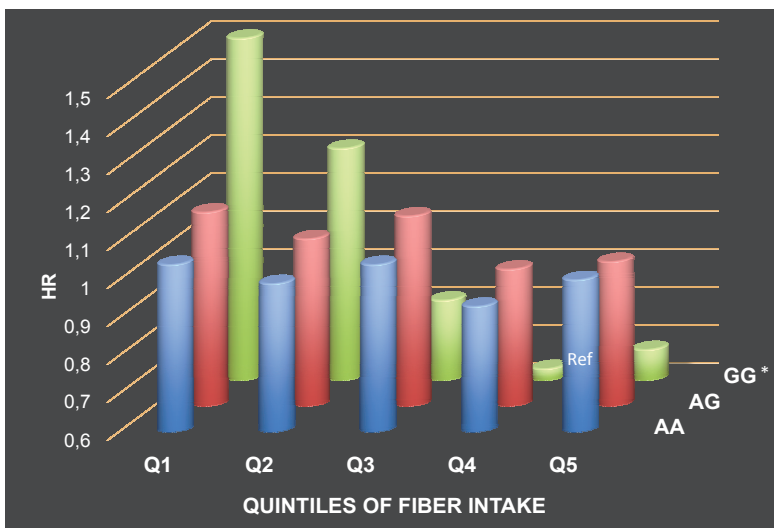

Figure 13. HR of T2D in quintiles of fiber intake by the *ZBED3* rs4457053 genotype
* indicates a significant association between fiber intake and T2D among different *ZBED3* genotypes ($P < 0.05$) or a significant association between fiber intake and T2D among different *ZBED3* genotypes ($P < 0.05$)

Study III

Dietary fiber, TCF7L2, and the metabolic syndrome

The *TCF7L2* rs7903146 variant did not associate with the prevalence nor incidence of the metabolic syndrome in study III. Of the metabolic syndrome related traits, the *TCF7L2* risk T-allele associated with elevated baseline levels of FPG (Table 4). Table 5 shows the characteristics of MDC-CC by tertiles of fiber intake. Higher fiber intake did not associate with prevalence of the metabolic syndrome, however, it associated with smaller WC (P = 0.05), lower body fat percentage (P = 0.02), lower FPG (P = 0.04), lower FPI (P = 0.0006) and lower HOMA-IR ($P = 2\times10^{-6}$).

Table 4. Characteristics of participants of MDC-CC by the *TCF7L2* rs7903146 genotype

	TCF7L2 rs7903146			OR (95%CI)	
	CC	CT	TT	or β (SE)[a]	P_{trend}
n	2534	1745	327	–	–
Sex (male), *n* (%)	1039 (41.0)	685 (39.4)	122 (36.9)	–	–
Age (years)	57.55 ± 0.12	57.43 ± 0.14	57.42 ± 0.33	−0.09 (0.14)	0.51
MetS, *n* (%)	708 (27.6)	466 (26.2)	95 (28.5)	0.99 (0.89-1.10)	0.88
BMI (kg/m^2)	25.62 ± 0.08	25.53 ± 0.09	25.41 ± 0.21	−0.10 (0.09)	0.29
WC (cm)	83.22 ± 0.20	83.24 ± 0.24	82.76 ± 0.55	−0.11 (0.23)	0.63
Body fat (%)	27.10 ± 0.10	26.98 ± 0.11	26.94 ± 0.26	−0.10 (0.11)	0.38
FPG (mmol/L)	5.61 ± 0.02	5.66 ± 0.02	5.72 ± 0.04	0.05 (0.02)	0.005
FPI (pmol/L)	46.0 ± 0.92	47.1 ± 1.11	44.6 ± 2.55	0.13 (1.09)	0.79
SBP (mmHg)	140.8 ± 0.4	141.0 ± 0.4	141.3 ± 9.8	0.21 (0.42)	0.62
DBP (mmHg)	86.90 ± 0.18	86.69 ± 0.22	86.66 ± 0.50	−0.16 (0.22)	0.46
TC (mmol/L)	6.17 ± 0.02	6.17 ± 0.03	6.13 ± 0.06	−0.01 (0.03)	0.68
LDLC (mmol/L)	4.18 ± 0.02	4.17 ± 0.02	4.12 ± 0.05	−0.02 (0.02)	0.52
HDLC (mmol/L)	1.39 ± 0.01	1.40 ± 0.01	1.40 ± 0.02	0.003 (0.008)	0.68
TG (mmol/L)	1.33 ± 0.01	1.33 ± 0.02	1.34 ± 0.04	0.005 (0.016)	0.55
HOMA-IR	1.59 ± 0.03	1.60 ± 0.03	1.59 ± 0.07	0.007 (0.030)	0.76
Total energy intake (kcal)	2325 ± 12	2333 ± 14	2344 ± 32	9 (14)	0.51
Fiber (g/1000 kcal)	9.36 ± 0.06	9.38 ± 0.07	9.37 ± 0.15	0.01 (0.07)	0.83

Data presented as mean ± SEM

[a]Adjusted for age and sex

MetS, Metabolic Syndrome; BMI, Body Mass Index; WC, Waist Circumference; FPG, Fasting Plasma Glucose; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; TC, Total Cholesterol; LDLC, Low Density Lipoprotein Cholesterol; HDLC, High Density Lipoprotein Cholesterol; TG, Triglycerides; FPI, Fasting Plasma Insulin; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance

Table 5. Characteristics of participants of MDC-CC by tertiles of fiber intake

	Fiber intake tertiles			OR (95%CI)	
	Low	Medium	High	or β (SE)[a]	P_{trend}[a(b)]
n	1535	1536	1535	–	–
Sex (male), n (%)	829 (54.0)	624 (40.6)	393 (25.6)	–	–
Age (years)	57.13 ± 0.15	57.46 ± 0.15	57.91 ± 0.15	0.39 (0.11)	0.0003
MetS, *n* (%)	454 (29.6)	416 (27.1)	377 (24.6)	0.95 (0.87-1.04)	0.29
BMI (kg/m^2)	25.51 ± 0.10	25.74 ± 0.10	25.46 ± 0.10	−0.02 (0.08)	0.75
WC (cm)	83.37 ± 0.26	83.56 ± 0.25	82.64 ± 0.26	−0.37 (0.19)	0.05 (0.001)
Body fat (%)	27.19 ± 0.13	27.16 ± 0.12	26.78 ± 0.13	−0.21 (0.09)	0.02 (0.003)
FPG (mmol/L)	5.68 ± 0.02	5.62 ± 0.02	5.62 ± 0.02	−0.03 (0.02)	0.04 (0.04)
FPI (pmol/L)	47.3 ± 1.24	47.1 ± 1.18	44.5 ± 1.23	−1.44 (0.91)	0.00006 (0.00001)
SBP (mmHg)	140.7 ± 0.47	140.9 ± 0.45	141.1 ± 0.47	0.22 (0.35)	0.53 (0.48)
DBP (mmHg)	86.98 ± 0.24	86.80 ± 0.23	86.63 ± 0.24	−0.17 (0.18)	0.33 (0.36)
TC (mmol/L)	6.18 ± 0.03	6.16 ± 0.03	6.17 ± 0.03	−0.007 (0.02)	0.67 (0.68)
LDLC (mmol/L)	4.18 ± 0.03	4.16 ± 0.03	4.18 ± 0.03	−0.001 (0.019)	0.84 (0.86)
HDLC (mmol/L)	1.39 ± 0.01	1.40 ± 0.01	1.39 ± 0.01	0.002 (0.007)	0.74 (0.80)
TG (mmol/L)	1.35 ± 0.02	1.33 ± 0.02	1.32 ± 0.02	−0.01 (0.01)	0.19 (0.20)
HOMA-IR	1.67 ± 0.03	1.62 ± 0.03	1.49 ± 0.03	−0.09 (0.03)	2×10^{-6} (8×10^{-8})
Total energy intake (kcal)	2483 ± 15	2332 ± 15	2173 ± 15	−156 (11)	1×10^{-44} (1×10^{-44})
Fiber intake (g/1000 kcal)	6.60 ± 0.02	9.02 ± 0.02	12.49 ± 0.06	–	–

Data presented as mean ± SEM

[a]Adjusted for age, sex, total energy intake, season, method, physical activity, alcohol intake, smoking status, and education as needed

[b]Additional adjustment for BMI

MetS, Metabolic Syndrome; BMI, Body Mass Index; WC, Waist Circumference; FPG, Fasting Plasma Glucose; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; TC, Total Cholesterol; LDLC, Low Density Lipoprotein Cholesterol; HDLC, High Density Lipoprotein Cholesterol; TG, Triglycerides; FPI, Fasting Plasma Insulin; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance

In MDC-CC (*n* = 4,606), the association between fiber intake and baseline metabolic syndrome (*n* = 1,269) was modified by *TCF7L2* rs7903146 genotype ($P_{interaction}$ = 0.02). Higher fiber intake associated with lower prevalence of the metabolic syndrome among non-risk CC genotype carriers (OR = 0.87; 95% CI: 0.78-0.98 per tertile) but not among CT and TT genotypes carriers (*P* = 0.38 and 0.55, respectively) (Figure 14A). In the re-investigation sample of MDC-CC (*n* = 2,337), higher fiber intake associated with lower risk of developing the metabolic syndrome (*n* = 755) among CC genotype carriers (OR = 0.84; 95% CI: 0.72-0.98) but not among CT and TT genotype carriers (*P* = 0.75 and 0.33, respectively) (Figure 14B). However, the test for interaction did not reach statistical significance ($P_{interaction}$ = 0.20).

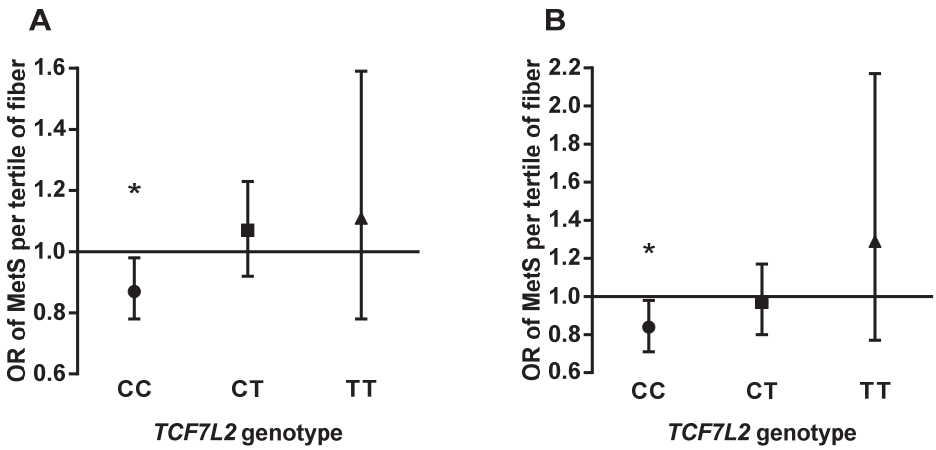

Figure 14. Association between fiber intake and prevalence (A) and incidence (B) of the metabolic syndrome by the *TCF7L2* rs7903146 genotypes

Dietary fiber intake also showed significant interactions with the *TCF7L2* rs7903146 variant on several metabolic traits. Higher fiber intake associated with a smaller WC among CC genotype carriers (β = –0.71 cm per tertile, $P = 0.006$) but not among CT and TT genotype carriers (0.77 and 0.31, respectively) ($P_{interaction} = 0.008$) (Table 6). The *TCF7L2* genotype modified the associated effect of fiber intake on body fat percentage ($P_{interaction} = 0.03$), TC ($P_{interaction} = 0.007$), LDLC ($P_{interaction} = 0.018$), and tended to modify the effect on FPI ($P_{interaction} = 0.09$) and HOMA-IR ($P_{interaction} = 0.06$). Higher fiber intake associated with lower body fat percentage (β –0.34 % per tertile, $P = 0.007$), lower FPI levels (β = –1.66 % per tertile, $P = 0.00005$) and lower HOMA-IR (β –0.12 per tertile, $P = 2\times10^{-06}$) only among CC genotype carriers. Further, higher fiber intake was associated with elevated baseline levels of TC (β 0.20 mmol/L per tertile, $P = 0.01$) and higher LDLC levels only among TT genotype carriers (β = 0.20 mmol/L per tertile, $P = 0.007$) (Table 7).

Table 6. Mean values of metabolic syndrome components in tertiles of fiber intake by *TCF7L2* rs7903146 genotype in the MDC-CC

Fiber intake	*TCF7L2* rs7903146 genotype			Effect Size (SE)[a]	P_{trend}[a]	$P_{interaction}$[b(c)]
	CC	CT	TT			
WC (cm)						
Low	83.9 ± 0.34	83.1 ± 0.42	80.3 ± 1.15	−1.12 (0.42)	0.008	0.008 (0.001)
Medium	83.7 ± 0.34	83.3 ± 0.40	84.3 ± 0.98	0.31 (0.39)	0.42	
High	82.5 ± 0.36	82.9 ± 0.40	81.9 ± 1.14	0.44 (0.39)	0.26	
Effect size (SE)[b]	−0.71 (0.26)	−0.09 (0.30)	0.88 (0.87)			
P_{trend}[b]	0.006	0.77	0.31			
HDLC (mmol/L)						
Low	1.39 ± 0.01	1.39 ± 0.01	1.42 ± 0.04	0.001 (0.01)	0.77	0.76 (0.99)
Medium	1.39 ± 0.01	1.42 ± 0.01	1.39 ± 0.03	0.008 (0.01)	0.49	
High	1.39 ± 0.01	1.38 ± 0.01	1.41 ± 0.04	−0.001 (0.01)	0.71	
Effect size (SE)	0.004 (0.009)	−0.003 (0.01)	−0.007 (0.03)			
P_{trend}	0.53	0.81	0.62			
TG (mmol/L)						
Low	1.37 ± 0.03	1.32 ± 0.03	1.28 ± 0.08	−0.03 (0.03)	0.56	0.15 (0.24)
Medium	1.34 ± 0.02	1.30 ± 0.03	1.41 ± 0.06	0.006 (0.03)	0.88	
High	1.29 ± 0.03	1.37 ± 0.03	1.29 ± 0.07	0.04 (0.03)	0.10	
Effect size (SE)	−0.04 (0.02)	0.03 (0.02)	0.004 (0.06)			
P_{trend}	0.02	0.49	0.80			
FPG (mmol/L)						
Low	5.67 ± 0.03	5.69 ± 0.04	5.78 ± 0.08	0.03 (0.04)	0.40	0.43 (0.61)
Medium	5.59 ± 0.03	5.64 ± 0.04	5.73 ± 0.06	0.07 (0.03)	0.02	
High	5.59 ± 0.03	5.65 ± 0.04	5.63 ± 0.07	0.06 (0.03)	0.055	
Effect size (SE)	−0.04 (0.02)	−0.02 (0.03)	−0.07 (0.06)			
P_{trend}	0.07	0.45	0.22			
SBP (mmHg)						
Low	141.3 ± 0.6	139.6 ± 0.8	141.6 ± 1.8	−0.61 (0.73)	0.41	0.47 (0.59)
Medium	140.4 ± 0.6	141.6 ± 0.7	141.4 ± 1.5	1.01 (0.70)	0.15	
High	141.1 ± 0.6	141.4 ± 0.8	140.3 ± 1.7	0.26 (0.73)	0.72	
Effect size (SE)	−0.12 (0.46)	0.88 (0.57)	−0.58 (1.33)			
P_{trend}	0.79	0.12	0.66			
DBP (mmHg)						
Low	87.3 ± 0.3	86.7 ± 0.4	86.4 ± 0.9	−0.49 (0.39)	0.22	0.59 (0.76)
Medium	86.8 ± 0.3	86.8 ± 0.4	86.9 ± 0.8	0.14 (0.37)	0.71	
High	86.8 ± 0.3	86.5 ± 0.4	86.3 ± 0.9	−0.11 (0.36)	0.76	
Effect size (SE)	−0.27 (0.24)	−0.05 (0.29)	−0.07 (0.69)			
P_{trend}	0.26	0.86	0.92			

All data is presented as mean ± SEM

[a]Adjusted for age and sex

[b] Adjusted for age, sex, method, season, total energy, leisure time physical activity, alcohol intake, smoking and education level

[c]Additional adjustment for BMI

WC, Waist Circumference; HDLC, High Density Lipoprotein Cholesterol; TG, Triglycerides; FPG, Fasting Plasma Glucose; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure

Table 7. Mean values of other metabolic traits in tertiles of fiber intake by *TCF7L2* rs7903146 genotype in the MDC−CC

Fiber intake	*TCF7L2* rs7903146 genotype			Effect Size (SE)[a]	P_{trend}[a]	$P_{interaction}$[b(c)]
	CC	CT	TT			
BMI (kg/m^2)						
Low	25.6 ± 0.1	25.4 ± 0.2	24.9 ± 0.4	−0.32 (0.16)	0.04	0.29
Medium	25.8 ± 0.1	25.7 ± 0.2	26.0 ± 0.4	0.04 (0.15)	0.82	
High	25.5 ± 0.1	25.5 ± 0.2	25.2 ± 0.4	−0.02 (0.16)	0.88	
Effect size (SE)[b]	−0.07 (0.10)	0.03 (0.12)	0.14 (0.33)			
P_{trend}[b]	0.48	0.82	0.67			
Body Fat (%)						
Low	27.3 ± 0.2	27.1 ± 0.2	26.6 ± 0.5	−0.52 (0.20)	0.01	0.03 (0.049)
Medium	27.0 ± 0.2	27.0 ± 0.2	28.1 ± 0.4	0.05 (0.19)	0.79	
High	26.6 ± 0.2	27.0 ± 0.2	26.8 ± 0.5	0.17 (0.19)	0.37	
Effect size (SE)	−0.34 (0.13)	−0.06 (0.14)	0.14 (0.39)			
P_{trend}	0.007	0.69	0.73			
TC (mmol/L)						
Low	6.20 ± 0.04	6.19 ± 0.05	5.99 ± 0.11	−0.06 (0.04)	0.21	0.007 (0.009)
Medium	6.20 ± 0.04	6.14 ± 0.04	6.03 ± 0.10	−0.08 (0.04)	0.05	
High	6.12 ± 0.04	6.20 ± 0.04	6.38 ± 0.11	0.10 (0.04)	0.01	
Effect size (SE)	−0.04 (0.03)	0.007 (0.03)	0.20 (0.04)			
P_{trend}	0.14	0.85	0.01			
LDLC (mmol/L)						
Low	4.19 ± 0.04	4.20 ± 0.04	3.99 ± 0.10	−0.05 (0.04)	0.30	0.018 (0.023)
Medium	4.20 ± 0.03	4.12 ± 0.04	3.99 ± 0.09	−0.09 (0.04)	0.018	
High	4.14 ± 0.04	4.19 ± 0.04	4.38 ± 0.10	0.09 (0.04)	0.016	
Effect size (SE)	−0.02 (0.03)	−0.002 (0.030)	0.20 (0.08)			
P_{trend}	0.34	0.86	0.007			
FPI						
Low	47.8 ± 1.5	47.4 ± 2.3	42.9 ± 3.0	−1.80 (1.58)	0.16	0.09 (0.21)
Medium	46.1 ± 1.5	48.7 ± 2.2	47.0 ± 2.5	1.66 (2.28)	0.45	
High	44.4 ± 1.6	44.9 ± 2.2	42.6 ± 2.8	0.45 (1.70)	0.23	
Effect size (SE)	−1.66 (1.13)	−1.31 (1.67)	−0.09 (2.20)			
P_{trend}	0.00005	0.14	0.88			
HOMA−IR						
Low	1.72 ± 0.04	1.64 ± 0.06	1.54 ± 0.12	−0.10 (0.05)	0.09	0.06 (0.19)
Medium	1.58 ± 0.04	1.65 ± 0.06	1.73 ± 0.10	0.08 (0.06)	0.24	
High	1.48 ± 0.04	1.52 ± 0.06	1.46 ± 0.11	0.03 (0.04)	0.26	
Effect size (SE)	−0.12 (0.03)	−0.06 (0.05)	−0.04 (0.09)			
P_{trend}	2×10^{-06}	0.10	0.48			

All data is presented as mean ± SE

[a]Adjusted for age and sex

[b]Adjusted for age, sex, method, season, total energy, leisure time physical activity, alcohol intake, smoking and education level

[c]Additional adjustment for BMI

BMI, Body Mass Index; TC, Total Cholesterol; LDLC, Low Density Lipoprotein Cholesterol; FPI, Fasting Plasma Insulin; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance

Study IV

Risk of CVD by Chromosome 9p21 and Vegetable and Wine Intake

The risk G allele of rs4977574 associated with higher incidence of CVD (HR = 1.16; 95% CI: 1.10–1.22 per G-allele) in MDCS (Table 8). Higher intakes of vegetables, wine, and total alcohol associated with a lower incidence of CVD ([HR = 0.95; 95% CI: 0.91–0.996], 0.91 [0.86–0.96], and 0.92 [0.86–0.98], respectively).

Both vegetable and wine intakes modified the elevated risk of CVD by the rs4977574 G-allele ($P_{interaction}$ = 0.043 and 0.029, respectively). No interactions were observed with fruit or total alcohol intakes ($P_{interaction}$ = 0.69 and 0.73, respectively). The elevated risk of CVD by the rs4977574 G allele was restricted to the medium and high tertiles of vegetable intake ($P_{trend} = 6 \times 10^{-8}$ and 0.0004, respectively) and to non- and low consumers of wine ($P_{trend} = 4 \times 10^{-7}$ and 0.0004, respectively). The lower risk of CVD by higher vegetable intake was restricted to individuals carrying no risk alleles (P_{trend} = 0.001), while the lower risk of CVD by higher wine consumption was restricted to carriers of the risk G-allele (P_{trend} = 0.00009) (AG and GG genotypes, P_{trend} = 0.01 and 0.001, respectively) (Figure 15).

The rs4977574 variant was not associated with any of the baseline levels of known risk markers of CVD (Table 8). However, we observed interactions between rs4977574 and vegetable intake on baseline levels of HbA_{1C} ($P_{interaction}$ = 0.015), as well as smoking status on baseline levels of HDLC ($P_{interaction}$ = 0.049). The rs4977574 G allele associated with higher baseline levels of HbA_{1C} among individuals in the lowest tertile of vegetable intake (P_{trend} = 0.009), and with lower HDLC only among never-smokers (P_{trend} = 0.045). When stratified by the rs4977574 genotype, higher vegetable intake associated with lower HbA_{1C} levels only among G allele carriers (P_{trend} = 0.0002). Smoking associated strongly with lower baseline levels of HDLC in MDC-CC ($P_{trend} = 1 \times 10^{-10}$), and the magnitude of this associated effect was strongest among individuals carrying the non-risk AA genotype ($P_{trend} = 1 \times 10^{-7}$) (Figure 16).

Table 8. Characteristics of participants in MDCS (*n* = 23,949) by chromosome 9p21 rs4977574 genotype

	rs4977574 genotype			HR (95%CI)	
	AA	AG	GG	or β (SE)[a]	P_{trend}
Total Number	7325	11777	4847		
Sex (%women)	62.6	62.3	62.3		
Incident CVD N (%)	863 (11.8)	1562 (13.3)	739 (15.3)	1.16 (1.10-1.22)	4×10^{-09}
Age (years)	57.9 ± 7.7	57.9 ± 7.6	57.8 ± 7.7	−0.04 (0.07)	0.60
BMI (kg/m^2)	25.7 ± 3.9	25.6 ± 3.9	25.7 ± 3.9	0.001 (0.035)	0.98
Waist (cm)	83.6 ± 15.6	83.4 ± 12.8	83.7 ± 16.7	0.02 (0.11)	0.87
SBP (mmHg)	141 ± 20	141 ± 20	141 ± 20	0.21 (0.17)	0.21
DBP (mmHg)	85 ± 10	85 ± 10	86 ± 10	0.03 (0.09)	0.76
FPG (mmol/L) [b]	5.63 ± 0.84	5.62 ± 0.72	5.66 ± 0.91	0.009 (0.016)	0.59
HbA_{1C} (mmol/mol) [b]	39.6 ± 4.94	39.6 ± 4.82	39.9 ± 5.32	0.13 (0.10)	0.19
LDLC (mmol/L) [b]	4.15 ± 1.00	4.19 ± 0.98	4.19 ± 0.97	0.02 (0.02)	0.33
HDLC (mmol/L) [b]	1.39 ± 0.37	1.40 ± 0.38	1.39 ± 0.36	0.001 (0.007)	0.85
Triglycerides (mmol/L) [b]	1.35 ± 0.73	1.32 ± 0.71	1.37 ± 0.90	0.002 (0.015)	0.48
hsCRP [b]	0.26 ± 0.43	0.24 ± 0.39	0.27 ± 0.48	0.003 (0.009)	0.67
Energy intake (kcal/day)	2280 ± 652	2274 ± 653	2276 ± 653	−3.90 (5.26)	0.46
Vegetables (g/day)	180 ± 98	182 ± 100	180 ± 99	−0.18 (0.90)	0.85
Fruits (g/day)	196 ± 127	193 ± 125	197 ± 127	0.29 (1.14)	0.80
Wine (g/day)	41.23 ± 58.24	42.77 ± 60.97	42.53± 60.55	0.70 (0.55)	0.20
Alcohol (g/day)	10.6 ± 12.3	10.9 ± 12.7	10.8 ± 12.8	0.13 (0.11)	0.22

Data represented as mean ± standard deviation

[a] Multivariable Cox proportional hazards model for incidence of cardiovascular disease (CVD) during follow-up adjusting for age and sex with HR referring to hazard ratio per risk G allele using an additive genetic model

[b] Linear regression analyses of rs4977574 G allele using an additive genetic model with quantitative traits or characteristics at baseline adjusting for age and sex when appropriate with β referring to associated effect estimate per risk G allele

[c] In MDC-CC only, N=4,828 (AA N=1,460; AG N=2,381; GG N=987)

CI, Confidence Interval; CVD, cardiovascular disease; SE, standard error; BMI, Body Mass Index; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; FPG, Fasting Plasma Glucose; HbA_{1C}, Hemoglobin A_{1C}; LDLC, Low-Density Lipoprotein Cholesterol; HDLC, High-Density Lipoprotein Cholesterol; hsCRP, High-Sensitivity C-Reactive Protein

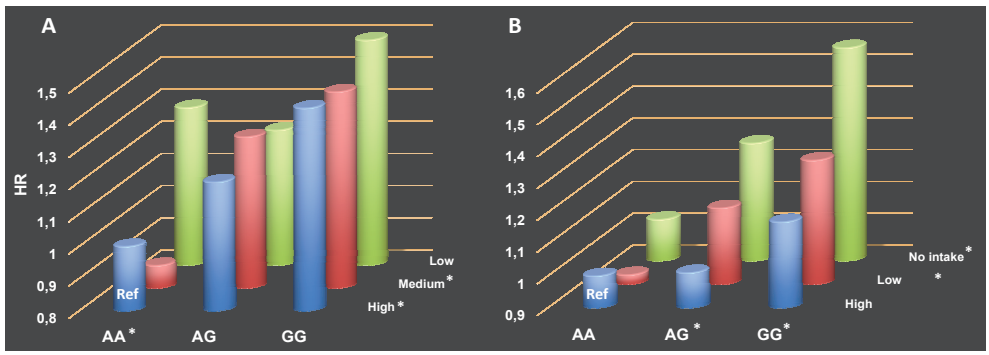

Figure 15. HR of CVD by chromosome 9p21 rs4977574 genotype in tertiles of vegetable intake (A) and categories of wine consumption (B)
* indicates a significant association between 9p21 rs4977574 genotype and CVD in categories of vegetable (A) or wine (B) intake ($P < 0.05$); or a significant association of vegetable (A) or wine (B) intake with CVD among different 9p21 rs4977574 genotypes ($P < 0.05$)

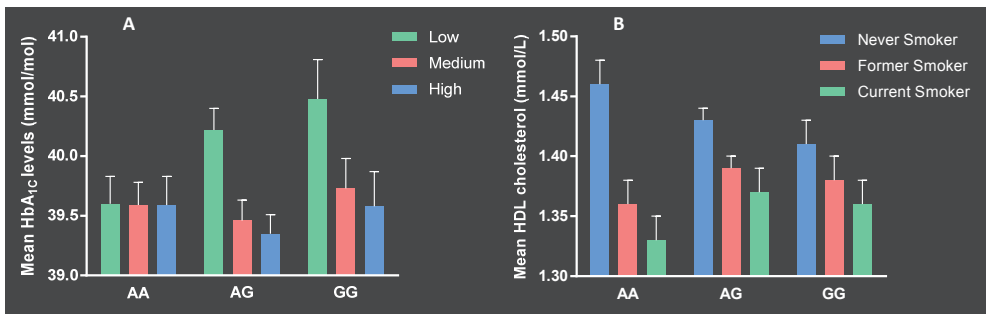

Figure 16. (A) Mean baseline HbA_{1C} levels in tertiles of vegetable intake by the rs4977574 genotype and (B) Mean baseline HDLC levels in smoking status categories by the rs4977574 genotype
(A) The G allele was associated with elevated HbA_{1C} levels only among individuals in the lowest tertile of vegetable intake (β = 0.48 mmol/mol, SE = 0.18 per G allele, $P = 0.009$). Higher vegetable intake was associated with lower HbA_{1C} only among individuals with AG (β = −0.28 mmol/mol, SE = 0.12 per risk tertile, $P = 0.019$) and GG genotypes (β = −0.43 mmol/mol, SE = 0.20 per risk tertile, $P = 0.032$). (B) The G allele was associated with lower levels of HDLC only among never-smokers (β = −0.02 mmol/L, SE = 0.008 per G allele, $P = 0.045$). Higher risk categories of smoking were associated with lower HDLC in all genotypes. The magnitude of this association was largest among individuals with AA genotypes (β = −0.05 mmol/L, SE = 0.01 per higher risk category, $P = 1 \times 10^{-7}$) compared with AG (β = −0.03 mmol/L, SE = 0.009 per higher risk category, $P = 0.0004$) and GG (β = −0.02 mmol/L, SE = 0.01 per higher risk category, P = 0.024) genotypes.

Study V

Mendelian Randomization for Cardio-Metabolic Traits in T2D and CHD

Mendelian randomization analyses in MDCS (n = 28,589) were performed. A total of 4,427 individuals had T2D and 2,997 had CHD, of these, 3,257 individuals had developed T2D and 2428 had developed CHD during a 15.4-year follow-up period (December 2010).

Cardio-Metabolic Genetic Risk Scores

Table 9 shows the associations between the different cardio-metabolic GRSs and their respective traits. All the GRSs were strongly associated with their traits, however, the variance of the traits explained by these GRSs varied. The explained variances of BMI and SBP by their GRSs was the lowest, while the explained variance of LDLC by its GRS was the highest.

Table 9. Association between genetic risk scores and their respective traits

GRS	β (SE)	*P*	Variance explained (%)
GRS_{BMI}	0.090 (0.006)	2×10^{-52}	0.8
GRS_{SBP}[b]	0.063 (0.005)	2×10^{-32}	0.5
GRS_{LDLC}[a, c]	0.269 (0.013)	8×10^{-90}	7.2
GRS_{HDLC}[a, c]	0.245 (0.012)	9×10^{-88}	5.7
GRS_{TG}[a, c]	0.222 (0.013)	2×10^{-66}	4.3
GRS_{FPG}[a, d]	0.110 (0.013)	3×10^{-16}	2.2

Effect per 1 SD of GRS on 1 SD of the Ln transformed corresponding trait.
[a]Data available only in the MDC-CC (n = 5,432)
[b]Adjusted for antihypertensive treatment
[c]Adjusted for lipid-lowering treatment
[d]Excluding individuals with diabetes at baseline

Trait and Instrumental Variable Effects on Incidence of Type 2 Diabetes

Figure 17 shows associations between the baseline levels of various cardio-metabolic traits with the incidence of T2D and the instrumental variable (IV) associations with incident and overall T2D. The baseline levels of all tested cardio-metabolic traits associated with incidence of T2D. The IV analyses indicated that the predicted elevations of BMI and FPG by their GRSs were directly associated with T2D incidence (HR: 1.904; 95% CI: 1.292-2.804 and HR: 2.669; 1.948-3.657, per SD of BMI and FPG, respectively) and with overall T2D (OR: 2.455; 1.703-3.539 and OR: 2.716; 2.019-3.652, per SD of BMI and FPG, respectively). In contrast to LDLC trait association, IV analyses suggested that the predicted elevation of LDLC by its GRS was inversely associated with incidence

of T2D (HR: 0.876; 0.770-0.996, per SD of LDLC) and overall T2D (OR: 0.879; 0.778-0.992, per SD of LDLC).

In a separate analysis using GRS_{LDLC} as a predictor of ApoB, the values of which were available for the entire MDCS cohort, the IV analyses indicated that the predicted elevation of ApoB by GRS_{LDLC} was associated with both lower incidence of T2D (HR: 0.866; 0.753-0.996, per SD of ApoB) and lower overall T2D risk (OR: 0.869; 0.762-0.991, per SD of ApoB). No evidence was observed for association of genetic predisposition for elevated SBP, HDLC or TG with T2D.

In sensitivity analyses we used a multivariable Mendelian randomization to correct for the possible bias caused by pleiotropic associations. Consistent with IV results, an inverse association of SNP β coefficients on LDLC with SNP β coefficients on T2D ($\beta = -0.22$; $P = 0.008$) was observed after adjusting for SNP β coefficients of all the other traits. Furthermore, we observed direct associations between SNP β coefficients on BMI and FPG with SNP β coefficients on T2D ($\beta = 0.48$; $P = .027$ and $\beta = 0.45$; $P = .012$, respectively) (Table 10).

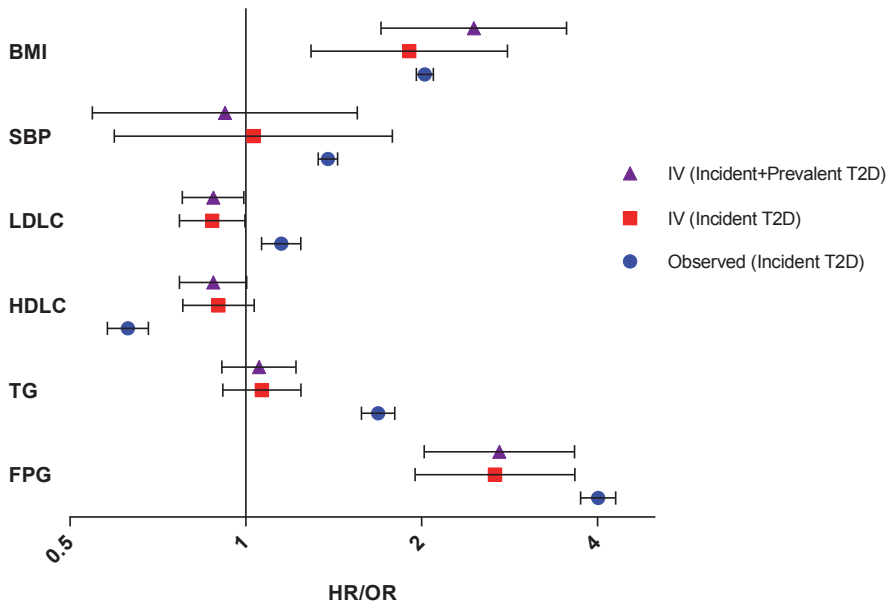

Figure 17. Trait and instrumental variable associations with T2D in MDCS
Blue circle designates the observed HR of incident T2D per 1 SD of the trait; red square designates the HR of incident T2D per 1 SD of the trait using its GRS as an instrumental variable; purple triangle designates the OR of overall T2D (incident + prevalent) per 1 SD of the trait using its GRS as an instrumental variable

We also performed similar analyses using data from the most recent GLGC and DIAGRAM meta-analyses.[60,486] Using the β coefficients of the 185 LDLC, HDLC, and TG SNPs on these three traits from GLGC, and the β coefficients of the same SNPs for T2D from DIAGRAM, we observed an inverse association between SNP β coefficients on LDLC and SNP β coefficients on T2D (β = −0.20; $P = 5 \times 10^{-7}$) adjusting for β coefficients of the same SNPs on HDLC and TG. In addition, we observed an inverse association between SNP β coefficients on LDLC with SNP β coefficients on HOMA-IR (β = −0.022; $P = 0.008$) but not with HOMA-B (Table 11).

Table 10. Multivariable Mendelian randomization analyses of cardio-metabolic traits and the risk of T2D and CHD using MDCS data

	β_{T2D}			β_{CHD}		
Predictors	β	SEM	*P*	β	SEM	*P*
β_{BMI}	0.48	0.22	.027	0.47	0.21	.027
β_{SBP}	−0.15	0.28	.59	0.05	0.27	.85
β_{LDLC}[a]	−0.22	0.08	.008	0.22	0.08	.007
β_{HDLC}[a]	−0.09	0.10	.34	0.005	0.09	.96
β_{TG}[a]	0.05	0.10	.60	−0.007	0.10	.95
β_{FPG}[a]	0.45	0.18	.012	−0.15	0.17	.39

[a]Data available only in the MDC-CC (*n* = 5,432)

Trait and Instrumental Variable Effects on Incidence of Coronary Heart Disease

Figure 18 shows associations between baseline levels of various cardio-metabolic traits with the incidence of CHD and IV associations with incident and overall CHD. The baseline levels of all tested cardio-metabolic traits associated with incidence of CHD. IV analyses indicated that the predicted elevations in LDLC and TG by their GRSs were directly associated with CHD incidence (HR: 1.305; 1.125-1.515 and HR: 1.312; 1.097-1.568, per SD of LDLC and TG, respectively) and with overall risk of CHD (OR: 1.246; 1.074-1.445 and OR: 1.221; 1.022-1.458, per SD, respectively). Similarly, the predicted elevation in baseline ApoB by GRS_{LDLC} was directly associated with incidence of CHD (HR: 1.336; 1.136-1.571, per SD of ApoB) and overall risk of CHD (OR: 1.270; 1.081-1.492, per SD of ApoB). In contrast with trait associations, IV analyses did not provide significant evidence for association of genetic predisposition for elevated BMI, SBP, HDLC or FPG with CHD.

Multivariable Mendelian randomization sensitivity analysis indicated direct associations of SNP β coefficients for LDLC with SNP β coefficients on CHD (β = 0.22; $P = 0.007$) after adjusting for SNP β coefficients of all the other traits. In addition, we observed direct association between SNP β coefficients on BMI with SNP β coefficients on CHD (β = 0.47; $P = 0.027$) using the same analysis (Table 10).

Table 11. Multivariable Mendelian randomization analyses of LDLC and T2D, HOMA-B, and HOMA-IR using GWAS data of GLGC, DIAGRAM and MAGIC

		β_{T2D}			β_{HOMA-B}			$\beta_{HOMA-IR}$		
Predictor	Adjustment	β	SEM	P value	β	SEM	P value	β	SEM	P value
β_{LDLC}		−0.18	0.04	8×10^{-6}	0.000	0.007	.98	−0.018	0.008	.036
	β_{HDLC}	−0.20	0.04	6×10^{-7}	−0.002	0.007	.73	−0.022	0.008	.007
	β_{TG}	−0.18	0.04	8×10^{-6}	0.000	0.007	1	−0.018	0.008	.030
	β_{HDLC}, β_{TG}	−0.20	0.04	5×10^{-7}	−0.002	0.007	.73	−0.022	0.008	.008

Multivariable linear regression models using 185 lipid associated SNPs from GLGC GWAS.[486] β coefficients of the same SNPs on T2D were obtained from the DIAGRAM GWAS[60] and on HOMA-B and HOMA-IR from the MAGIC GWAS.[282]

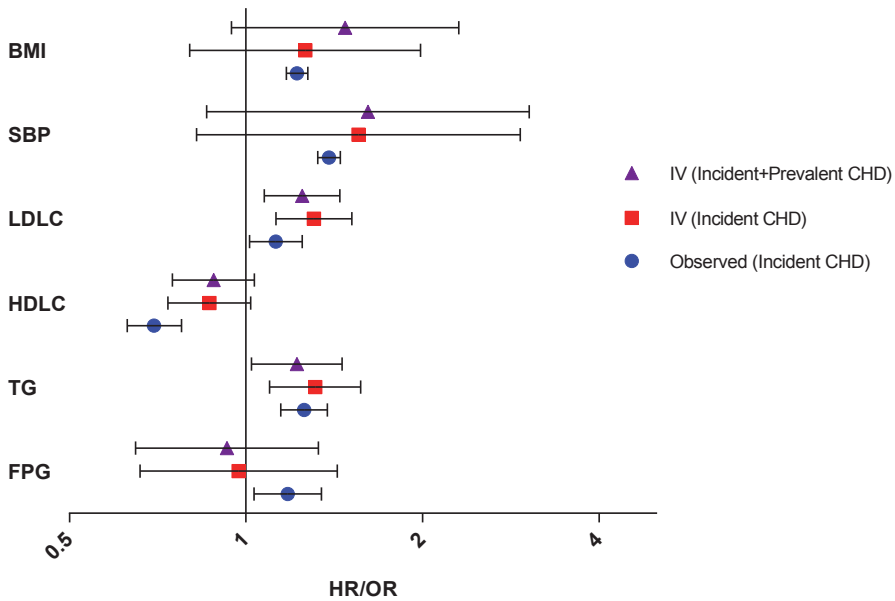

Figure 18. Trait and instrumental variable associations with CHD in MDCS
Blue circle designates the observed HR of incident CHD per 1 SD of the trait; red square designates the HR of incident CHD per 1 SD of the trait using its GRS as an instrumental variable; purple triangle designates the OR of overall CHD (incident + prevalent) per 1 SD of the trait using its GRS as an instrumental variable

Discussion

This thesis revolves around two major topics. The first theme aims to understand how interactions between genetic variations and lifestyle factors influence the risk of T2D and CVD. The second theme aims to understand the causal nature of the relationship of common cardio-metabolic biomarkers with T2D and CHD using genetic variations. To study gene-lifestyle interactions we have examined the variants in the *TCF7L2* and chromosome 9p21 which have so far been the strongest in association with T2D and CVD, respectively. To study causality we have utilized the Mendelian randomization approach that benefits from the random allocation of alleles at conception and thus eliminates biases by confounding and reverse causation.

Gene-Lifestyle Interactions and T2D (Study I-III)

TCF7L2-Fiber Interactions and T2D

The *TCF7L2* rs7903146 risk T-allele was associated with 44% elevated risk of T2D in MDCS, which is consistent with findings from previous studies.[259,286-288] However, the magnitude of the risk increase by the T-allele was significantly accentuated by increasing dietary fiber intake. Similar observations were obtained with cross-sectional analyses of HbA_{1C} levels showing that the magnitude of the association between the risk T-allele and HbA_{1C} increased with higher fiber intake. On the other hand, no interactions between the *TCF7L2* rs7903146 and carbohydrate, fat or protein intake on the risk of T2D were observed.

The association between higher fiber intake and T2D risk was also modified by the *TCF7L2* genotype. Our results indicate that the protective associated effect of higher fiber intake is limited to the non-risk CC genotype carriers of rs7903146 and such associated effect is lacking among T-allele carriers who comprise around 45% of the MDCS study population. Similar observations have been reported in the prospective EPIC-Potsdam case-control study and the Stockholm Diabetes Prevention Program. In the EPIC study, whole grain intake was associated with lower risk of T2D among carriers of the non-risk CC genotype, while carriers of the risk T-allele lacked such protection.[335] In the Stockholm Diabetes Prevention

Program, the protective associated effect of both whole grains and cereal fibers on T2D was restricted to non-risk allele carriers of the *TCF7L2* rs7903146 and rs4506565 variants.[336]

In the Nurses' Health Study, the magnitude of the increased risk of T2D by the *TCF7L2* rs12255372 risk allele was observed to be more pronounced among individuals consuming diets with high glycemic index and glycemic load.[334] Since diets with high glycemic index and -load are in general assumed to be low in their fiber content, these results could be in contrast to our observation of a more pronounced risk by the risk allele among individuals with high fiber intake. However, a meta-analysis of 3 large cohorts has previously shown the protective associated effect of cereal fibers to be independent of the glycemic index and glycemic load.[155] This observation indicates that the interactions observed with *TCF7L2* and glycemic index and -load in the Nurses' Health Study may be independent of dietary fiber.

The cross-sectional interaction between dietary fiber and *TCF7L2* genotype on HbA_{1C} levels is in line with the interaction observed prospectively on incidence of T2D. The *TCF7L2* T-allele was associated with higher HbA_{1C} levels only among individuals with high fiber intake. A high fiber intake was strongly associated with lower HbA_{1C} levels among CC genotype carriers, this association was weaker among CT genotype carriers, while TT genotype carriers completely lacked this association. Dietary fiber intake did not interact with *TCF7L2* genotype on FPG levels, which is in line with a large meta-analysis of 14 cohorts (including MDC-CC) that found no interaction with whole grain intake on FPG levels.[548]

WNT Signaling and Dietary Fiber

TCF7L2 is a principal transcription factor in the WNT signaling pathway. This highlights the important role this pathway could play in the pathogenesis of T2D. We have therefore investigated whether variants in other T2D loci linked to the WNT pathway could interact with fiber intake to influence the risk of T2D. The annotation of 51 previously reported T2D gene loci resulted in identification of seven genes that associated with WNT signaling including *TCF7L2* (rs7903146 and rs12255372), *HHEX* (rs1111875), *HNF1A* (rs7957197), *NOTCH2* (rs10923931), *TLE4* (rs13292136), *ZBED3* (rs4457053) and *PPARG* (rs1801282 and rs13081389).

We have observed novel interactions between dietary fiber and variants in the *NOTCH2* and *ZBED3* loci on the risk of T2D. In contrast to the *TCF7L2* findings, higher fiber intake was associated with lower risk of T2D only among risk allele carriers of the *NOTCH2* rs10923931 and homozygotes for the risk allele of the *ZBED3* rs4457053. Although the *NOTCH2* and *ZBED3* variants did not show any association with the risk of T2D in the MDCS population, the risk alleles of these

variants showed tendencies for higher risk of T2D among individuals in the lower categories of fiber intake and tendencies for lower risk among those in the higher categories of fiber intake.

The reason for the opposite direction of interaction effect of the variants in *NOTCH2* and *ZBED3* with fiber intake as compared to *TCF7L2* variants could be due to the complexity through which the WNT signaling operates. Previous studies have indicated that the WNT pathway should not be viewed as having an additive effect, but rather as a continuum, as both high and low levels of WNT activity have been shown to promote colorectal cancer cell apoptosis.[549,550] In addition, TCF7L2 plays a dual role in the WNT pathway as it can act as a transcriptional activator in the presence of β-catenin while it functions as a repressor in the absence of β-catenin.[551,552]

TCF7L2-Fiber Interactions and the Metabolic Syndrome

The interaction between the *TCF7L2* variant and dietary fiber were further explored in relation to the metabolic syndrome and related quantitative traits in MDC-CC. The cross-sectional associations of dietary fiber intake with prevalence of the metabolic syndrome and with baseline WC were observed to be modified by the *TCF7L2* genotype. Furthermore, the *TCF7L2* genotype modified the association of dietary fiber with several quantitative traits related to the metabolic syndrome including body fat percentage, TC, LDLC, and HOMA-IR. These observations are in line with the TULIP intervention study which reported that among individuals in the intervention group a higher fiber intake was associated with greater weight loss among homozygotes for the *TCF7L2* non-risk allele as compared to risk allele carriers.[337]

Our findings suggest that higher fiber intake associates with lower prevalence of the metabolic syndrome only among the *TCF7L2* non-risk CC genotype carriers. This finding is also supported by our prospective analysis in which the protective association with incidence of the metabolic syndrome was restricted to non-risk CC genotype carriers although the test for interaction did not reach statistical significance. Our results also suggest that only among non-risk allele carriers, higher fiber intake associates with smaller WC, lower TG levels, lower body fat percentage, and lower HOMA-IR. Furthermore, the TT genotype carriers not only lacked the association with metabolically healthier phenotypes but also had higher TC and LDLC associated with high fiber intake.

Potential Mechanisms for Interactions

The mechanisms by which fiber intake may protect against T2D include effects on satiety, body weight, hormonal responses, insulin sensitivity, inflammation and through fermentation products of dietary fiber.[553] Fiber intake has previously been associated with lower post-prandial glucose and insulin concentrations, which have been mainly attributed to slower intestinal absorption of nutrients [554]. In addition, dietary fiber has previously demonstrated improvement in insulin sensitivity[555-557] and favorable effects on lipid traits.[558,559] The relationship between fiber intake and incretin hormone responses has been inconsistent across different studies and this could be attributed to differences in the studied fiber types, the limited number of individuals studied, and/or the short period of the studies.[560,561] However, high fiber intake among hyperinsulinemic subjects was associated with elevated plasma levels of GLP-1 and SCFAs after 9–12 months, pointing to a long term effect of dietary fiber on the regulation of incretin hormones and glucose homeostasis.[562] SCFAs are the most common products of colonic fermentation of dietary fiber by microbiota. These fermentation products, including butyrate, acetate and propionate, have been extensively studied in relation to colon cancer. *In vitro* studies have indicated that SCFAs, and particularly butyrate, induce apoptosis in colon cancer cell lines through hyperactivation of the WNT pathway.[563] Therefore, the interactions observed in our study of fiber intake with WNT associated genetic variants on T2D and other cardio-metabolic traits could reflect molecular interactions between SCFAs and WNT signaling in various metabolically important tissues.

SCFAs have been associated with increased expression of the proglucagon gene and secretion of GLP-1 in rat intestinal cells.[564-566]. Furthermore, SCFAs have been shown to induce GLP-1 secretion via the FFA receptor 2 (ffar2) in mice.[567] These observations along with the previously mentioned study, that reported elevated GLP-1 and SCFA plasma levels after long term fiber intervention, suggest that fiber intake could stimulate GLP-1 secretion through SCFA. At least part of the protective association of dietary fiber with T2D could therefore be mediated by SCFAs through increased GLP-1 release. Most of the investigations concerning the functional effect of the *TCF7L2* risk variant have so far focused on β-cell dysfunction, mainly attributed to impaired incretin effect associated with the risk allele.[304,568] Therefore, it can be speculated that carriers of the *TCF7L2* risk allele could suffer from some degree of incretin resistance, leading to a lack of or less benefit from higher GLP-1 levels associated with SCFAs from higher fiber intake.

Dietary fiber intake has been associated with elevated plasma levels of SCFAs.[569,570] Therefore, the effects of SCFAs may not be restricted to the intestinal epithelium but may influence other tissues or cells as the pancreatic islets. Butyrate has previously been reported to be the most potent histone deacetylase inhibitor (HDACi) among SCFAs.[571] This is particularly relevant as the rs7903146 risk variant sequence has been reported to confer an islet-specific open chromatin state translating to an elevated enhancer effect on *TCF7L2*

transcription.[311] It can thus be speculated that butyrate could play a role in further propagating the open chromatin state among the rs7903146 T-allele carriers via histone hyperacetylation, which could result in further enhanced transcription of the already over-expressed risk transcript.

The mechanism by which dietary fiber may increase insulin sensitivity is not fully understood.[572] *In vivo* studies have previously indicated that SCFAs may increase insulin sensitivity by inhibiting adipose tissue lipolysis and decreasing the levels of circulating FFAs.[573,574] In addition, another insulin sensitizing effect could be through the HDACi activity of SCFAs and particularly butyrate that has been previously shown to stimulate adipocyte differentiation.[575] Therefore, the insulin sensitizing effects of dietary fiber may at least partially be mediated through SCFAs and/or GLP-1 which also has insulin sensitizing effects.[576] In addition, GLP-1 has been shown to stimulate preadipocyte proliferation and inhibit apoptosis.[577]

Activation of the WNT signaling pathway has previously been reported to have an inhibitory effect on preadipocyte differentiation and adipogenesis.[578] Failure of preadipocytes to differentiate into mature adipocytes may lead to hypertrophic growth of adipocytes that is associated with increased insulin resistance.[579] In a previous study, the *TCF7L2* rs7903146 risk T-allele has been shown to be negatively correlated with splice variants retaining exon 13a in human subcutaneous adipose tissue which predominate early during adipocyte differentiation.[580]. This provides some evidence for attenuation of adipocyte differentiation among carriers of the *TCF7L2* risk allele. It can therefore be assumed that carriers of the risk allele may lack at least part of the beneficial effect of SCFAs on adipocyte differentiation. This may partially explain the lack of beneficial effects of fiber intake on T2D, metabolic syndrome and insulin resistance among risk allele carriers of *TCF7L2*.

Chromosome 9p21 and CVD (Study IV)

In a previous multi-ethnic study, the chromosome 9p21 variants were reported to interact with a prudent dietary pattern score on the risk of MI and CVD.[502] Our aim was to analyze if similar interactions with vegetable and fruit intake could be observed in MDCS. In addition, we studied interactions with alcohol and wine consumption as, similar to vegetables and fruits, these beverages include bioactive substances with anti-oxidant and cardio-protective properties.

In MDCS, individuals carrying the risk G-allele of the 9p21 rs4977574 had elevated risk of CVD while those who reported higher levels of vegetable intake and moderate or high wine and alcohol consumption had lower risk of CVD. The associated elevated risk of CVD by the rs4977574 G allele was observed to be

modified by vegetable and wine intake. The elevated risk of CVD by the risk G-allele was observed only among individuals who reported medium or high vegetable intake, and to those reporting zero or low consumption of wine.

The associated lower risk with higher vegetable and wine intake was also modified by the rs4977574 genotype. The associated protective effect of high vegetable consumption on CVD was only observed among carriers of the non-risk AA genotype (31% of the population) while the associated protective effect of wine consumption on CVD was only observed among the risk G-allele carriers.

Consistent with results from earlier studies, the 9p21 variant did not associate with any of the traditional clinical risk factors of CVD at baseline. However, after stratification by vegetable intake, we observed that the risk G-allele was cross-sectionally associated with elevated HbA_{1C} levels among individuals in the lowest tertile of vegetable intake. Additional stratification by smoking status revealed that the risk G-allele was cross-sectionally associated with lower HDLC levels among never-smokers. These nominally significant interactions could indicate that the mechanisms by which the 9p21 locus may exert its risk could involve derangements in glucose and lipid metabolism.

We have observed that the associated increased risk by the G-allele among individuals already at high risk for CVD, due to low vegetable intake, was attenuated. This type of interaction is concordant with our previous study in which the associated increased risk of CHD by the 9p21 rs4977574 G-allele was attenuated among individuals who are at high risk of CVD due to smoking.[503] The interactions observed with vegetable intake are also in line with an earlier study that reported an interaction between the 9p21 variants and a prudent diet score. However, contrary to our findings, the associated effect of the risk allele was strongest among individuals in the lowest tertile of the prudent diet score.[502] On the other hand, we observed that the associated risk increase of CVD by the G-allele was attenuated among consumers of wine. In separate interaction analyses on CHD and stroke, similar tendencies for interactions were observed with both vegetable and wine intake. However, statistical significance could not be reached, which could be due to lower power in these subgroup analyses.

Due to the correlation between vegetable and wine intakes, and as an attempt to exclude potential confounding, we mutually adjusted the analyses for these factors and the results remained similar. The interaction between the 9p21 variant and wine consumption on CVD risk was independent of total alcohol consumption indicating that other bioactive substances as resveratrol in wine could be driving this observation. Furthermore, interactions between rs4977574 and vegetable or wine intake were adjusted for smoking behavior, indicating that these interactions were independent of smoking.

The mechanisms by which the 9p21 locus confers an increased risk of CVD remain incompletely understood. The SNPs in this locus are located an LD block

that lacks any protein-coding genes.[476,492] However, the large noncoding RNA *ANRIL* has been previously mapped to the risk interval.[494,495] Although there is no conclusive evidence about the potential connection between *ANRIL* and CVD, several studies suggest a possible role in the epigenetic regulation of gene expression.[581-584] Both vegetables and wine are rich with bioactive substances that have epigenetic effects such as resveratrol (in red grapes), sulforaphane (in broccoli and sprouts), butyrate (a fermentation product of dietary fiber), and genistein (in fava beans and soya beans).[585-587] The epigenetic mechanisms through which the 9p21 locus may influence the risk for CVD could thus potentially be modified by nutritional or environmental factors through gene-environment interactions.

Cardio-Metabolic Traits and Risk of T2D and CHD (Study V)

In study V we have performed Mendelian randomization analyses to investigate whether genetic predisposition for cardio-metabolic traits is associated with T2D and CHD, and whether there are differences in the causal connections of the cardio-metabolic traits with T2D and CHD. Our findings indicate that genetic predisposition for lower plasma levels of LDLC associates with elevated risk for T2D, suggesting an opposite role for LDLC in the development of T2D as compared with CHD. In addition, our results support a causal role for elevated BMI in both T2D and CHD.

Causal Inference in T2D

First, we used the IV approach to investigate whether genetic predisposition for elevated BMI, SBP, LDLC, HDLC, TG, and FPG translates to higher risk for T2D. Consistent with earlier results from RCTs and Mendelian randomization studies, we observed significant evidence for a causal relationship between elevated FPG and BMI and higher risk of T2D. Importantly, and in contrast to the observed trait associations in MDC-CC, we found evidence for an inverse causal association between LDLC and T2D, which is in line with the reported increased risk of T2D by LDLC-lowering observed in statin trials.

Second, we used a multivariable Mendelian randomization analysis with adjustments for potential bias by pleiotropic associations of the genetic variants with more than one of the different cardio-metabolic traits. This sensitivity analysis confirmed the direct causal association of BMI and FPG with T2D and the inverse causal association of plasma LDLC levels with T2D.

Third, we performed another multivariable Mendelian randomization analysis for lipid traits using the GWAS meta-analysis data of GLGC, DIAGRAM and MAGIC[60,486] rather than MDCS data. This sensitivity analysis confirmed the causal association of lower LDLC with increased risk of T2D. This analysis further indicated an inverse causal association between LDLC and HOMA-IR, but not with HOMA-B, suggesting that the effects of LDLC on T2D could be through insulin sensitivity rather than secretion.

Similar to our results, the direct causal relationship between BMI and T2D has also been reported in other Mendelian randomization studies.[130,131] These observations emphasize the role of increased adiposity in the pathogenesis of T2D and highlight the important role of weight loss, regardless of how it is achieved, as a crucial factor in the prevention of T2D.

Statins and T2D

The inverse causal relationship between LDLC and T2D observed in our study could explain at least part of the increased risk of new-onset diabetes associated with statin therapy.[588-593] The JUPITER (Justification for the Use of Statins in Primary Prevention: an Intervention Trial Evaluating Rosuvastatin) trial has reported an increased risk of diabetes associated with rosuvastatin.[591] Similar findings have been reported in meta-analyses of RCTs, suggesting that statins confer elevated risk of diabetes.[589,590,592] In addition, a recent population-based cohort study indicated that the level of risk increase of diabetes is concordant with reported adherence to statin therapy.[588]

Although the mechanistic explanation for the association of statins with increased risk of diabetes has been much debated, no clear explanation has yet been suggested. Most of the studies investigating the mechanisms have focused on the putative deleterious effects of statins rather than a potential role of LDLC-lowering *per se*. In a recent large study, genetic variants in the 3-hydroxy-3-methylglutaryl-CoA reductase gene (*HMGCR*) that associate with lower LDLC and lower hepatic HMGCR expression were found to be associated with increased risk of T2D and increased body weight.[593] One important conclusion of that study was that genetic inhibition of HMGCR could explain the increased risk of T2D by statin therapy. Our study adds to that conclusion by suggesting that the increased risk of T2D can in fact be a result of the lowering of plasma-LDLC levels, and as such, may not be restricted to pharmacological or genetic inhibition of HMGCR. In addition, the adjustment for the pleiotropic associations of the SNPs with other cardio-metabolic traits, which may be particularly important for BMI, indicated that the inverse association between LDLC and T2D is not mediated by BMI. Furthermore, a recent study reported a lower prevalence of diabetes in patients with familial hypercholesterolemia compared with their unaffected relatives.[594] This observation further supports the inverse causal association between plasma levels of LDLC and T2D observed in our study.

Causal Inference in CHD

The same Mendelian randomization approaches were applied to CHD. IV analysis indicated direct causal associations of both higher LDLC and TG with CHD, which is consistent with earlier studies.[414,425,426,467,595] Further, multivariable sensitivity analyses, adjusting for pleiotropic effects of the SNPs, suggested an association between genetic predisposition to elevated BMI and increased risk of CHD, and confirmed the direct causal association of elevated LDLC with CHD.

The direct causal effects of LDLC and TG on CHD observed in our IV analyses are supported by results in previous clinical trial and Mendelian randomization studies.[414,426,467,595] The causal effect of LDLC on CHD is also consistent with recent evidence from the IMPROVE-IT trial that showed additional benefits of ezetimibe, on top of statin therapy, on LDLC-lowering and cardiovascular outcomes.[596]

In the multivariable sensitivity analyses we observed direct causal associations of both LDLC and BMI with CHD after taking into account other known pleiotropic effects. A previous large Mendelian randomization study using *FTO* SNP as an instrument reported no causal associations between BMI and CHD.[130] In this context, it is important to remember that in all Mendelian randomization analyses, including our study, it is very important to consider the limited strength of the instrumental variables as only a modest fraction of the variance of most traits can be explained by current available GRSs, and in particular single SNPs. However, although the variance of BMI explained by the GRS used in our study was only 0.8%, our findings supported direct causality between BMI and CHD, which is in line with a couple of other Mendelian randomization studies.[131,391]

The lack of a significant association between TG and CHD using the multivariable approach is in contrast with the significant association in our IV analysis, and in contrast with the earlier and larger study by Do *et al*.[425] To investigate if the TG result turned non-significant in our multivariable analysis due to the additional incorporation of non-lipid traits in the multivariable model, as compared to the Do *et al*. study, we did an additional analysis with only LDLC, HDLC, and TG included in the multivariable analysis. However, TG remained not significantly associated with CHD, probably due to low power, underscoring the weakness of the used instrumental variable. Finally, the lack of an inverse causal association of HDLC with CHD in both the IV and multivariable analyses is consistent with previous clinical trial and Mendelian randomization studies.[424-426,597]

Conclusions

We have investigated gene-lifestyle interactions in T2D and CVD using their strongest susceptibility genetic variants in *TCF7L2* and chromosome 9p21, respectively. We have also investigated the causal relationship between common cardio-metabolic traits and T2D and CHD using Mendelian randomization.

We can conclude that:

- The associated lower risk of T2D with higher fiber intake was restricted to around 55% of the population who were non-carriers of the *TCF7L2* risk allele, while carriers of the risk allele lacked the protective association with higher fiber intake
- Other variants in genes linked to the WNT pathway may also modify the associated lower risk of T2D by higher fiber intake. The protective association of higher fiber intake with T2D was restricted to risk allele carriers of the *NOTCH2* and to carriers of two copies of the risk allele of the *ZBED3*. This may suggest that dietary fiber may at least partially exert its protective associated effects on T2D through modulation of the WNT pathway.
- Carriers of the *TCF7L2* risk allele lack the beneficial effect of dietary fiber on the metabolic syndrome and several of the traits related to it. This indicates that the *TCF7L2* risk variant may exert its diabetogenic and dysmetabolic effects not only through defects in β-cells, but also through effects in other organs and tissues, possibly including the adipose tissue, liver, and/or intestine.
- The lower risk of CVD by higher vegetable intake was restricted to non-carriers of the 9p21 risk allele, while wine consumption appeared to lower the risk of CVD only among carriers of the risk allele. Furthermore, the observed interactions of the 9p21 with vegetable intake and smoking on HbA_{1C} and HDLC levels, respectively, provide evidence for that the risk increase by 9p21 may include derangements at the level of glucose and lipid metabolism
- Genetic predisposition for elevated LDLC have opposite consequences for the risk of T2D and CHD. The increased risk of T2D by decreasing LDLC may not be restricted to pharmacological or genetic inhibition of 3-

hydroxy-3-methylglutaryl-CoA reductase, indicating that uniform lowering of LDLC may translate into increased risk of T2D. In addition, BMI may play a causal role in both T2D and CHD pathogenesis.

Populärvetenskaplig Sammanfattning

Förekomsten av typ 2-diabetes (T2D) och hjärt-kärlsjukdom (CVD) ökar i epidemiska proportioner världen över. Denna epidemi svarar för en stor hälsomässig och ekonomisk belastning och är en ledande orsak till sjuklighet och dödlighet. Utvecklingen av dessa sjukdomar är mycket komplex och involverar både genetiska och livsstilsrelaterade faktorer. Övergången till en stillasittande livsstil och ökat kaloriintag leder till fetma, vilket tillsammans med cigarettrökning är de största bovarna bakom denna epidemi. Den genetisk komponent som bidrar till orsakssambanden av dessa tillstånd framgår också tydligt till följd av familjär aggregering och skillnader mellan etniska grupper. Gen-livsstils interaktioner kan också antas vara en viktig faktor till orsakssambanden för dessa sjukdomar.

Att förstå gen-livsstils interaktioner är viktigt och kan bidra till förståelsen av komplexa sjukdomar som T2D och CVD. Enkelt uttryckt finns interaktioner när storleken på sambandet mellan en viss livsstilsfaktor och sjukdom förändras bland människor utifrån deras genetiska bakgrund. Förståelse för dessa samspel kan hjälpa oss att identifiera biologiska mekanismer genom vilka livsstilsfaktorer och genetiska faktorer påverkar risken för sjukdom. Detta kan öppna dörrarna till att identifiera bättre läkemedel för att förebygga och behandla dessa sjukdomar. Att studera interaktioner kan också hjälpa oss att anpassa strategier för individanpassad förebyggande och behandling av människor beroende på deras genetiska bakgrund. Detta är fortfarande ett relativt nytt kunskapsområde och mycket arbete återstår för att förstå dessa samspel.

Denna avhandling syftar till att undersöka gen-livsstils interaktioner i T2D och CVD genom att studera de gener som tidigare identifierats som starkt associerade med dessa sjukdomar. Vi har utgått från Malmö Kost Cancer Studien som omfattar mer än 30 000 individer. Vi har observerat att interaktioner mellan den starkaste genetiska varianten för T2D (TCF7L2) och kostfiberintag påverkar risken för denna sjukdom. Tidigare studier har genomgående visat att ett högre fiberintag skyddar mot T2D. Vi har observerat detta skyddande samband endast bland bärare av den icke-risk CC genotyp som utgör cirka 55 % av befolkningen, till skillnad från bärare av riskgenotyper (CT och TT) där fiberintag inte skyddar mot diabetes. Dessutom modifierade TCF7L2-genotypen på liknande vis sambandet mellan fiberintag och metabolt syndrom. Individer med metabolt syndrom visar ofta en anhopning av olika riskfaktorer för T2D och CVD, som fetma (särskilt bukfetma),

högt blodtryck, höga blodnivåer av triglycerider och glukos, eller låga nivåer av HDL-kolesterol.

Vanliga genvariationer i över 50 ställen på det mänskliga genomet har förknippats med ökad risk för T2D. TCF7L2 är ett protein som reglerar andra geners uttryck på en cellulär signalväg som kallas WNT signaleringsväg. För att förstå om fiberintag påverkar T2D genom WNT signalvägen, undersökte vi mer än 51 T2D associerade genvarianter för koppling till denna signalväg och identifierade totalt 7 sådana varianter. Förutom TCF7L2, observerade vi interaktioner mellan två genetiska varianter i NOTCH2 och ZBED3 generna, vilket indikerar att fiberintag kunde utöva sin skyddande verkan genom WNT-signalväg.

Vi har också observerat att interaktion mellan den starkaste genetiska varianten för CVD (på kromosom 9p21) och intag av grönsaker respektive vin, påverkar risken för CVD. Det skyddande sambandet mellan högt grönsaksintag och CVD begränsades till bärare av icke-risk AA genotypen. Det skyddande sambandet mellan vinkonsumtion och CVD var begränsad till bärare av riskgenotyper (AG och GG).

Det främsta målet i epidemiologiska studier är att uppnå kausalitet d.v.s. att förstå orsakssamband. Emellertid är systematiska fel ett stort problem i alla observationsstudier, och dessa fel kan bero på flera olika orsaker. En av de största utmaningarna för dessa studier är olika s.k. störfaktorer, dvs. förekomsten av olika variabler som gör det svårt att urskilja vilka variabler som har ett orsakssamband. Denna snedvridning kan elimineras i randomiserade kontrollerade studier genom att balansera störfaktorer i jämförelsegrupper genom randomisering. Eftersom vi vet att genetiska varianter fördelas slumpvis vid befruktningen, kan dessa också användas som medel för att balansera störfaktorer och på så vis uppnå kausalitet. I vår studie har vi använt genetiska varianter som associerar med övervikt, blodtryck, blodsocker, triglycerider och kolesterol, för att studera orsakssamband mellan dessa egenskaper och T2D och CVD. Vår viktigaste observation var orsakssambandet mellan genetiskt lägre LDL-kolesterol (det onda kolesterolet) och ökad risk för T2D. Dock finns orsakssamband mellan lägre LDL-kolesterol och minskad risk för CVD. Detta är första gång i världen som man upptäckt detta orsakssamband och våra resultat är viktiga eftersom de väcker frågan om vi bör utveckla nya läkemedel för att ytterligare minska LDL-kolesterol i blodet på grund av en möjlig ökad risk för att utveckla T2D.

I min avhandling har jag framlagt bevis för att det finns gen-livsstils interaktioner i T2D och CVD och dessa behöver följas upp i framtida studier. Våra resultat visar också att minskning av LDL-kolesterol i blodet kan ge en ökad risk för typ 2-diabetes, och en viktig fråga för framtiden är att förstå mekanismerna bakom detta samband mellan LDL-kolesterol och T2D.

Popular Summary

The prevalence of type 2 diabetes (T2D) and cardiovascular disease (CVD) is increasing in epidemic proportions around the globe. This epidemic accounts for huge health and economic burdens as a leading cause for morbidity and mortality. The development of these diseases is very complex and involves both genetic and lifestyle factors. The shift to sedentary lifestyles and increased caloric intake leading to obesity along with cigarette smoking are the main culprits behind this epidemic. The genetic component contributing to the etiology of these conditions is also evident due to familial aggregation and differences among ethnic groups. Gene-lifestyle interactions are also believed to be an important factor in the etiology of these diseases.

Understanding gene-lifestyle interactions is believed to be important and may contribute to the understanding of complex diseases as T2D and CVD. In simple terms interactions exist when the magnitude of the association between a certain lifestyle factor and the disease changes among people based on their genetic background. Understanding interactions may help us in identifying biologic mechanisms through which lifestyle factors and genetic factors affect the risk of disease. This can open the door into identifying better drugs for treating and preventing these diseases. Studying interactions may also help us to personalize prevention and treatment strategies in people according to their genetic background. This discipline is still relatively new and much work is still needed to understand interactions.

This thesis aims to investigate gene-lifestyle interactions in T2D and CVD using the strongest genes previously identified to associate with these diseases. We have studied the Malmö Diet and Cancer Study that includes more than 30,000 individuals. We have observed interactions between the strongest T2D genetic variant (TCF7L2) and dietary fiber intake influencing the risk of this disease. Previous studies have consistently reported that higher fiber intake protects against T2D. We have observed this protective association only among carriers of the non-risk CC genotype who constitute around 55% of the population, while among carriers of the risk genotypes (CT and TT) fiber intake did not protect against diabetes. In addition, TCF7L2 genotype similarly modified the association of fiber intake with the metabolic syndrome. Individuals with the metabolic syndrome usually have a clustering of different T2D and CVD risk factors, as obesity, high blood pressure, high blood levels of cholesterol and glucose.

More than 50 locations on the human genome have been associated with increased risk for T2D. TCF7L2 is a transcription factor in a cellular pathway called the WNT signaling pathway. To understand if fiber intake affects T2D through the WNT signaling pathway, we investigated more than 51 T2D genes for connections to this pathway and identified 7 such connections. In addition to TCF7L2, we observed interactions between 2 genetic variants in the NOTCH2 and ZBED3 genes, indicating that fiber intake could exert its protective actions through this pathway.

We have also observed interactions between the strongest CVD genetic variant (chromosome 9p21) with both vegetable and wine intakes influencing the risk of CVD. The protective association between high vegetable intakes and CVD was restricted to carriers of the non-risk AA genotype. The protective association between wine consumption and CVD was restricted to carriers of the risk genotypes (AG and GG).

The ultimate goal in epidemiological studies is to obtain causality. However, observational studies are often biased due to several reasons. One of the main challenges for these studies is called confounding that happens due to the correlation of different factors that makes it difficult to pinpoint the causal one. This bias can be eliminated in randomized controlled trials through balancing the confounders between comparison groups by randomization. Since we know that genetic variants are randomly allocated at conception, they can also be used as means for balancing confounders and obtaining causality. In our study we have used genetic variants in genes that affect obesity, blood pressure, blood glucose and cholesterol to study the causal relationship between these traits and T2D and CVD. Our main observation was the causal association between lower LDL cholesterol (the bad cholesterol) and increased risk of T2D. However, lower LDL cholesterol is causally associated with lower risk of CVD. Our study is first in the world to connect lower LDL-cholesterol to increased risk of T2D. Our results are important because they raise the question if we should develop new drugs to further reduce LDL cholesterol in blood due to the possible increased risk of developing T2D.

To summarize, this thesis has provided novel evidence for gene-lifestyle interactions in T2D and CVD which need to be followed-up in future studies. Our results raise concerns for increased risk of T2D associated with lower levels of LDL cholesterol. Future studies need to be performed to understand the mechanisms that connect LDL-cholesterol to T2D.

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Paper I

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ARTICLE

Role of *TCF7L2* risk variant and dietary fibre intake on incident type 2 diabetes

G. Hindy · E. Sonestedt · U. Ericson · X.-J. Jing · Y. Zhou · O. Hansson · E. Renström · E. Wirfält · M. Orho-Melander

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Abstract
Aims/hypothesis The T allele of transcription factor 7-like 2 gene variant, *TCF7L2* rs7903146, increases the risk of type 2 diabetes by 40–50%. As *TCF7L2* rs7903146 has been associated with diminished incretin effect we investigated whether interaction between dietary intake of carbohydrate, fat, protein or fibre and this variant affects the risk of type 2 diabetes.
Methods A cohort of 24,799 non-diabetic individuals from the Malmö Diet and Cancer Study (MDCS), with dietary data obtained by a modified diet history method, were followed up for 12 years, with 1,649 recordings of incident type 2 diabetes made. Risk of type 2 diabetes in strata of diet quintiles was analysed prospectively adjusting for potential confounders. Cross-sectional analyses were performed on baseline fasting glucose and HbA_{1c} levels in a subset of 5,216 randomly selected individuals from the MDCS.
Results The elevated risk of type 2 diabetes with rs7903146 (OR 1.44, 95% CI 1.33, 1.56, $p=4.6\times10^{-19}$) increased with higher intake of dietary fibre (OR 1.24, 95% CI 1.04, 1.47 to OR 1.56, 95% CI 1.31, 1.86 from the lowest to highest quintile; $p_{interaction}=0.049$). High intake of dietary fibre was inversely associated with diabetes incidence only among CC genotype carriers (OR 0.74, 95% CI 0.58, 0.94 per quintile, $p=0.025$). The T allele was associated with 0.027% elevated HbA_{1c} ($p=0.02$) and this effect increased with higher intake of fibre (from −0.021% to 0.079% for the lowest to the highest quintile, $p_{interaction}=0.02$). Each quintile of higher fibre intake was associated with lower HbA_{1c} levels among CC and CT but not among TT genotype carriers (−0.036%, $p=6.5\times10^{-7}$; −0.023%, $p=0.009$; and 0.012%, $p=0.52$, respectively).
Conclusions/interpretation Our study suggests that dietary fibre intake may modify the association between *TCF7L2* rs7903146 and incidence of type 2 diabetes, and that higher fibre intake may associate with protection from type 2 diabetes only among non-risk allele carriers.

Keywords Diet · Gene · Gene–environment interaction · Transcription factor 7-like 2 (TCF7L2) · Type 2 diabetes

Abbreviations

%E	Percentage of non-alcohol energy intake
EI	Energy intake
EPIC	European Prospective Investigation into Cancer and Nutrition
GLP-1	Glucagon-like peptide 1
GWAS	Genome-wide association studies
HDACi	Histone deacetylase inhibitor
MDC-CC	Malmö Diet and Cancer Study, cardiovascular cohort
MDCS	Malmö Diet and Cancer Study
PAL	Physical activity level
SCFA	Short-chain fatty acid
TCF7L2	Transcription factor 7-like 2

G. Hindy · E. Sonestedt · U. Ericson · X.-J. Jing · Y. Zhou · O. Hansson · E. Renström · E. Wirfält · M. Orho-Melander
Department of Clinical Sciences in Malmö, Lund University, Clinical Research Center, Malmö, Sweden

M. Orho-Melander (✉)
Diabetes and Cardiovascular Disease, Genetic Epidemiology, Department of Clinical Sciences, Lund University Diabetes Centre, Lund University, Clinical Research Centre 91:12, Jan Waldenströms gata 35, 20502 Malmö, Sweden
e-mail: Marju.Orho-Melander@med.lu.se

Introduction

Transcription factor 7-like 2 gene (*TCF7L2*) rs7903146 to this date remains the strongest and most widely replicated type 2 diabetes susceptibility locus [1, 2]. In addition to the increased risk of type 2 diabetes, the *TCF7L2* rs7903146 T allele has been associated with increased fasting glucose and HbA_{1c} levels in genome-wide association studies (GWAS) [3, 4].

As a principal transcription factor in the wingless-type MMTV integration site (WNT) signalling pathway [5], TCF7L2 has been reported to be involved in the induction of transcription of the proglucagon gene through heterodimerisation with β-catenin and synthesis of glucagon-like peptide 1 (GLP-1) [6]. In line with this, several studies have reported an attenuated insulin response to oral glucose in individuals with the *TCF7L2* risk variant, pointing to the possibility of a defective incretin system [7, 8].

Levels of incretin hormones are modified by macronutrient intake [9, 10] and several previous studies have tested for interactions between *TCF7L2* risk variants and diet. In the Diabetes Prevention Program, the TT genotype of *TCF7L2* rs7903146 showed a tendency towards being more strongly associated with type 2 diabetes in the placebo group compared with the intervention group but the results did not reach statistical significance [11]. In the European Prospective Investigation into Cancer and Nutrition (EPIC) Potsdam cohort [12] higher whole-grain intake was found to be protective against type 2 diabetes among rs7903146 CC genotype carriers but not among T allele carriers. Still another study, a large meta-analysis of 14 cohorts, investigating fasting glucose levels instead of incident type 2 diabetes, did not detect any interaction between the *TCF7L2* risk allele and whole-grain intake on that phenotype [13]. In addition, the *TCF7L2* risk allele was reported to have a stronger association with type 2 diabetes among individuals with higher dietary glycaemic load and glycaemic index [14]. Finally, a recent report from the Tübingen Lifestyle Intervention Program (TULIP) described an interaction between dietary fibre and the *TCF7L2* rs7903146 risk variant with regard to successful weight loss after a lifestyle intervention [15].

In this study we hypothesised that different dietary intakes, in particular the relative intake levels of carbohydrates, fats, proteins or fibres, could modify the risk associated with the *TCF7L2* rs7903146 T allele in incident type 2 diabetes.

Methods

Study population The Malmö Diet and Cancer Study (MDCS) is a population-based prospective cohort study based in the city of Malmö, Sweden. In 1991 the source population of the MDCS was defined to include all individuals born between 1926 and 1945 and living in Malmö. In 1995 this was extended to include all women born between 1923 and 1950 and men born between 1923 and 1945. This resulted in a source population of 74,138 individuals. Study participants were recruited through public advertisements or personal letters. Mental disability and limited Swedish language skills were used as the sole exclusion criteria. Study participants were invited to visit the screening centre twice during the baseline examination period, which extended from March 1991 to October 1996. During the first visit, participants were divided into groups of six to eight individuals and received instructions on how to record meals in the menu book. They were also instructed on how to fill in the diet questionnaire and the extensive questionnaire covering socioeconomic and lifestyle factors, to be completed at home. Approximately 10 days later, participants returned for a dietary history interview. By the end of the baseline examination period, we had complete dietary, anthropometric and lifestyle data on 28,098 individuals. Details of the recruitment procedures are described elsewhere [16].

From this population we excluded 909 individuals with prevalent type 2 diabetes, identified as individuals with a self-reported diabetes diagnosis or on a self-reported glucose-lowering regimen. After exclusion of prevalent type 2 diabetes patients we were left with 27,189 individuals, 24,799 of whom had available DNA samples and were genotyped successfully for *TCF7L2* rs7903146 and composed our study population. Of these, 15,010 were women (mean [±SD] age 57.3±7.9 years, BMI 25.3±4.2 kg/m^2) and 9,789 were men (age 59.1±3.4 years, BMI 26.2±3.4 kg/m^2).

Altogether 6,103 individuals were randomly selected from the MDCS to participate in a cardiovascular subcohort (MDC-CC). Additional measurements were obtained for these individuals, including analysis of fasting blood glucose and HbA_{1c} levels. For the analyses in the MDC-CC, we excluded cases of prevalent type 2 diabetes, and included 5,216 individuals with complete diet, fasting glucose and genotype information: 3,067 women (age 57.3±5.9 years, BMI 25.3±4.2 kg/m^2) and 2,149 men (age 57.5±6.0 years, BMI 26.1±3.4 kg/m^2).

The MDCS was approved by the Ethical Committee at Lund University (LU 51-90). All participants provided written informed consent.

Incident type 2 diabetes We studied the incidence of type 2 diabetes until December 2006 (mean follow-up time 11.8±3.0 years). Incident cases were identified using the Swedish

National Diabetes Register [17] and the Diabetes 2000 register of Skåne region [18]; both registers included only individuals diagnosed by a physician according to established guidelines. To identify cases that were not diagnosed at the hospital, we used the local HbA_{1c} register, which contains data from institutional and non-institutional care in Malmö since 1988 [19]. Individuals with at least two HbA_{1c} values above 6.0%, using the Swedish Mono-S standardisation system (corresponding to 6.9% using the US National Glycohemoglobin Standardization Program and 52 mmol/mol using International Federation of Clinical Chemistry and Laboratory Medicine units) [20, 21], were categorised as diabetes cases. In our study population (n=24,799) a total of 1,649 incident cases of type 2 diabetes occurred during the follow-up period.

Dietary assessment An interview-based, modified dietary history method specially designed for the MDCS was used consisting of: (1) a 7-day menu book where lunch, dinner meals and cold beverages, including alcohol, were recorded; and (2) a dietary 168-item questionnaire to assess meal patterns, consumption frequencies and portion sizes of regularly consumed foods. Medicinal drugs, natural remedies and nutrient supplements were recorded in the menu book. A 48-page booklet was used to help participants at home estimate the portion sizes for recording information in the questionnaire. This was followed by interviews performed by trained interviewers. Portion sizes and dishes in the menu book were estimated during the interview using a more extensive book with photographs. Participants were also asked about their meal pattern, cooking methods and food choices.

Data from the menu book and diet questionnaire were used to calculate the average daily intake of foods. The average daily food intake was converted to energy and nutrient intakes using the Malmö Diet and Cancer Food and Nutrient Database, which was designed for the MDCS and was derived from PC KOST2-93 of the Swedish National Food Administration [22, 23].

A slight alteration of the coding routines for dietary data was introduced in September 1994 [23]. A method variable, classifying data collected before and after September 1994, along with a four-category season variable (i.e. winter, spring, summer and autumn) was created and used as a covariate to adjust for variation in data collection over time.

Dietary variables used in our analysis included total energy intake (EI) (kJ), carbohydrate, fat and protein intake as percentages of non-alcohol EI (%E), and fibre intake as grams (g) per 4,184 kJ (1,000 kcal). The relative validity of the dietary assessment method used in the MDCS has previously been evaluated in a sample of 50- to 69-year-old Malmö residents, 105 women and 101 men. The reference method used was 18 days' weighed food records (3 days every second month) collected over 1 year. Energy-adjusted Pearson correlation coefficients for fat, carbohydrate, protein and fibre intake were in the range of 0.54–0.74 [24].

Individuals with potentially inaccurate reports of EI (n=4,548) were identified as having a ratio of EI to the basal metabolic rate outside the 95% CI limits of the physical activity level (PAL) calculated for each individual as total energy expenditure. This procedure is described in detail elsewhere [25].

Individuals with a change in their dietary habits in the past (n=5,540) due to illness or other factors were identified by one questionnaire item [22].

Other variables used as potential confounders Leisure-time physical activity was assessed by an extensive lifestyle questionnaire adapted from the Minnesota Leisure Time Physical Activity Questionnaire. Participants had to estimate the number of minutes per week for each season they spent performing each of 17 different physical activities. The duration was multiplied by an intensity factor to create a physical activity score that was divided into tertiles. Participants were classified as current smokers, ex-smokers and never-smokers. Alcohol intake was classified into four categories based on grams of alcohol consumed per day: zero, low (<15 g/day in women or <20 g/day in men), medium (15–30 g/day in women or 20–40 g/day in men) and high consumers (>30 g/day in women or >40 g/day in men). The education variable was created by classifying participants according to their highest educational level (≤8 years, 9–10 years and 11–13 years at school, and university degree).

Genotyping TCF7L2 rs7903146 was genotyped using the TaqMan PCR method (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The ABI Prism Sequence Detection System ABI 7900HT (Applied Biosystems) was used for post-PCR allelic discrimination by measuring allele-specific fluorescence. The concordance rate was >99% in 325 randomly repeated samples. Genotyping success rate was 96%. The genotypes were in Hardy–Weinberg equilibrium (p=0.16); 13,571 (54.7%) individuals carried the CC genotype, 9,488 (38.3%) the CT genotype and 1,740 (7.0%) the TT genotype.

Statistical analysis Assuming an additive model, logistic regression was used to calculate the OR of incident type 2 diabetes associated with the *TCF7L2* T risk allele in the MDCS, adjusting for age, sex and BMI. A similar analysis was done within quintiles of relative intakes of carbohydrate, fat, protein and fibre. Interactions between *TCF7L2* genotypes and quintiles of different dietary intakes and type 2 diabetes incidence were analysed by introducing a multiplicative factor of genotype and dietary quintiles as continuous variables and

also adding these variables to the equation. Interactions were analysed using a basic adjustment model for age, sex, BMI, total EI, method and season. For the sensitivity analyses, we excluded inaccurate reporters of EI and in the prospective analysis of incident type 2 diabetes, we further excluded individuals reporting a change in their dietary habits.

In the MDC-CC subcohort we performed cross-sectional analysis using linear regression to calculate the effect sizes per each risk T allele on baseline fasting plasma glucose and HbA_{1c} in quintiles of fibre intake, adjusting for age, sex and BMI. Interactions between quintiles of dietary fibre intake and *TCF7L2* genotype on fasting plasma glucose and HbA_{1c} were analysed by introducing a multiplicative factor of genotype and dietary quintiles as continuous variables using the same adjustment model as described for the MDCS above. For the sensitivity analyses, we excluded individuals potentially reporting inaccurate EI.

QUANTO (http://hydra.usc.edu/gxe/ accessed 1 March 2012) was used to calculate the statistical power for the gene–diet interaction with incident type 2 diabetes and baseline HbA_{1c} levels [26, 27]. Assuming an OR of 0.90 per fibre quintile (additive model) and an OR of 1.44 per *TCF7L2* T allele (26% allele frequency, additive model) on type 2 diabetes incidence, and an effect of −0.028% per fibre quintile and 0.027% per *TCF7L2* T allele on HbA_{1c} levels, we had 80% power to detect an interaction OR of at least 1.08 in type 2 diabetes incidence, and an interaction effect of at least 0.022% on HbA_{1c} levels.

Our analyses showed similar results after further adjustments for potential confounders as physical activity, alcohol intake, smoking habits and level of education.

We used IBM SPSS Statistics, version 19 (SPSS Inc., Chicago, IL, USA), for the analyses. Two-sided p values of <0.05 were considered significant.

Results

The *TCF7L2* rs7903146 T allele was associated with a 44% (95% CI 33, 56) increased risk of incident type 2 diabetes ($p=4.6\times10^{-19}$) in the MDCS. In the MDC-CC subcohort, each additional rs7903146 T allele was associated with 0.059 mmol/l higher fasting plasma glucose ($p=0.004$) and 0.027% (0.27 mmol/mol) higher HbA_{1c} ($p=0.02$) level (Table 1). Different genotype carriers reported similar mean intakes of total energy, carbohydrates, fats, protein and fibre (Table 1).

No significant interactions were found between rs7903146 and quintiles of carbohydrate, fat or protein intake ($p=0.91$, 0.47 and 0.70, respectively) and incident type 2 diabetes (Table 2). However, the risk of type 2 diabetes with the

Table 1 Characteristics of the MDCS cohort by *TCF7L2* genotype

Characteristic	*TCF7L2* genotype			OR (95% CI)[a] or β (SE)[b]	p_{trend}[c]
	CC	CT	TT		
n	13,571	9,488	1,740		
Incident T2DM (%)	741 (5.5)	757 (8.0)	151 (8.7)	1.44 (1.33, 1.56)[a]	4.6×10^{-19}
FPG (mmol/l)[d]	5.6±0.9	5.7±0.9	5.7±0.8	0.06 (0.02)	0.004
FPI (pmol/l)[d]	46.8±43.6	47.6±52.6	44.1±27.2	0.22 (1.00)	0.83
HbA_{1c} (%)[d]	4.8±0.5	4.8±0.5	4.9±0.5	0.03 (0.01)	0.02
HbA_{1c} (mmol/mol)[d]	39.7±5.2	40.0±5.3	40.1±4.8	0.27 (0.12)	0.02
Age (years)	58.1±7.6	58.0±7.6	58.0±7.6	−0.08 (0.07)	0.30
BMI (kg/m^2)	25.7±3.9	25.7±3.9	25.5±3.8	−0.08 (0.04)	0.06
Energy (kJ)	9,548±2,728	9,535±2,711	9,569±2,874	5.06 (24.2)	0.28
Carbohydrate (%E)	45.2±6.0	45.2±6.1	45.2±5.9	0.01 (0.06)	0.99
Fat (%E)	39.1±6.1	39.0±6.2	39.1±6.0	−0.02 (0.06)	0.75
Protein (%E)	15.7±2.6	15.8±2.5	15.8±2.5	0.01 (0.03)	0.79
Fibre (g/4,184 kJ)	9.0±2.7	9.0±2.7	9.1±2.8	0.003 (0.025)	0.91

Data are means ± SD unless otherwise stated

No. of individuals included in MDCS cohort, $n=24,799$

[a] Logistic regression model assuming an additive genetic model adjusting for age, sex and BMI

[b] β represents the difference generated by each additional T allele

[c] General linear model, assuming an additive genetic model adjusting for age, sex, BMI, season and method where appropriate

[d] Data available only for the MDC-CC, $n=5,216$

FPG, fasting plasma glucose; FPI, fasting plasma insulin; T2DM, type 2 diabetes mellitus

Table 2 OR of incident type 2 diabetes by *TCF7L2* rs7903146 genotype and quintiles of different dietary intakes in the MDCS

Dietary component	Mean intake	OR (95% CI)[a]				p_{trend}[a]	$p_{interaction}$[a,b]
		CC	CT	TT	Additive model		
Carbohydrate (%E)							0.91 (0.44)
Q1	36.9	1.00 (ref)	1.54 (1.21, 1.95)	2.09 (1.40, 3.11)	1.49 (1.25, 1.77)	8.3×10^{-6}	
Q2	42.1	1.00 (0.79, 1.26)	1.36 (1.07, 1.73)	1.62 (1.05, 2.51)	1.32 (1.10, 1.58)	2.9×10^{-3}	
Q3	45.2	0.92 (0.72, 1.17)	1.62 (1.28, 2.05)	1.85 (1.24, 2.76)	1.53 (1.29, 1.82)	9.5×10^{-7}	
Q4	48.2	0.87 (0.68, 1.11)	1.37 (1.07, 1.76)	1.71 (1.12, 2.60)	1.47 (1.23, 1.76)	3.0×10^{-5}	
Q5	53.7	0.91 (0.71, 1.16)	1.47 (1.16, 1.88)	1.34 (0.83, 2.17)	1.37 (1.14, 1.64)	9.0×10^{-4}	
p_{trend}		0.30	0.74	0.19			
Fat (%E)							0.47 (0.17)
Q1	30.5	1.00 (ref)	1.55 (1.23, 1.96)	1.63 (1.06, 2.51)	1.38 (1.16, 1.64)	3.0×10^{-4}	
Q2	35.9	0.73 (0.57, 0.94)	1.29 (1.01, 1.66)	2.03 (1.36, 3.03)	1.69 (1.41, 2.03)	1.9×10^{-8}	
Q3	39.0	0.98 (0.77, 1.24)	1.47 (1.15, 1.86)	1.55 (1.03, 2.35)	1.36 (1.14, 1.61)	5.8×10^{-4}	
Q4	42.2	0.88 (0.69, 1.12)	1.45 (1.14, 1.85)	1.53 (0.99, 2.38)	1.45 (1.21, 1.73)	5.1×10^{-5}	
Q5	47.6	0.98 (0.77, 1.24)	1.37 (1.07, 1.75)	1.66 (1.07, 2.58)	1.36 (1.13, 1.64)	9.4×10^{-4}	
p_{trend}		0.64	0.61	0.55			
Protein (%E)							0.70 (0.52)
Q1	12.5	1.00 (ref)	1.57 (1.21, 2.05)	1.81 (1.15, 2.86)	1.44 (1.19, 1.74)	2.2×10^{-4}	
Q2	14.4	1.12 (0.87, 1.45)	1.87 (1.45, 2.41)	1.69 (1.06, 2.69)	1.40 (1.17, 1.68)	2.3×10^{-4}	
Q3	15.6	1.02 (0.79, 1.32)	1.65 (1.27, 2.13)	2.02 (1.29, 3.15)	1.49 (1.24, 1.80)	3.0×10^{-5}	
Q4	16.9	1.25 (0.97, 1.60)	1.66 (1.28, 2.14)	2.18 (1.42, 3.33)	1.33 (1.12, 1.59)	1.5×10^{-3}	
Q5	19.4	1.24 (0.97, 1.60)	2.09 (1.62, 2.68)	2.63(1.78, 3.89)	1.51 (1.29, 1.77)	3.5×10^{-7}	
p_{trend}		0.12	0.10	0.04			
Fibre (g/4,184 kJ)							0.049 (0.006)
Q1	5.8	1.00 (ref)	1.35 (1.08, 1.70)	1.33 (0.86, 2.05)	1.24 (1.04, 1.47)	1.4×10^{-2}	
Q2	7.5	0.74 (0.58, 0.93)	1.09 (0.86, 1.38)	1.39 (0.90, 2.16)	1.43 (1.18, 1.72)	2.0×10^{-4}	
Q3	8.7	0.80 (0.63, 1.01)	1.29 (1.03, 1.63)	1.70 (1.15, 2.50)	1.52 (1.28, 1.80)	1.8×10^{-6}	
Q4	10.1	0.73 (0.57, 0.93)	1.14 (0.90, 1.46)	1.48 (0.98, 2.26)	1.49 (1.24, 1.80)	2.1×10^{-5}	
Q5	13.1	0.74 (0.58, 0.94)	1.41 (1.11, 1.79)	1.44 (0.94, 2.22)	1.56 (1.31, 1.86)	8.3×10^{-7}	
p_{trend}		0.029	0.78	0.64			

No. of individuals included in MDCS cohort, n=24,799

[a] Basic model with adjustments for age, sex, BMI, total EI, season and method

[b] Sensitivity analysis after excluding inaccurate reporters of EI using the basic model

CC, CT, TT denotes *TCF7L2* genotype; ref denotes reference value

TCF7L2 T allele increased from 24% to 56% from the lowest (mean intake: 5.8±0.8 g/4,184 kJ) to the highest (mean intake: 13.1±2.2 g/4,184 kJ) quintile of fibre intake ($p_{interaction}$=0.049). In the sensitivity analysis excluding potential inaccurate reporters of EI (18.3% of the study sample), the interaction between rs7903146 and quintiles of fibre intake was more evident (p=0.006) (Table 2). The interaction remained significant after further exclusion of individuals who reported a dietary change in the past (resulting in exclusion of 35.9% of the study sample) (p=0.046).

Since in several earlier studies fibre intake has been associated with protection against type 2 diabetes, we next analysed the effect of fibre intake on the risk of type 2 diabetes among different *TCF7L2* genotype carriers. When comparing the extreme groups of fibre intake (i.e. the highest quintile vs the lowest) separately within each genotype group, we found that higher fibre intake was associated with protection against type 2 diabetes among CC genotype carriers (OR 0.74, 95% CI 0.58, 0.94, p_{trend}=0.025), but not among CT or TT genotype carriers (CT: OR 1.03, 95% CI 0.80, 1.32, p_{trend}=0.77; TT: OR 1.13, 95% CI 0.62, 2.07, p_{trend}=0.60) (Fig. 1).

We next performed cross-sectional interaction analyses of the quantitative traits of fasting glucose and HbA_{1c} that have been reported to be associated with the *TCF7L2* variant in GWAS (Table 3). In the MDC-CC we did not detect any significant interaction between quintiles of fibre intake and *TCF7L2* genotype and baseline fasting plasma glucose

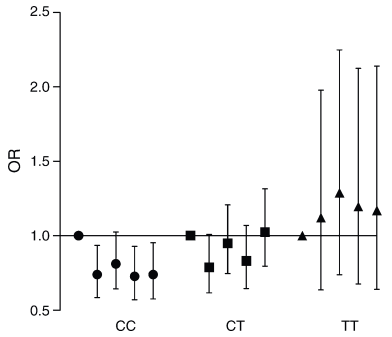

Fig. 1 ORs of type 2 diabetes in quintiles of fibre intake in strata of *TCF7L2* genotype in MDCS (n=24,799). We used the first quintile as a reference (OR 1) and adjusted for age, sex, BMI, total EI, season and method. Comparing the highest and lowest quintiles, a higher fibre intake was only protective among CC (circle) genotype carriers (p_{trend}=0.025). Higher fibre intakes were not associated with type 2 incidence among CT (square) (p_{trend}=0.77) and TT (triangle) (p_{trend}=0.60) genotype carriers. The error bars denote the 95% CI

levels (p=0.20). However, the association with elevated baseline HbA_{1c} levels increased significantly with higher fibre intake (effect size −0.021% (−0.21 mmol/mol) to 0.079% (0.80 mmol/mol) per T allele from the lowest to highest quintile, $p_{interaction}$=0.020), and the T allele was significantly associated with higher HbA_{1c} levels only in the highest quintile of fibre intake (p_{trend}=0.002) (Fig. 2). This result remained significant in a sensitivity analysis after excluding inaccurate reporters of EI ($p_{interaction}$=0.033).

We then analysed the association between fibre intake and HbA_{1c} levels among different *TCF7L2* genotype carriers (Table 3). Among all MDC-CC individuals, higher fibre intake was associated with lower HbA_{1c} levels (−0.03% [−0.3 mmol/mol], −0.04% [−0.4 mmol/mol] to −0.02% [−0.2 mmol/mol] per quintile of fibre intake, $p_{trend}=1.7\times10^{-4}$). This protective association was strongest among CC genotype carriers (−0.036% [−0.37 mmol/mol] per quintile, $p=6.5\times10^{-7}$), while a higher fibre intake was not associated with HbA_{1c} levels among TT genotype carriers (0.012% [0.13 mmol/mol], p=0.52) and carriers of both alleles appeared as an intermediate group (−0.023% [−0.24 mmol/mol], p=0.009) (Fig. 2).

As *TCF7L2* rs7903146 has been, in several studies, shown to associate more strongly with risk of type 2 diabetes among lean as compared with overweight individuals, we were concerned that some potential confounding could still be present after adjusting for BMI. In the MDCS the risk of type 2 diabetes associated with the *TCF7L2* T allele decreased from 86% to 31% from the lowest to highest BMI

Table 3 Mean fasting plasma glucose and HbA_{1c} by quintiles of fibre intake and *TCF7L2* genotype

Quintiles	*TCF7L2* genotype			Effect size[a]	p_{trend}[a]	$p_{interaction}$[b]
	CC	CT	TT			
Mean fasting glucose (mmol/l)						
Fibre (g/4,184 kJ)						
Q1	5.85	5.84	5.73	−0.006	0.91	0.20
Q2	5.65	5.70	5.83	0.09	0.03	
Q3	5.60	5.69	5.74	0.10	0.01	
Q4	5.59	5.60	5.67	0.009	0.80	
Q5	5.53	5.63	5.71	0.12	0.006	
Effect size[b]	−0.04	−0.03	−0.03			
p_{trend}[b]	0.0002	0.047	0.39			
Mean HbA_{1c} % (mmol/mol)						
Fibre (g/4,184 kJ)						
Q1	4.92 (40.8)	4.90 (40.6)	4.85 (40.1)	−0.021 (−0.21)	0.49	0.02
Q2	4.79 (39.5)	4.82 (39.4)	4.87 (40.3)	0.032 (0.33)	0.16	
Q3	4.81 (39.7)	4.86 (40.2)	4.85 (40.1)	0.033 (0.33)	0.21	
Q4	4.78 (39.4)	4.82 (39.7)	4.78 (39.4)	0.011 (0.12)	0.60	
Q5	4.75 (39.1)	4.80 (39.6)	4.93 (40.9)	0.079 (0.80)	0.002	
Effect size[b]	−0.036 (−0.37)	−0.023 (−0.24)	0.012 (0.13)			
p_{trend}[b]	6.5×10^{-7}	0.009	0.52			

Data are taken from n=5,216 individuals

[a] Basic model with adjustments for age, sex and BMI

[b] Basic model with adjustments for age, sex, BMI, total EI, season and method

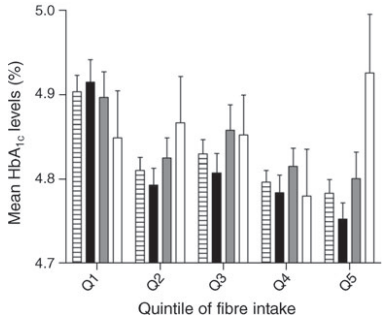

Fig. 2 Mean HbA_{1c} levels in quintiles of fibre intake, by the *TCF7L2* rs7903146 genotypes, in the MDC-CC (n=5,216). The associated effect size (β) per T allele on HbA_{1c} level increased in higher fibre intake groups ($p_{interaction}$=0.020) and the risk allele was associated with higher HbA_{1c} levels only in the highest quintile of fibre intake (β=0.08%, p=0.002). Individuals in the highest quintile of fibre intake had significantly lower HbA_{1c} levels compared with those in the lowest intake group (p<0.001). This association was driven by the strong associated effect per fibre intake quintile among the CC genotype carriers (β=−0.036%, $p=6.5\times10^{-7}$). No such association was observed among TT genotype carriers (β=0.012, p=0.52) while carriers of both alleles appeared as an intermediate group (β=−0.023%, p=0.009). To convert values for HbA_{1c} in % into mmol/mol, multiply by 10.11 and subtract 8.94. Genotype carriers: hatched bar, all; black bar, CC; grey bar, CT; white bar, TT. The error bars denote the SEM

quintile ($p_{interaction}$=0.009). However, dietary fibre intake and BMI did not correlate (r^2=−0.006, p=0.88) and separate interaction analyses of BMI tertiles indicated similar results in each BMI category.

Finally, each quintile of higher leisure-time PAL was associated with 6.4% reduced risk of type 2 diabetes (p=0.0003). However, PAL did not interact with *TCF7L2* genotype on type 2 diabetes incidence (p=0.46), and the interaction between fibre intake and *TCF7L2* genotype on type 2 diabetes incidence remained similar after further adjustment for leisure-time physical activity.

Discussion

Although type 2 diabetes is thought to result from a complex interplay between genetic predisposition and an unfavourable environment, very little is known about the interactions involved. We observed the risk increase of type 2 diabetes with the rs7903146 T allele to be significantly accentuated by increasing dietary fibre intake. Analyses of HbA_{1c} levels supported this observation as the rs7903146 T allele was only associated with higher HbA_{1c} levels among individuals with the highest fibre intake.

Several previous studies have reported a protective association between a high fibre intake and type 2 diabetes [28, 29]. Our results indicate that the protective effect of higher fibre intake is dependent on the genetic background of the individual, being limited to *TCF7L2* non-risk CC genotype carriers. This is in line with a recent report from the prospective EPIC-Potsdam case-control study reporting that the association between whole-grain intake and protection against type 2 diabetes is dependent on *TCF7L2* rs7903146 genotype; a high whole-grain intake was associated with protection among CC genotype carriers while individuals carrying one or two T alleles lacked such protection [12]. Our analyses of HbA_{1c} levels by *TCF7L2* genotype and fibre intake further support such an interaction, as the *TCF7L2* T allele was associated with higher HbA_{1c} levels only among individuals with higher fibre intake. Consistent with the observed dissimilar effects of fibre intake on type 2 diabetes incidence among different *TCF7L2* genotype carriers, a high fibre intake was strongly associated with lower HbA_{1c} levels among CC genotype carriers, while this association was completely lacking among TT genotype carriers. We did not find any interaction between dietary fibre intake and *TCF7L2* variant on fasting glucose levels, which is in line with a large meta-analysis of 14 cohorts (including MDC-CC), which reported no interaction between whole-grain intake and *TCF7L2* variant and fasting glucose levels [13].

The major strengths of our study include the high relative validity of our dietary assessment method, the combination of a diet diary with a questionnaire, the large sample size, the prospective design and the ability to identify inaccurate reporters of energy intake and individuals who had changed their diet in the past. In addition, the obtained association between higher dietary fibre intake and lower risk of type 2 diabetes and HbA_{1c} levels suggests that the dietary and type 2 diabetes incidence measures of the MDCS are adequate. Still, our study suffers from limitations including projection of the baseline diet data to the whole follow-up period in the prospective analyses (type 2 diabetes) and the limited causal inference in the cross-sectional analyses (HbA_{1c} levels). In addition, we did not correct the statistical analyses for multiple comparisons as the dietary variables are correlated and we had the possibility of repeating the test of interaction between *TCF7L2* rs7903146 and fibre intake on HbA_{1c} levels. Despite these limitations our interaction data from the prospective analyses were supported by the data obtained using a cross-sectional design. However, we need to keep in mind that the observed significance levels of the interactions were not robust and thus the possibility of false-positive findings cannot be excluded and therefore our results need to be replicated in other studies.

In our study, the protective association of higher dietary fibre intake with type 2 diabetes incidence was restricted to around 55% of the population who were non-carriers of the *TCF7L2* risk allele, while TT genotype carriers completely lacked such protection and CT carriers appeared as an intermediate group. Fibre intake has been associated with lower postprandial glucose and insulin concentrations, which have

been mainly attributed to slower intestinal absorption of nutrients [30]. In our study this was reflected among the CC genotype carriers who had a significantly lower HbA_{1c} level as well as a significantly lower incidence rate of type 2 diabetes when reporting high fibre intake. Dietary fibre has, in previous studies, been associated with inconsistently affected GLP-1 response, which could be due to differences in studied fibre types, the limited number of individuals studied and/or the short duration of the studies [31, 32]. However, it has been shown in hyperinsulinaemic individuals that after 9–12 months, higher fibre intake was associated with elevated plasma short-chain fatty acids (SCFAs), which are products of colonic fermentation of dietary fibre, and higher GLP-1 levels [33], pointing to a long-term effect of dietary fibre on glucose homeostasis. Several animal studies have shown that SCFAs are associated with increased expression of the proglucagon gene and GLP-1 secretion in rat intestinal cells [34–36]. At least part of the protective association of dietary fibre with the risk of type 2 diabetes could therefore be mediated by SCFAs through increased GLP-1 release. Since the *TCF7L2* T allele has previously been associated with an impaired incretin effect [7], it can be speculated that carriers of this risk allele could suffer from some degree of incretin resistance, leading to a lack of benefit from higher GLP-1 levels associated with SCFAs from higher fibre intake. This could be of clinical relevance, especially as many type 2 diabetes patients are on incretin-based treatment regimens and the risk allele carriers may benefit from these drugs to a lesser extent.

However, as systemic plasma SCFAs have previously been reported to increase after fermentable dietary fibre intake [37, 38], SCFAs may affect other tissues, such as pancreatic islets. Among the different SCFAs, butyrate has been identified as the most potent histone deacetylase inhibitor (HDACi) [39], which may be of interest because the rs7903146 risk variant sequence has been reported to confer an islet-specific open chromatin state translating to an elevated enhancer effect on *TCF7L2* transcription [40]. Butyrate as a fermentation product of dietary fibre could therefore play a role in further propagating the previously reported difference between the rs7903146 T allele carriers and the non-carriers via histone hyperacetylation, which may result in further enhanced transcription of the already overexpressed risk transcript. Another possibility could be the ability of an HDACi to increase the levels of active β-catenin [41].

To conclude, our study suggests that the *TCF7L2* risk variant modifies the protective association of dietary fibre intake with type 2 diabetes incidence and HbA_{1c} levels. Although our epidemiological observations cannot be translated into dietary advice for carriers of the *TCF7L2* risk allele, our results question whether a fibre-rich diet is protective against type 2 diabetes in all individuals, and by which mechanisms such protection may be lost in T allele carriers. Further studies are needed to answer these questions and to understand the mechanisms by which the *TCF7L2* risk variant increases the risk of type 2 diabetes.

Acknowledgements Of the 28,098 participants in the MDCS cohort, 1,758 incident diabetes cases and 1,758 controls have been included in the EPIC InterAct Consortium for the study of genetic factors and gene–lifestyle interactions with regard to incident diabetes. Being a large cohort study, the MDCS represents a different study design, compared with the case-control study design of EPIC InterAct. The dietary data used in EPIC InterAct have been harmonised between several study centres, and many details found in the MDCS dietary data are lacking in these harmonised data. Therefore, different study designs, different study sizes and unequal dietary data ensure the uniqueness of the present study vs the pooled analyses performed within EPIC InterAct.

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M. Orho-Melander is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of the data and the accuracy of the analysis. Part of this work was presented as an oral presentation at the EASD meeting in Lisbon, Portugal, in 2011.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement GH performed the statistical analyses, was involved in interpretation of the results, drafted the first version of the manuscript and revised the paper according to co-workers' advice. ES was involved in planning the study, as well as in statistical analyses, interpretation of the results and reviewing the manuscript. UE was involved in planning the study, as well as in statistical analyses, interpretation of the results and reviewing the manuscript. XJJ, YZ, OH and ER were involved in interpretation of the results, and reviewed the manuscript. EW was involved in planning the study and the statistical analyses, interpretation of the results and reviewed the revised and last version of the manuscript. MOM was involved in the planning of the study, the statistical analyses and interpretation of the data. MOM helped to draft the first version of the manuscript and reviewed the revised and last version of the manuscript. The authors provided their approval of the final version of the manuscript.

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Paper II

Type 2 diabetes associated variants in genes annotated to WNT signaling interact and dietary fiber in relation to incidence of type 2 diabetes

George Hindy, Ines G. Mollet, Gull Rukh, Ulrika Ericson, Marju Orho-Melander

Department of Clinical Sciences in Malmö, Skåne University Hospital, Lund University, Sweden

Corresponding author:

Professor Marju Orho-Melander
Department of Clinical Sciences in Malmö
Lund University
Clinical research center, 91:12, Jan Waldenströms gata 35
SE-205 02 Malmö
Sweden
Phone: +46 40 39 12 10
Fax: +46 40 39 12 22
E-mail: marju.orho-melander@med.lu.se

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Abstract

Background and aims: Short chain fatty acids such as butyrate are fermentation products of dietary fiber. Butyrate is a potent histone deacetylase inhibitor that can up-regulate WNT activity. TCF7L2 is a central transcription factor in the canonical WNT signaling pathway and genetic variants in *TCF7L2* has earlier been found to interact with dietary fiber intake on type 2 diabetes (T2D) incidence. Here we investigate whether other GWAS identified T2D genes may be involved in the WNT signaling pathway upstream of TCF/LEF interact and if single nucleotide polymorphisms (SNPs) in such genes may with dietary fiber on T2D incidence.

Materials and methods: We included 26,930 individuals without diabetes from the Malmö Diet and Cancer Study (MDCS) population based cohort. Diet data was collected at baseline (1991-1996) using a modified diet history method including an extensive food frequency questionnaire, a 7-day food diary, and an interview. Altogether 51 gene loci (58 SNPs) were analyzed for putative links to WNT-signaling using annotations from Gene Ontology, Kioto Encyclopedia of Genes and Genomes, Biocarta, Panther pathways and Literature integrated within the DAVID-ABR. Over a mean follow up period of 14 years 2,860 incident cases of T2D were recorded. COX proportional hazards regression was used to analyze association with incident T2D adjusted for age and sex and interaction between genotypes and quintiles of fiber intake adjusting for age, sex, BMI, total energy intake, season, diet method version, physical activity, smoking, alcohol intake, and education.

Results: Seven genes (9 SNPs) were annotated as involved in WNT-signaling upstream of TCF/LEF including *TCF7L2* (rs7903146 and rs12255372), *HHEX* (rs1111875), *HNF1A* (rs7957197), *NOTCH2* (rs10923931), *TLE4* (rs13292136), *ZBED3* (rs4457053) and *PPARG* (rs1801282 and rs13081389). SNPs in *TCF7L2*, *HHEX* and *HNF1A* loci associated significantly with incidence of T2D [HR 95% CI: 1.32 (1.24-1.39), 1.23 (1.16-1.30), 1.07 (1.01-1.12), and 1.14 (1.07-1.22) per risk allele]. SNPs in *TCF7L2*, *NOTCH2* and *ZBED3* showed significant interactions with fiber intake on T2D incidence ($P_{interaction}$ = 0.04, 0.007, 0.01, and 0.003, respectively). Higher fiber intake associated with lower T2D risk only among 1) homozygotes for the non-risk alleles of *TCF7L2* SNPs [HR 95% CI 0.95 (0.91-0.99) and 0.94 (0.90-0.98) per fiber intake quintile], 2) risk allele carriers of *NOTCH2* rs10923931 [HR 95% CI: 0.90 (0.84-0.97) and

0.70 (0.50-0.99) per fiber intake quintile]; 3) homozygotes for the risk allele of *ZBED3* rs4457053 [N=1643: HR 95% CI: 0.84 (0.75-0.94)].

Conclusion: Our results suggest that several T2D susceptibility SNPs in genes involved in WNT-signaling may interact with dietary fiber intake on T2D incidence. The putative mechanisms by which fiber intake could modify WNT signaling through these variants in T2D pathogenesis include effects on beta cell survival, adipogenesis, adipocyte proliferation, and/or GLP-1 production in intestinal L-cells.

Introduction

Type 2 diabetes is a multifactorial disease and both genetic and environmental factors, and their complex interactions, are thought to contribute to the disease development. Recently, more than 80 type 2 diabetes associated genetic variants have been discovered mainly through genome-wide association studies [1, 2]. Of these, the transcription factor 7-like 2 gene (*TCF7L2*) variant rs7903146 shows the strongest association with type 2 diabetes [3].

TCF7L2 is a principal transcription factor in the canonical wingless-type MMTV integration site (WNT) signaling pathway [4]. This highlights the importance of the canonical WNT pathway in the pathogenesis of type 2 diabetes. Many studies demonstrated an essential role of WNT signaling pathway in pancreatic islet beta cell genesis and proliferation [5]. Glucagon-like peptide -1 (GLP-1) mediated proliferation of rat beta cell line (INS-1) has been shown to occur through activation of the WNT pathway [6]. In addition, WNT signaling has been reported to mediate the expression of the pro-glucagon gene in the intestinal L cells and thus the synthesis of GLP-1 [7]. Moreover, activation of the WNT pathway has been observed to prevent pre-adipocyte differentiation [8].

Several studies have reported a protective association between higher dietary fiber intake and type 2 diabetes [9, 10]. Short chain fatty acids (SCFA), the by-products of colonic fermentation of dietary fibers, have been reported to hyper-activate WNT signaling in a colorectal cell line [11]. Fiber intake could thus exert its protective effect at least partially by modifying WNT activity through SCFAs. SCFAs have previously been associated with increased insulin secretion [12], promotion of adipocyte differentiation [13], and induction of GLP-1 secretion via the free fatty acid receptor 2 (FFAR2) [14]. Biologic interactions could thereby occur between fiber intake through SFCAs, and the WNT pathway on type 2 diabetes risk at the level of pancreatic beta cells, intestinal L cells, or adipocytes.

We have previously reported that the protective associated effect of higher fiber intake may be restricted to the non-risk CC genotype carriers of *TCF7L2* rs7903146 [15]. Similar results were also reported in the EPIC-Potsdam case-control study showing a protective association between higher whole grain intake and type 2 diabetes only among the non-risk allele carriers [16]. In line with these results, lower risk of type 2 diabetes by higher whole grain and cereal

fiber intake was restricted to the non-risk allele carriers in a subgroup of men in the Stockholm Diabetes Prevention Program [17]. Further, another lifestyle intervention demonstrated a greater weight loss by higher fiber intake after a 9-month intervention among individuals carrying the non-risk CC genotype compared to risk allele carriers [18].

As association between *TCF7L2* and type 2 diabetes has highlighted an important role for WNT signaling in type 2 diabetes pathogenesis we therefore used pathway analysis tools to evaluate if other type 2 diabetes associated loci are connected to this pathway. As several studies have indicated that dietary fiber or whole grain intake may modify the association between *TCF7L2* variants and type 2 diabetes or weight loss, we hypothesized that additional type 2 diabetes susceptibility genes in the WNT pathway may interact with fiber intake on type 2 diabetes incidence.

Methods

Study population

The Malmö Diet and Cancer Study (MDCS) is a population-based prospective cohort in the city of Malmö, in Sweden. The source population (74,138 individuals) was defined in 1991 to include all individuals living in Malmö and born between the years 1926 and 1945. In 1995 this definition was modified to include all men born between 1923 and 1945 and women born between 1923 and 1950 [19, 20]. Study participants were recruited through public advertisements or personal letters. Individuals with mental disability and limited Swedish language skills were excluded. Study participants visited the screening center twice during the baseline examination period between March 1991 and October 1996. During the first visit, participants received instructions on recording their meals in the menu book and on filling in the diet questionnaire and the extensive questionnaire covering socio-economic and lifestyle factors. Approximately 10 days later, subjects returned for a dietary history interview. By the end of the baseline examination period, complete dietary, anthropometric and lifestyle data was collected for 28,098 participants. Details of the recruitment procedures are described elsewhere [21].

After exclusion of prevalent type 2 diabetes patients, identified as individuals with a self-reported diabetes diagnosis or on a self-reported anti-diabetic regimen, we were left with 26,930 individuals for our prospective analyses. From the MDCS, 6,103 individuals were randomly selected to participate in a cardiovascular sub-cohort (MDC-CC). Additional measurements were

obtained for these individuals, including analysis of fasting blood glucose, fasting plasma insulin, and HbA_{1C} levels. After excluding prevalent cases of type 2 diabetes and individuals with no diet data this sub-cohort consisted of 5,507 individuals.

The MDCS was approved by the Ethical Committee at Lund University (LU 51-90). All participants provided written informed consent.

Incident type 2 diabetes

The Swedish National Diabetes Register (NDR) [22] and the Diabetes 2000 register of Skåne region [23] were used to identify incident cases of type 2 diabetes. Both of these registers only include individuals diagnosed by a physician according to established guidelines. We additionally used the local HbA_{1C} register, which contains data from institutional and non-institutional care in Malmö since 1988, to identify additional cases that were not diagnosed at the hospital [24]. Individuals were categorized as having diabetes if they had at least two recorded HbA_{1C} values above 6.0% using the Swedish Mono-S standardization system [corresponding to 6.9% with the U.S. National Glycohemoglobin Standardization Program (NGSP) and 52 mmol/mol with the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) units] [25, 26]. A total of 2,860 incident cases of type 2 diabetes were identified during the mean follow-up period of 14.3 years.

Dietary assessment

A modified dietary history method specially designed for the MDCS was used consisting of a 7-day menu book, a dietary 168-item questionnaire, and an interview. In the menu book participants were asked to record lunch and dinner meals, cold beverages including alcohol medicinal drugs, natural remedies, and nutrient supplements. The 168-item questionnaire was used to assess meal patterns, consumption frequencies and portion sizes of regularly consumed foods. A 48-page booklet was used to help participants at home estimate the portion sizes for the questionnaire. During the interview a more extensive book with photographs was used to estimate portion sizes and dishes in the menu book. Participants were also asked about their meal pattern, cooking methods, and food choices.

The average daily intake of foods was calculated using data from the menu book and diet questionnaire. The MDC Food and Nutrient Database, which was derived from PC KOST2-93 of

the Swedish National Food Administration, was used to convert the average daily food intake to energy and nutrient intakes [27, 28].

A slight alteration of the coding routines for dietary data was introduced in September 1994 [28]. A method variable, classifying data collected before and after September 1994, along with a four-category season variable (i.e. winter, spring, summer and autumn) was created and used as covariate to adjust for variation in data collection over time.

Dietary variables used in our analysis included total energy intake (kcal) and fiber intake as grams (g) per 1000 kcal. The relative validity of the dietary assessment method in MDCS has previously been evaluated in a sample of 50–69-year-old Malmö residents, 105 women and 101 men. The reference method used was 18 days' weighed food records (3 days every second month) collected over 1 year. Energy-adjusted Pearson correlation coefficients for fiber intake was 0.69 for women and 0.74 for men [29].

Other variables used as potential confounders

An extensive lifestyle questionnaire adapted from the Minnesota Leisure Time Physical Activity Questionnaire was used to assess leisure-time physical activity. Participants had to estimate the number of minutes per week for each season they spent performing each of 17 different physical activities. The duration was multiplied by an intensity factor to create a physical activity score that was divided into tertiles. Alcohol intake was classified into four categories based on grams of alcohol consumed per day: zero-consumers, low (<15 g/d in women or <20 g/d in men), medium (15–30 g/d in women or 20–40 g/d in men) and high consumers (>30 g/d in women or >40 g/d in men). Participants were classified as current smokers, ex-smokers and never-smokers. The education variable was created by classifying participants according to their highest educational level (≤8 years, 9–10 years, and 11–13 years at school, and university degree).

Genotyping

A total of 58 previously reported type 2 diabetes associated single nucleotide polymorphisms were selected for genotyping in the MDCS [1, 2]. Genotyping was performed by MassARRAY iPLEX (Sequenom, San Diego, CA, USA) or Taqman 5' nuclease (Applied Biosystems, Foster City, CA, USA) assays according to the manufacturers' instructions. The concordance rate was >99% in 5,500 samples which were genotyped using Human Omni Express Exome Bead Chip

Kit (Illumina, San Diego, CA, USA). Genotyping success rate ranged between 96-99%. All genotypes were in Hardy-Weinberg equilibrium (P>0.0009).

Gene annotation

A list of 51 unique gene loci, corresponding to 58 SNPs (MDC cohort) associated with T2D, were analyzed for links to WNT signaling using a combination of annotation from the Gene Ontology [30], the Kioto Encyclopedia of Genes and Genomes (http://www.kegg.jp/kegg/, accessed on the 15th of March 2013), Biocarta pathways (http://www.biocarta.com/genes/index.asp, accessed on the 15th of March 2013), Panther pathways [31, 32] and Literature integrated within the DAVID Annotation Bioinformatics Resource (http://david.abcc.ncifcrf.gov, accessed on the 15th of March 2013) .

Statistical analysis

Assuming an additive model, Cox proportional hazards regression was used to calculate the hazards ratio (HR) of incident type 2 diabetes associated with each risk allele in the MDCS adjusting for age and sex. After stratification to quintiles of relative intakes of fiber (g/1000kcal), interactions between the SNP genotypes and quintiles of fiber intakes on type 2 diabetes incidence were analyzed by introducing a multiplicative factor of the genotype and dietary quintiles as continuous variables in addition to these variables to the equation. Interactions were analyzed adjusting for age, sex, BMI, total energy intake, method and season as a basic model. Additional adjustments for physical activity, alcohol intake, smoking habits, and level of education were included in a full multivariable model. In the MDC-CC subcohort we performed cross-sectional analysis using linear regression to calculate the effect sizes of each WNT-annotated risk allele on baseline fasting plasma glucose, fasting plasma insulin, HOMA-IR, and HbA_{1C} adjusting for age and sex.

We used IBM SPSS Statistics, version 19 (SPSS Inc., Chicago, IL, USA), for the analyses. Two-sided p-values of <0.05 were considered significant.

Results

Association between fiber intake and type 2 diabetes incidence and related quantitative traits

The characteristics of the study population in quintiles of dietary fiber intake are shown in Table 1. A total of 2,860 incident cases of type 2 diabetes were recorded after a mean follow-up time of 14.3 years. The risk of type 2 diabetes was significantly lower among individuals in the higher quintiles of fiber intake (HR: 0.96; CI: 0.94-0.99; *P*: 0.01 per quintile). Higher fiber intake was observed to strongly associate ($P<0.001$) with reduced baseline fasting plasma glucose (FPG), fasting plasma insulin (FPI), homeostasis model for assessment of insulin resistance (HOMA-IR), and HbA_{1C} (Table 1).

Annotation of type 2 diabetes associated genes to the WNT pathway

Annotation of 51 gene loci corresponding to 58 SNPs associated with T2D resulted in identification of 7 genes with earlier detected connections to WNT signaling: *TCF7L2* (rs7903146 and rs12255372), *HHEX* (rs1111875), *HNF1A* (rs7957197), *NOTCH2* (rs10923931), *TLE4* (rs13292136), *ZBED3* (rs4457053) and *PPARG* (rs1801282 and rs13081389).

Association between WNT-annotated gene variants and type 2 diabetes and related traits

A total of 9 type 2 diabetes associated genes were annotated to the WNT signaling pathway and of these 7 genes (9 SNPs) were annotated upstream of TCF7L2 and selected for further analyses (Supplementary figure). These included *TCF7L2* (rs7903146 and rs12255372), *HHEX* (rs1111875), *HNF1A* (rs7957197), *NOTCH2* (rs10923931), *TLE4* (rs13292136), *ZBED3* (rs4457053) and *PPARG* (rs1801282 and rs13081389). Both *TCF7L2* variants (rs7903146 and rs12255372), *HHEX* rs1111875, and *HNF1A* rs7957197 were associated with increased risk of type 2 diabetes in MDCS (Table 2). In addition, the *TCF7L2* variants and the *HNF1A* variant associated with elevated baseline FPG. The *HHEX* variant associated with lower baseline FPI and the *TCF7L2* variants and the *ZBED3* variant associated with elevated baseline HbA_{1C} (Table 2).

Interaction between WNT-annotated gene variants and fiber intake on incidence of type 2 diabetes

In line with our previous report, *TCF7L2* rs7903146 interacted with quintiles of fiber intake on type 2 diabetes incidence ($P_{interaction} = 0.04$). However, our present study included 2,860 type 2 diabetes cases instead of 1,649 cases in our previous study [15]. Additionally, another *TCF7L2* variant, rs12255372, showed a somewhat stronger interaction with fiber intake on type 2 diabetes

incidence ($P_{interaction}$ = 0.007) (Table 3). The protective associated effect of higher fiber intake on type 2 diabetes incidence was restricted to homozygotes for the non-risk allele (GG genotype) (HR: 0.94; CI: 0.90-0.98; *P* = 0.005), while risk allele carriers lacked such protection. Moreover, individuals in the lowest fiber quintile did not show any increased risk of type 2 diabetes by the risk (T) allele (Table 4).

Of the WNT annotated SNPs (in genes upstream of TCF7L2) we found, in addition to *TCF7L2*, the SNPs in *NOTCH2* and *ZBED3* loci (Table 4) to significantly interact with fiber intake on type 2 diabetes incidence . In addition, none of the type 2 diabetes SNPs in genes not annotated to the WNT pathway showed significant interactions with fiber intake.

The *NOTCH2* locus was observed to interact with fiber intake on type 2 diabetes incidence ($P_{interaction}$ = 0.01). Higher fiber intake was observed to associate with lower risk of type 2 diabetes only among carriers of the risk (T) allele; GT (HR: 0.90; CI: 0.84-0.97; *P* = 0.004) and TT (HR: 0.70; CI: 0.50-0.99; *P* = 0.045). The *NOTCH2* risk allele did not associate with type 2 diabetes incidence in the whole population (HR: 1.00; CI: 0.91-1.09; *P* = 0.96); however it was associated significantly with decreased risk of type 2 diabetes in the fourth fiber intake quintile (HR: 0.72; CI: 0.57-0.92; *P* = 0.009) (Table 4). Another interaction was observed between quintiles of fiber intake and *ZBED3* rs4457053 on type 2 diabetes incidence ($P_{interaction}$ = 0.003). Higher fiber intake was observed to associate with lower risk of type 2 diabetes only among homozygotes for the risk (G) allele (HR: 0.84; CI: 0.74-0.94; *P* = 0.002). Although the *ZBED3* risk allele did not associate with type 2 diabetes incidence in the whole population it showed a tendency for increased risk in the lower quintiles of fiber intake and decreased risk in the upper quintiles (Table 4).

Discussion

In this prospective study we observe interactions between dietary fiber intake and variants in 3 genes annotated to the canonical WNT signaling pathway on type 2 diabetes incidence in a large population-based cohort. This indicates a putative role of the WNT pathway in the pathogenesis of type 2 diabetes and the putative effect of dietary fibers through this pathway.

Annotation of the 51 gene loci corresponding to 58 SNPs associated with type 2 diabetes resulted in identification of seven genes having known connections with WNT signaling. Novel interactions between dietary fiber and two type 2 diabetes associated variants in the *NOTCH2* and *ZBED3* loci were observed. Contrary to the *TCF7L2* findings, higher fiber intake was observed to be protective only among risk allele carriers of the *NOTCH2* variant and homozygotes for the risk allele of the *ZBED3* variant. Although none of the *NOTCH2* and *ZBED3* variants showed any association with type 2 diabetes in the MDCS, their risk alleles showed tendencies for increased type 2 diabetes risk in the lower quintiles of fiber intake and tendencies for decreased risk in the higher quintiles.

The WNT pathway may play a central role in the pathogenesis of type 2 diabetes. Pancreatic beta cell genesis and GLP-1 mediated proliferation have been shown to be mediated through activation of the WNT pathway [5, 6]. In addition to effects on beta cells, the WNT pathway mediates pro-glucagon gene expression in intestinal L cells and thus the synthesis of glucagon-like peptide 1 (GLP-1) [7]. Activation of the WNT pathway has been shown to keep pre-adipocytes in their undifferentiated state and could eventually result in ectopic fat accumulation and insulin resistance [8]. Recently, a liver specific knock out of *tcf7l2* in mice was linked to reduced hepatic glucose production [33]. WNT transcriptional activity has been reported to be hyper-activated by butyrate which is one of the SCFAs produced by microfloral fermentation of dietary fibers leading to apoptosis of colorectal cancer cells [11, 34]. Fiber intake is mainly linked to elevated luminal concentrations of SCFAs in the intestines. In addition, it has been linked to elevated SCFAs in plasma after 1 year of high fiber intervention [35]. Fiber intake could thus at least partially exert its systemic anti-diabetogenic effects through modulation of the WNT signaling pathway by SCFAs.

In this study we observe that the direction of the interaction effects were in opposite direction with variants in *NOTCH2* and *ZBED3* as compared to the *TCF7L2* variants. Reports from colon cancer research show that the WNT pathway should not be viewed as having an additive effect but rather as a continuum since both high and low WNT activity has been shown to promote colorectal cancer cell apoptosis [11, 34]. The dual role played by TCF7L2 adds to this complexity where it can act as a transcriptional activator in the presence of beta catenin but instead as a repressor in the absence of β-catenin [36, 37]. This highlights the complexity by

which WNT signaling may operate in the pathogenesis of type 2 diabetes and could explain the directional discrepancies observed in the interactions with fiber.

TCF7L2 risk allele is the most important common type 2 diabetes susceptibility variant. Most of the studies so far have reported beta cell dysfunction and an attenuated incretin effect associated with the risk allele [38, 39]. A 1-year high fiber intervention was linked to elevated plasma SCFAs and GLP-1 in hyperinsulinemic subjects [35]. Furthermore, SCFAs have been shown to induce GLP-1 secretion via the FFA receptor 2 (FFAR2) in mice [14]. Therefore, the carriers of the *TCF7L2* risk allele may suffer from beta cell incretin resistance and thus could be less prone to the beneficial associated effects of higher fiber intake through increased GLP-1 secretion. SCFAs, and particularly butyrate, are potent HDACis and this is particularly important as *TCF7L2* variant has been linked to an islet specific open chromatin state which could be exacerbated with higher fiber intake [40]. ZBED3 has been recently identified as an axin binding protein that modulates the WNT signaling pathway [41]. Binding of the ZBED3 to axin prevents the formation of the destruction complex that normally phosphorylates beta catenin and targets it for proteasomal degradation. *ZBED3* risk allele has been shown to be associated with increased ZBED3 expression in adipose tissue and this could translate to elevated WNT activity [42]. The *NOTCH2* gene encodes a transmembrane protein which physically associates with active beta catenin and decreases its levels leading to lower WNT activity in both stem and colon cancer cells [43]. However, whether the *NOTCH2* variant associates with NOTCH2 expression is not yet known.

The high relative validity of our dietary assessment method, the combination of a diet diary with a questionnaire, the large sample size, and the prospective design with a long follow-up period are the major strengths of our study. However, our study has several limitations that need to be discussed. Although our study population is large, it still has a limited power for detecting interactions which could result in many false negative outcomes. Therefore, replication of our results is important in other studies with reasonable power and good quality of diet data. We did not correct for multiple testing as our study is hypothesis driven and we believe that the selected WNT-associated genes are biologically dependent and the interactions with the *NOTCH2* and *ZBED3* variants may reflect essentially the interactions with *TCF7L2*. Finally, our

diet data was collected at baseline and may fail to detect the effect of the changes in dietary habits over the follow-up period.

Our results indicate that several T2D susceptibility SNPs in genes involved in WNT-signaling may interact with dietary fiber intake on T2D incidence. The putative mechanisms by which fiber intake could modify WNT signaling through these variants in T2D pathogenesis include effects on beta cell survival, adipogenesis and adipocyte proliferation, and/or GLP-1 production in intestinal L-cells. In addition, functional studies are needed to better understand the putative role played by dietary fiber in type 2 diabetes through the WNT pathway.

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Table 1. Characteristics of MDCS in quintiles of fiber intake

		Quintiles of fiber intake						
	Total n = 26,930	Q1 n = 5386	Q2 n = 5386	Q3 n = 5386	Q4 n = 5386	Q5 n = 5386	B (SE) or HR (CI)	P-value[a]
Incident T2D	2860 (10.6%)	610 (11.3%)	594 (11.0%)	612 (11.4%)	515 (9.56%)	529 (10.9%)	0.96 (0.94-0.99)	0.01
Sex (% males)	10,443 (39%)	3,042 (56%)	2428 (45%)	2064 (38%)	1,640 (30%)	1,269 (24%)		5×10^{-83}
Age (years)	57.98 ± 7.62	57.40 ± 7.47	57.73 ± 7.66	58.19 ± 7.73	58.33 ± 7.72	58.26 ± 7.50		4×10^{-88}
BMI	25.65 ± 3.91	25.49 ± 3.95	25.65 ± 3.81	25.83 ± 3.94	25.73 ± 3.87	25.53 ± 3.98	0.02 (0.02)	0.20
FPG[b]	5.65 ± 0.84	5.82 ± 1.05	5.69 ± 0.81	5.63 ± 0.79	5.58 ± 0.74	5.56 ± 0.81	-0.04 (0.008)	3×10^{-07}
FPI[b]	46.50 ± 46.22	50.80 ± 43.44	46.33 ± 33.68	47.51 ± 50.49	45.08 ± 59.28	43.93 ± 38.91	-1.09 (0.46)	2×10^{-07}
HOMA-IR[b]	1.60 ± 1.22	1.75 ± 1.32	1.64 ± 1.06	1.65 ± 1.62	1.53 ± 1.04	1.49 ± 1.00	-0.06 (0.01)	9×10^{-09}
HbA_{1C}[b]	4.82 ± 0.50	4.90 ± 0.59	4.82 ± 0.45	4.82 ± 0.53	4.80 ± 0.43	4.77 ± 0.49	-0.03 (0.005)	3×10^{-10}

Data are means ± SD unless otherwise stated

Number of individuals included in MDCS cohort, *N*=26,930

[a] Adjusted for age, sex, bmi, total energy intake, dietary method, and season where appropriate

[b] Data available only for the MDC-CC, *N*=5,507

Table 2. Association with type 2 diabetes and related traits in MDCS

	Incident T2D[a]		FPG (mmol/L)[b]		FPI (pmol/L)[b]		HbA_{1C}[b]	
	HR (95% CI)	P-value	Beta (SE)	P-value	Beta (SE)	P-value	Beta (SE)	P-value
TCF7L2 rs7903146	1.32 (1.24-1.39)	9×10^{-21}	0.05 (0.02)	0.01	0.04 (0.18)	0.73	0.03 (0.01)	0.03
TCF7L2 rs12255372	1.23 (1.16-1.30)	5×10^{-12}	0.04 (0.02)	0.04	-0.11 (0.18)	0.69	0.02 (0.01)	0.03
HHEX rs1111875	1.07 (1.01-1.12)	0.02	-0.02 (0.02)	0.22	-0.51 (0.17)	0.015	0.01 (0.01)	0.27
HNF1A rs7957197	1.14 (1.07-1.22)	0.0002	0.05 (0.02)	0.02	0.19 (0.20)	0.52	0.02 (0.01)	0.22
NOTCH2 rs10923931	1.00 (0.91-1.09)	0.96	0.03 (0.03)	0.30	-0.03 (0.29)	0.72	0.01 (0.02)	0.43
TLE4 rs13292136	1.00 (0.91-1.10)	0.94	0.004 (0.03)	0.88	0.28 (0.29)	0.41	0.02 (0.02)	0.26
ZBED3 rs4457053	1.02 (0.96-1.08)	0.61	0.02 (0.02)	0.22	-0.23 (0.19)	0.18	0.03 (0.01)	0.03
PPARG rs1801282	1.06 (0.98-1.14)	0.17	0.03 (0.02)	0.16	0.20 (0.23)	0.39	0.00 (0.01)	1.00
PPARG rs13081389	0.99 (0.89-1.09)	0.81	0.04 (0.03)	0.26	0.28 (0.30)	0.32	0.01 (0.02)	0.60

[a] Adjusted for age and sex, *N*=26,930

[b] Adjusted for age and sex; data available only for the MDC-CC, *N*=5,507

Table 3. Interaction between SNPs in WNT associated genes and quintiles of fiber intake on type 2 diabetes incidence

	RAF	Total	Cases	HR	95% CI	$P_{interaction}$
TCF7L2 rs7903146	0.26	24596	2664	1.04	1.00-1.09	0.04
TCF7L2 rs12255372	0.27	24979	2657	1.06	1.01-1.10	0.007
HHEX rs1111875	0.59	25649	2712	1.03	0.99-1.08	0.11
HNF1A rs7957197	0.80	26003	2761	0.99	0.95-1.04	0.74
NOTCH2 rs10923931	0.10	25197	2645	0.92	0.86-0.98	0.01
TLE4 rs13292136	0.08	26064	2768	1.00	0.93-1.07	0.96
ZBED3 rs4457053	0.26	24450	2605	0.94	0.90-0.98	0.003
PPARG rs1801282	0.86	25835	2742	0.96	0.91-1.01	0.13
PPARG rs13081389	0.93	24832	2661	1.03	0.95-1.10	0.40

Adjusted for age, sex, bmi, total energy intake, dietary method, and season

Table 4. Risk of type 2 diabetes by genotype and quintiles of fiber intake

		Genotype					
		xx	xR	RR	HR per RA	P_{trend}	$P_{interaction}$[a(b)]
TCF7L2 rs7903146							
	Q1	1.29 (1.07-1.55)	1.75 (1.45-2.10)	1.63 (1.16-2.30)	1.20 (1.06-1.37)	0.004	0.06 (0.04)
	Q2	1.20 (1.00-1.44)	1.65 (1.37-1.99)	2.22 (1.64-3.02)	1.30 (1.14-1.47)	1×10^{-6}	
	Q3	1.21 (1.01-1.46)	1.67 (1.39-2.01)	2.19 (1.65-2.92)	1.36 (1.20-1.54)	1×10^{-6}	
	Q4	1.09 (0.90-1.31)	1.37 (1.13-1.67)	1.74 (1.27-2.38)	1.24 (1.08-1.42)	0.003	
	Q5	1 (ref)	1.75 (1.45-2.11)	2.08 (1.53-2.83)	1.50 (1.31-1.70)	1×10^{-9}	
	HR per Q	0.95 (0.91-0.99)	0.98 (0.94-1.02)	1.01 (0.92-1.12)			
	P_{trend}	0.01	0.31	0.81			
TCF7L2 rs12255372							
	Q1	1.36 (1.14-1.63)	1.58 (1.31-1.90)	1.34 (0.93-1.92)	1.05 (0.92-1.19)	0.5	0.009 (0.007)
	Q2	1.23 (1.03-1.48)	1.37 (1.13-1.66)	2.08 (1.56-2.77)	1.20 (1.06-1.36)	0.005	
	Q3	1.12 (0.93-1.34)	1.59 (1.33-1.91)	1.96 (1.48-2.61)	1.35 (1.19-1.53)	2×10^{-6}	
	Q4	1.10 (0.92-1.32)	1.26 (1.04-1.53)	1.60 (1.17-2.19)	1.16 (1.01-1.53)	0.04	
	Q5	1 (ref)	1.44 (1.19-1.74)	2.08 (1.53-2.84)	1.42 (1.24-1.63)	3×10^{-7}	
	HR per Q	0.94 (0.90-0.98)	0.97 (0.92-1.01)	1.02 (0.93-1.13)			
	P_{trend}	0.005	0.11	0.67			
NOTCH2 rs10923931							
	Q1	1.08 (0.94-1.24)	1.30 (1.04-1.62)	1.80 (0.90-3.61)	1.14 (0.94-1.38)	0.18	0.009 (0.013)
	Q2	1.05 (0.91-1.21)	1.12 (0.90-1.38)	0.95 (0.36-2.55)	1.10 (0.90-1.33)	0.35	
	Q3	1.08 (0.94-1.24)	1.13 (0.91-1.40)	0.99 (0.41-2.40)	1.04 (0.86-1.27)	0.66	
	Q4	0.99 (0.86-1.14)	0.67 (0.51-0.88)	0.83 (0.31-2.22)	0.72 (0.57-0.92)	0.009	
	Q5	1 (ref)	1.02 (0.81-1.29)	0.27 (0.04-1.95)	0.96 (0.77-1.20)	0.73	
	HR per Q	0.98 (0.95-1.01)	0.90 (0.84-0.97)	0.70 (0.50-0.99)			
	P_{trend}	0.26	0.004	0.045			
ZBED3 rs4457053							
	Q1	1.04 (0.88-1.23)	1.11 (0.93-1.33)	1.50 (1.10-2.03)	1.13 (0.99-1.29)	0.06	0.004 (0.003)
	Q2	0.99 (0.84-1.17)	1.04 (0.87-1.25)	1.21 (0.86-1.68)	1.07 (0.93-1.22)	0.36	
	Q3	1.04 (0.88-1.22)	1.10 (0.92-1.32)	0.81 (0.55-1.19)	1.00 (0.88-1.15)	0.97	

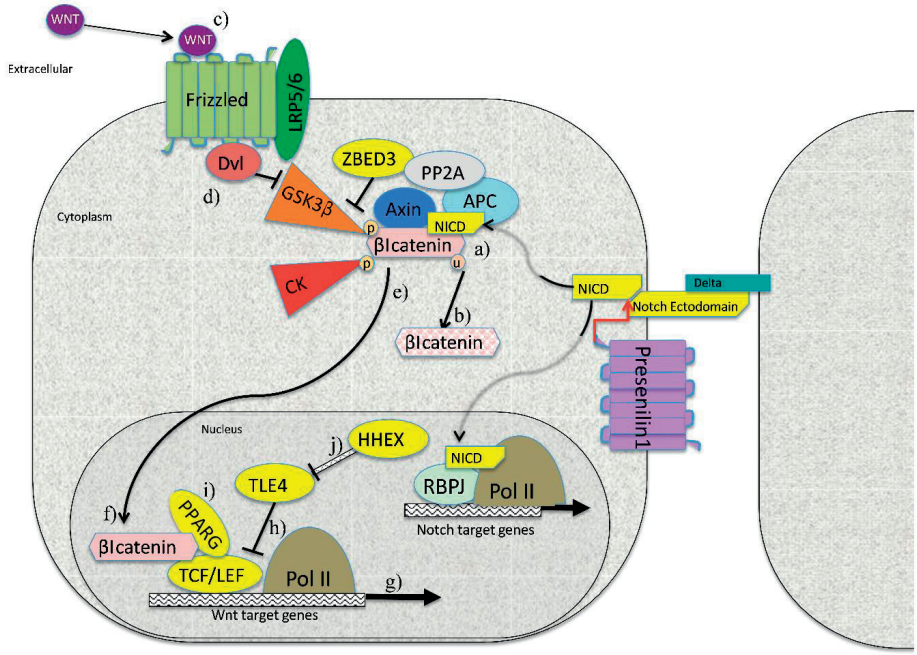
	Q4	0.93 (0.78-1.10)	0.96 (0.80-1.16)	0.63 (0.42-0.96)	0.96 (0.82-1.11)	0.55
	Q5	1 (ref)	0.98 (0.82-1.19)	0.68 (0.47-1.01)	0.92 (0.79-1.06)	0.25
	HR per Q	0.98 (0.94-1.02)	0.97 (0.93-1.02)	0.84 (0.74-0.94)		
	P_{trend}	0.36	0.23	0.002		

R = risk increasing allele

[a] Adjusted for age, sex, bmi, total energy intake, dietary method, and season

[b] Adjusted for age, sex, bmi, total energy intake, dietary method, season, physical activity, alcohol intake, smoking, and education

Supplementary figure:



Functional positioning of ZBED3, TLE4, TCF7L2, HNF1A, HHEX, PPARG and NOTCH2 within the Wnt Canonical pathway

Functional positioning of these genes to the WNT pathway is represented in Figure 9. When WNT receptor ligands are absent, transcriptional cofactor β-catenin is bound to a destruction complex established around the Axin/APC scaffolding complex (a), (only major components shown: GSK3β, Glycogen synthase kinase 3; CK, Casein kinases; PP2A, Protein phosphatase 2A; APC, Adenomatous polyposis coli). β-catenin is regulated not only through changes to its cytoplasmic concentration, but also through its cellular localization and extensive protein modification. The Axin degradation complex marks β-catenin for proteolysis (b) via phosphorylation and ubiquitination. When ligands bind to the Frizzled-LRP5/6 receptor complex (c), it activates Dishevelled (Dvl) in the cytoplasm of that cell (d). Dvl in turn promotes the attachment of the Axin/APC destruction complex to the Frizzled-LRP5/6 (LRP, Low density lipoprotein receptor-related protein) receptor complex followed by LRP5/6-mediated breakdown of Axin and concomitant release of β-catenin (e)[1, 2]. Zinc-finger BED domain-containing 3 (ZBED3)[3] binds to Axin leading to inhibition of GSK3β-mediated β-catenin phosphorylation and resulting in cytoplasmic accumulation of free β-catenin, that subsequently translocates to the nucleus where it binds to T-cell factor/lymphoid enhancer factor (TCF/LEF, e.g. TCF7L2 a.k.a. TCF4, HNF1A a.k.a TCF1)[4] family of transcription modulator complexes on WNT target gene promoters (f) to activate their transcription (g). Factors such as TLE1, TLE2, TLE3 and TLE4 repress the latter transactivation mediated by TCF/LEF complexes and β-catenin (h). Transcription factor PPARG interacts with β-catenin and TCF7L2 (i), but also appears to be a target of the WNT pathway in cancer cells.[5, 6] HHEX, on the other hand wields its enhancing action on WNT signaling by repression of

TLE4 expression (j).[7] The direct NOTCH-signaling pathway involves interaction of NOTCH with its ligand Delta, then NOTCH undergoes proteolytic cleavage by Presenilin releasing the Notch Intra-Cellular Domain (NICD),[8] which enters the nucleus and interacts with RBPJ to regulate transcription of specific NOTCH target genes. Convergence between NOTCH signaling and WNT signaling appears to mostly mediate cell fate.[8, 9] However, there are several pieces of evidence supporting interaction between WNT and NOTCH signaling pathways upstream of gene transcription through which NOTCH downregulates WNT signaling.[10-14] β-catenin interacts directly with, and is modulated by, the NOTCH receptor in a ligand independent manner, [10, 15] there is also evidence for a functional interaction between Axin and ACP in fine-tuning the intracellular traffic of NOTCH.[14, 16] Dvl also interacts with NOTCH [17] and GSK3 can also phosphorylate NOTCH.[18, 19] In addition to proteolytic processing of NOTCH there is evidence that presenilin 1 also associates with β-catenin.[20] This supports the notion that WNT and NOTCH signaling are an integrated functional module along with the adherens junctions/cadherin pathway (not shown) regulating β-catenin activity and localization and consequently influencing cell fate of physically adjacent cells.

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Paper III

TCF7L2 risk variant carriers lack the associated effect of dietary fiber intake on lower risk of metabolic syndrome

George Hindy, Ulrika Ericson, Emily Sonestedt, Peter M Nilsson, Olle Melander, Marju Orho-Melander

Diabetes and Cardiovascular Disease, Genetic Epidemiology, Department of Clinical Sciences in Malmö, Lund University, Sweden

Short Title: TCF7L2, dietary fibre intake and metabolic syndrome

Corresponding author:

Professor Marju Orho-Melander
Department of Clinical Sciences in Malmö
Lund University
Clinical research center, 91:12, Jan Waldenströms gata 35
SE-205 02 Malmö
Sweden
Phone: +46 40 39 12 10
Fax: +46 40 39 12 22

E-mail: marju.orho-melander@med.lu.se

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ABSTRACT

Dietary patterns rich in fibers have previously been associated with lower risk for type 2 diabetes (T2D) and the metabolic syndrome. The *TCF7L2* rs7903146 genotype has been reported to modify the associated effect of dietary fiber and whole grains on incidence of T2D. Our aim was to study if *TCF7L2* rs7903146 modifies the protective association of dietary fiber on metabolic syndrome and associated quantitative traits. We first performed a cross-sectional interaction analysis of the Malmö Diet and Cancer Cardiovascular Cohort (MDC-CC) of 4,606 individuals free from diabetes and cardiovascular disease and with dietary intake assessed using a combination of a 7-day diet diary, a questionnaire, and an interview. In total 1,247 individuals were classified as having metabolic syndrome using the NCEP ATP III criteria. Second, we performed a longitudinal interaction analysis among 2,337 individuals without metabolic syndrome or diabetes at baseline of which 775 fulfilled the criteria of metabolic syndrome after a mean follow-up time of 15 years. Higher fiber intake was associated with lower waist circumference, body fat percentage, fasting plasma glucose, fasting plasma insulin, and HOMA-IR at baseline. Among CC-genotype carriers, but not among T-allele carriers, higher fiber intake was associated with lower baseline prevalence and future incidence of metabolic syndrome and with beneficial levels of several associated quantitative traits with significant interactions observed on prevalence of metabolic syndrome and waist circumference at baseline. Our results suggest that the *TCF7L2* rs7903146 risk T-allele carriers may lack the beneficial associated effect of dietary fiber intake on the risk of metabolic syndrome and several associated features.

INTRODUCTION

TCF7L2 is the strongest reported type 2 diabetes (T2D) susceptibility locus (1). However, the role of TCF7L2 in the pathogenesis of T2D remains incompletely understood. While much of the evidence is pointing to beta-cell dysfunction and defective incretin action (2; 3), less is known about its role in other putatively affected functions like adipogenesis, inflammation, liver metabolism or adrenal and intestinal hormone secretion (5).

Several studies have reported a protective association between dietary fiber intake and T2D (17; 18). Further, dietary patterns rich in fruits and vegetables and poor in fatty foods and processed meat have been associated with favorable features of the metabolic syndrome (19) and in intervention studies higher consumption of diets rich in cereal fiber (20) and adherence to a Mediterranean-style diet (21) have been observed to be associated with lower prevalence of metabolic syndrome.

We have recently reported that carriers of the *TCF7L2* rs7903146 risk T-allele may lack the protective associated effect of higher dietary fiber intake on T2D incidence (7). Similar findings were earlier observed in the EPIC-Potsdam case-control study showing that the protective association between higher whole grain intake and T2D was restricted to individuals homozygous for the non-risk allele (CC-genotype carriers) (8). In line with these results, lower risk of T2D by higher whole grain and cereal fiber intake was restricted to CC-genotype carriers in a subgroup of men in the Stockholm Diabetes Prevention Program (9). In addition, the Tübingen Lifestyle Intervention Program (TULIP) reported an interaction between the rs7903146 variant and dietary fiber intake on weight loss: in contrast to risk-allele carriers, the CC-genotype carriers demonstrated significantly greater weight loss when committed to a high fiber diet (11).

A recent systematic review reported the *TCF7L2* rs7903146 T-allele to be more frequent among individuals with metabolic syndrome (12) and this allele has also been associated with an altered lipid profile and an impaired lipid metabolism (13; 14). As dietary fiber has been associated with lower, and the *TCF7L2* rs7903146 T-allele with higher risk of the metabolic syndrome, and as several studies have indicated an interaction between dietary fiber intake and *TCF7L2* risk variant on the risk of T2D or weight loss, our study was set up to test the hypothesis if *TCF7L2* rs7903146 may modify the protective association between dietary fiber and the metabolic syndrome and associated quantitative traits.

RESEARCH DESIGN AND METHODS

Study population. The Malmö Diet and Cancer Study (MDCS) is a population-based cohort in the city of Malmö, in southern Sweden. Baseline data collection was conducted during the period between 1991 and 1996 for women born between 1923 and 1950 and men born between 1923 and 1945 (22; 23). Recruitment was done through public advertisements or personal letters. Individuals with mental disability or limited Swedish language skills were excluded. At baseline dietary intake was assessed using a modified history method (give reference). Data on socioeconomic and lifestyle factors were collected using an extensive questionnaire. By the end of the baseline examination period, 28,098 participants had complete dietary, anthropometric and lifestyle data. Details of the recruitment procedures are described elsewhere (24). The MDCS was approved by the Ethical Committee at Lund University (LU 51-90) and all of the participants provided written informed consent.

Of the MDCS cohort, 6,103 individuals were randomly selected to participate in a cardiovascular sub-cohort (MDC-CC). These individuals underwent additional baseline measurements that included total cholesterol (TC), low-density lipoprotein cholesterol (LDLC), high-density lipoprotein cholesterol (HDLC), triglycerides (TG), and fasting blood glucose (FBG). From this population we excluded individuals with prevalent diabetes (identified as individuals with a self-reported diabetes diagnosis or on a self-reported anti-diabetic regimen), and prevalent cardiovascular disease (identified as individuals with a history of coronary event or stroke). After these exclusions, we included 4,606 individuals with complete data on genotype, diet, fasting blood tests, and blood pressure to the current study.

From MDC-CC, 3,734 individuals underwent a follow up re-examination between the years 2007 and 2010. A total of 2,337 individuals free from diabetes, cardiovascular disease, and metabolic syndrome (see below for definition) at baseline, and with complete baseline and follow-up data on genotype, diet, fasting blood tests, and blood pressure, were included in the prospective analyses.

Dietary measurements. In MDCS an interview-based, modified dietary history method was used. Participants were provided with a 7-day menu book for recording lunch and dinner meals and cold beverages, nutrient supplements, medicinal drugs, and natural remedies. In addition, a 168-item dietary questionnaire was used to assess portion sizes, consumption

frequencies and meal patterns of regularly consumed foods that were not covered by the menu book. This was followed by a 45 minutes interview performed by trained interviewers. Portion sizes and dishes in the menu book were estimated during the interview using a more extensive book with photographs.

The average daily food intakes obtained from the menu book and the diet questionnaire were converted to energy- and nutrient intakes using the MDC Food and Nutrient Database, which was designed for the MDCS and was derived from PC KOST2-93 of the Swedish National Food Administration (25; 26). The coding routines for dietary data were slightly modified in September 1994 (26). To adjust for variation in data collection over time, a method variable, classifying data collected before and after September 1994, along with a four-category season variable were created and used as covariates. The relative validity of the dietary assessment method has been previously evaluated using an 18 days weighed food records collected over 1 year. Energy-adjusted Pearson correlation coefficients for fiber intake was 0.69 for women and 0.74 for men (27).

Other variables used as potential confounders. An extensive lifestyle questionnaire adapted from the Minnesota Leisure Time Physical Activity Questionnaire was used to assess leisure-time physical activity. An estimate of the number of minutes per week for each season for 17 different physical activities was obtained for each participant. A physical activity score was created by multiplying the duration by an intensity factor and then ranked into tertiles. Participants were classified as current smokers, ex-smokers and never-smokers. Four categories of alcohol intake were created based on grams of alcohol consumed per day: zero-consumers, low (<15 g/d in women or <20 g/d in men), medium (15–30 g/d in women or 20–40 g/d in men) and high consumers (>30 g/d in women or >40 g/d in men). An education variable, classifying participants according to their highest educational level, was created (≤ 8 years, 9–10 years, and 11–13 years at school, and university degree).

Clinical measurements. A balance-beam scale was used to measure weight (kilograms) with subjects wearing light clothing and no shoes. A fixed stadiometer was used to measure height (centimeters). Waist circumference (WC) (centimeters) was measured midway between the lowest rib margin and iliac crest. BMI was measured as weight in kilograms divided by height in meters squared. Body composition was estimated using a bioelectric impedance analyzer (BIA 103; JRL Systems, Mt. Clemens, MI). Body fat percentage was calculated using an algorithm provided by the manufacturer.

Fasting serum lipids and whole blood glucose (FBG) were measured from blood samples drawn after an overnight fast. Samples were analyzed by standard methods at the Department of Clinical Chemistry, Malmö University Hospital. A routine hexokinase method was used to measure FBG at baseline and fasting plasma glucose (FPG) at the follow-up visit. FBG was converted to FPG by multiplying with 1.13. TG and TC were measured by a DAX 48 automatic analyzer (Bayer AB, Göteborg, Sweden) and HDLC after precipitation of LDLC and very low-density lipoprotein cholesterol with dextran sulphate. LDLC was calculated using the Friedewald formula (28). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the formula: (fasting plasma glucose × fasting plasma insulin (FPI))/22.5.

Metabolic syndrome was defined using the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) (29). Thus, an individual was classified as having the metabolic syndrome if at least three of the following criteria were met: (a) large WC (≥ 102 cm for men and ≥ 88 cm for women); (b) low plasma HDLC levels (< 1.0 mmol/l for men and < 1.3 mmol/l for women); (c) high plasma TG levels (plasma TG concentrations ≥ 1.7 mmol/l and/or lipid lowering treatment); (d) elevated blood pressure (≥ 130/85 mm Hg and/or antihypertensive treatment) and (e) elevated FPG (≥ 5.6 mmol/l) as suggested by the American Diabetes Association (ADA) (30). A total of 1,269 cases fulfilled the criteria of metabolic syndrome at baseline and 755 cases at follow-up.

Genotyping. TaqMan polymerase chain reaction (PCR) method (Applied Biosystems, Foster City, CA, USA), was used to genotype *TCF7L2* rs7903146 according to the manufacturer's instructions. Post-PCR allelic discrimination was done by measuring allele-specific fluorescence using the ABI Prism Sequence Detection System ABI 7900HT (Applied Biosystems). The concordance rate was >99% in randomly repeated 325 samples. Genotyping success rate was 96% and the genotypes were in Hardy-Weinberg equilibrium (P = 0.16). At baseline, 2,534 (55.0%) individuals carried the CC genotype, 1,745 (37.9%) the CT genotype, and 327 (7.1%) the TT genotype and among individuals with prospective data the corresponding frequencies were 54.0 %, 38.9 % and 7.1%, respectively.

Statistical Analyses. PASW statistics version 21 (SPSS Inc., Chicago, IL) was used for the statistical analyses. Logistic regression was used to obtain Odds Ratios (ORs) for metabolic syndrome per *TCF7L2* T-allele in all individuals and in strata of tertiles of fiber intake adjusting for age and sex, as well as per tertile of fiber intake in all individuals and in strata of

TCF7L2 genotype adjusting for age, sex, method, season, total energy intake, leisure time physical activity, alcohol intake, smoking status, and level of education. A general linear model was used to obtain the effect size per *TCF7L2* T-allele among all individuals and in strata of tertiles of fiber intake adjusting for age and sex, as well as per tertile of fiber intake among all individuals and in strata of *TCF7L2* genotypes on baseline WC, HDLC, TG, FPG, FPI, SBP, DBP, body fat percentage, BMI, TC, LDLC, and HOMA-IR adjusting for age, sex, method, season, and total energy intake, leisure time physical activity, alcohol intake, smoking status, and level of education. The values of HDLC, TG, TC, LDLC, and HOMA-IR were natural log transformed to achieve normality of distribution.

Tests for interactions were performed by including the tertiles of fiber intake, the *TCF7L2* genotype, and their multiplicative factor adjusting for age, sex, method, season, and total energy intake, leisure time physical activity, alcohol intake, smoking status, and level of education. Adjustments for BMI were done in an additional model. *P* values < 0.05 were considered statistically significant. All reported *P* values are two-sided.

RESULTS

Association between *TCF7L2* rs7903146 and fiber intake with metabolic syndrome and its component traits at baseline

The *TCF7L2* rs7903146 risk T-allele did not associate with metabolic syndrome at baseline (OR 0.99, 95% CI 0.89-1.10, p = 0.88). Of the individual component traits of metabolic syndrome the T-allele associated with higher baseline FPG (β 0.05 mmol/L, P = 0.005) (Table 1). Higher fiber intake was not associated with prevalence of the metabolic syndrome at baseline (OR 0.95, 0.87-1.04, p=0.29 per higher tertile of fiber intake). Each tertile of fiber intake associated with a smaller WC (β –0.37 cm, P = 0.05), lower body fat percentage (β –0.21 %, P = 0.02), lower FPG (β –0.03 mmol/L, P = 0.04), lower FPI (β –1.44 pmol/L, P = 0.00006) and lower HOMA-IR levels (β –0.09, P = 2×10^{-6}) (Table 2).

Interaction between *TCF7L2* rs7903146 and fiber intake on metabolic syndrome and its component traits at baseline

TCF7L2 rs7903146 genotype modified the associated effect of fiber intake on the prevalence of metabolic syndrome at baseline ($P_{interaction}$ = 0.02). After stratifying the baseline population by *TCF7L2* genotype, higher fiber intake associated with lower prevalence of the metabolic

syndrome only among homozygotes for the non-risk allele (CC genotype carriers) (OR 0.87, 0.78-0.98, P = 0.03 per tertile), but not among CT and TT genotype carriers (P = 0.38 and 0.55, respectively) (Fig. 1).

Of the individual components of the metabolic syndrome, *TCF7L2* genotype modified the associated effect of fiber intake on WC ($P_{interaction}$ = 0.001). Higher fiber intake associated with a smaller WC (β –0.71 cm per tertile, P = 0.006) among CC genotype carriers but not among CT and TT genotype carriers (P = 0.77 and 0.31, respectively) (Table 3). Furthermore, higher fiber intake associated with lower TG levels only among CC genotype carriers (β –0.04 mmol/l per tertile, P=0.02), but not among CT and TT genotype carriers (P = 0.49 and 0.80, respectively). However, the modified associated effect did not reach statistical significance ($P_{interaction}$ = 0.15) (Table 3). The result on lipid traits and blood pressure remained similar after additional adjustments for lipid lowering and antihypertensive treatments, respectively.

Interaction between *TCF7L2* rs7903146 and fiber intake on other traits related to the metabolic syndrome at baseline

Of the other traits related to the metabolic syndrome (BMI, body fat percentage, TC, LDLC, FPI and HOMA-IR), *TCF7L2* genotype was observed to modify the associated effect of fiber intake on body fat percentage ($P_{interaction}$ = 0.03), TC ($P_{interaction}$ = 0.007), LDLC ($P_{interaction}$ = 0.018), and tendencies for interaction was observed on FPI ($P_{interaction}$ = 0.09) and HOMA-IR ($P_{interaction}$ = 0.06) (Table 3). Restricted to CC-genotype carriers, higher fiber intake associated with lower body fat percentage (β –0.34 % per tertile, P = 0.007), lower FPI levels (β –1.66 % per tertile, P = 0.00005) and lower HOMA-IR (β –0.12 per tertile, P = 2×10^{-06}) (Table 4). In contrast, among TT genotype carriers higher fiber intake associated with higher TC levels (β 0.20 mmol/L per tertile, P = 0.01) and higher LDLC levels (β 0.20 mmol/L per tertile, P = 0.007) (Table 4).

***TCF7L2* rs7903146, fibre intake and risk of future metabolic syndrome**

In the prospective analyses of 2337 individuals not fulfilling the criteria for metabolic syndrome, nor having diabetes or cardiovascular disease at baseline, the *TCF7L2* rs7903146 T-allele did not associate with future risk of metabolic syndrome (OR 0.96, 0.84-1.10, P = 0.57), and higher fiber intake was not associated with the risk of future metabolic syndrome (OR 0.92, 0.82-1.04, P = 0.19 per tertile). In line with the results from the cross-sectional

analyses, higher fiber intake associated with lower incidence of future metabolic syndrome only among CC-genotype carriers (OR 0.84, 0.72-0.98, P = 0.03 per tertile), but not among CT and TT genotype carriers (P = 0.75 and 0.33, respectively) (Fig. 2). However, the test for interaction did not reach statistical significance (P = 0.20).

DISCUSSION

We have previously reported that the *TCF7L2* rs7903146 variant interacts with dietary fiber intake on T2D incidence such that higher fiber intake associated with lower T2D risk only among individuals not carrying any risk alleles (7). In addition, a similar modifying effect of fiber intake by the *TCF7L2* variant on its positive health effects has been seen in other studies (8; 9; 11).

In the present study we observe that the *TCF7L2* variant modifies the association of dietary fiber intake on prevalent metabolic syndrome and baseline WC. Furthermore, we observe an interaction on several quantitative traits related to the metabolic syndrome (body fat percentage, TC, LDLC, and HOMA-IR). Our findings suggest that higher fiber intake associates with lower prevalence of the metabolic syndrome only among individuals not carrying any *TCF7L2* risk alleles (CC genotype carriers). This finding is also supported by our prospective analysis in which the protective association on incident metabolic syndrome was restricted to non-risk allele carriers although the test for interaction did not reach statistical significance. Our results also suggest that only among non-risk allele carriers, higher fiber intake associates with smaller WC, lower TG levels, lower body fat percentage, and lower HOMA-IR. Furthermore, the TT genotype carriers not only lacked the association with metabolically healthier phenotypes but also had higher TC and LDLC associated with high fiber intake.

Dietary cereal fiber was previously associated with lower prevalence of the metabolic syndrome in the Framingham Offspring Cohort (20). Several studies have reported that higher consumption of dietary fiber has a beneficial effect on insulin sensitivity (31-33) and the lipid profile (34; 35). In our study we observe a strong beneficial association of higher fiber intake with insulin sensitivity (HOMA-IR). However, the mechanism by which dietary fiber may increase insulin sensitivity is not fully understood. High fiber intake was previously associated with elevated plasma levels of short chain fatty acids (SCFA), which are products of colonic fermentation of fibers, and GLP-1 (36). *In vivo* studies have indicated that SCFAs

may induce insulin sensitization by inhibiting adipose tissue lipolysis and decreasing levels of circulating free fatty acids (FFA) (37; 38). It has also been reported that HDACi activity of SCFAs and particularly butyrate stimulates adipocyte differentiation (39). A recent study provided evidence that SCFAs can induce GLP-1 secretion via the FFA receptor 2 (FFAR2) in mice (40). Therefore, the insulin sensitizing effects of dietary fiber may at least be partially mediated through SCFAs, which may decrease FFA and/or increase GLP-1, which has insulin sensitizing effects (41).

TCF7L2 risk allele is the most important common T2D susceptibility variant. Most of the studies so far have reported beta cell dysfunction and an attenuated incretin effect associated with the risk allele (2; 42). *TCF7L2* risk allele has been reported to be more prevalent among individuals with Metabolic syndrome (12), and to associate with an altered lipid profile (13) and impaired postprandial lipid metabolism (14). It is well known that TCF7L2 is a transcription factor in the WNT signaling pathway (4), which was previously reported to have an inhibitory effect on preadipocyte differentiation and adipogenesis (43). Failure of preadipocytes to differentiate may lead to hypertrophic growth of adipocytes which was linked to whole-body insulin resistance (44). *TCF7L2* rs7903146 risk T-allele has been shown to be negatively correlated with splice variants retaining exons 13a in human subcutaneous adipose tissue and these isoforms predominated at day 2 during adipocyte differentiation and then fell by 70% by day 10 (45). This provides some evidence that carrying *TCF7L2* risk allele may attenuate adipocyte differentiation. Thus carrying the risk genotype may interfere with the HDACi effect of SCFAs on adipocyte differentiation, thus explaining at least part of the interactions observed. Recently, GLP-1 was reported to promote preadipocyte proliferation and inhibit apoptosis which could lead to insulin sensitization and improved lipid homeostasis (46). This could explain part of the insulin sensitizing effects of dietary fiber mediated through GLP-1 after stimulation by SCFAs. This could also explain at least part of the interactions observed; in a sense similar to the incretin resistance in beta cells conferred by carrying the risk variant, the risk variant could attenuate preadipocyte differentiation, and lead to a lack of the beneficial effect of preadipocyte proliferation associated with GLP-1. Not only did individuals homozygous for the risk allele (TT) lack the beneficial effect of higher fiber intake but also had higher TC and LDLC as compared to CC genotype carriers.

Higher fiber intake has been linked with a favorable intestinal microbiota and lower plasma lipopolysaccharide (LPS) endotoxemia (47) which have been implicated in adipose tissue inflammation and insulin resistance (48). LPS may reach the circulation through increased intestinal permeability (48). A reduced *TCF7L2* expression has been previously linked to decreased Paneth cell α-defensins in Crohn's disease, and *TCF7L2* rs3814570 associated with ileal Crohn's disease (49). This may also explain part of the interactions observed where TCF7L2 may affect gut inflammation and leakiness of LPS.

The high relative validity of our dietary assessment and the combination of a diet diary and a diet history questionnaire are major strengths of our study. However, our study is limited as significant interactions were observed in the cross-sectional analysis of data that can limit causal inference. However, the similar modifying effect of fiber intake by the *TCF7L2* risk variant on incident metabolic syndrome support our results although it did not reach statistical significance which is probably due to the lower number of incident metabolic syndrome cases that may decrease the power. However, our present interactions are supported by previously reported interaction between the *TCF7L2* risk variant and fiber intake on T2D incidence in the Malmö Diet and Cancer prospective cohort (7) and by the results from the Tübingen Lifestyle Intervention Program (TULIP) where a greater weight loss was reported among individuals carrying the CC genotype as compared to carriers of the T-allele after a nine-month higher fiber intake intervention (11).

In conclusion, our study suggests that carriers of the risk variant may lack the beneficial effect of dietary fiber on several metabolic traits that characterize the metabolic syndrome. It also suggests that the *TCF7L2* risk variant may exert its diabetogenic and dysmetabolic effects not only through defects in beta cells, but also through effects in other organs and tissues, possibly including the adipose tissue, liver, and/or intestine.

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TABLE 1
Characteristics of the Malmö Diet and Cancer Study-Cardiovascular Cohort by *TCF7L2* rs7903146 genotype

	TCF7L2 rs7903146 genotype			OR (95%CI)	
	CC	CT	TT	or β (SE)*	P_{trend}
n	2534	1745	327		
Sex (male), *n* (%)	1039 (41.0)	685 (39.4)	122 (36.9)		
Age (years)	57.55 ± 0.12	57.43 ± 0.14	57.42 ± 0.33	-0.09 (0.14)	0.51
MetS, *n* (%)	708 (27.6)	466 (26.2)	95 (28.5)	0.99 (0.89-1.10)	0.88
BMI (kg/m^2)	25.62 ± 0.08	25.53 ± 0.09	25.41 ± 0.21	-0.10 (0.09)	0.29
WC (cm)	83.22 ± 0.20	83.24 ± 0.24	82.76 ± 0.55	-0.11 (0.23)	0.63
Body fat (%)	27.10 ± 0.10	26.98 ± 0.11	26.94 ± 0.26	-0.10 (0.11)	0.38
FPG (mmol/L)	5.61 ± 0.02	5.66 ± 0.02	5.72 ± 0.04	0.05 (0.02)	0.005
FPI	46.0 ± 0.92	47.1 ± 1.11	44.6 ± 2.55	0.13 (1.09)	0.79
SBP (mmHg)	140.8 ± 0.4	141.0 ± 0.4	141.3 ± 9.8	0.21 (0.42)	0.62
DBP (mmHg)	86.90 ± 0.18	86.69 ± 0.22	86.66 ± 0.50	-0.16 (0.22)	0.46
TC (mmol/L)	6.17 ± 0.02	6.17 ± 0.03	6.13 ± 0.06	-0.01 (0.03)	0.68
LDLC (mmol/L)	4.18 ± 0.02	4.17 ± 0.02	4.12 ± 0.05	-0.02 (0.02)	0.52
HDLC (mmol/L)	1.39 ± 0.01	1.40 ± 0.01	1.40 ± 0.02	0.003 (0.008)	0.68
TG (mmol/L)	1.33 ± 0.01	1.33 ± 0.02	1.34 ± 0.04	0.005 (0.016)	0.55
HOMA-IR	1.59 ± 0.03	1.60 ± 0.03	1.59 ± 0.07	0.007 (0.030)	0.76
Total energy intake (kcal)	2325 ± 12	2333 ± 14	2344 ± 32	9 (14)	0.51
Fiber (g/1000 kcal)	9.36 ± 0.06	9.38 ± 0.07	9.37 ± 0.15	0.01 (0.07)	0.83

All data is presented as mean ± SEM
*Adjusted for age and sex
MetS, Metabolic Syndrome; BMI, Body Mass Index; WC, Waist Circumference; FPG, Fasting Plasma Glucose; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; TC, Total Cholesterol; LDLC, Low Density Lipoprotein Cholesterol; HDLC, High Density Lipoprotein Cholesterol; TG, Triglycerides; FPI, Fasting Plasma Insulin; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance

TABLE 2
Characteristics of the Malmö Diet and Cancer Study-Cardiovascular Cohort by tertiles of fiber intake

	Fiber intake tertiles			OR (95%CI)	
	Low	Medium	High	or β (SE)*	P_{trend}*(†)
n	1535	1536	1535		
Sex (male), *n* (%)	829 (54.0)	624 (40.6)	393 (25.6)		
Age (years)	57.13 ± 0.15	57.46 ± 0.15	57.91 ± 0.15	0.39 (0.11)	0.0003
MetS, *n* (%)	454 (29.6)	416 (27.1)	377 (24.6)	0.95 (0.87-1.04)	0.29
BMI (kg/m^2)	25.51 ± 0.10	25.74 ± 0.10	25.46 ± 0.10	-0.02 (0.08)	0.75
WC (cm)	83.37 ± 0.26	83.56 ± 0.25	82.64 ± 0.26	-0.37 (0.19)	0.05 (0.001)
Body fat (%)	27.19 ± 0.13	27.16 ± 0.12	26.78 ± 0.13	-0.21 (0.09)	0.02 (0.003)
FPG (mmol/L)	5.68 ± 0.02	5.62 ± 0.02	5.62 ± 0.02	-0.03 (0.02)	0.04 (0.04)
FPI	47.3 ± 1.24	47.1 ± 1.18	44.5 ± 1.23	-1.44 (0.91)	0.00006 (0.00001)
SBP (mmHg)	140.7 ± 0.47	140.9 ± 0.45	141.1 ± 0.47	0.22 (0.35)	0.53 (0.48)
DBP (mmHg)	86.98 ± 0.24	86.80 ± 0.23	86.63 ± 0.24	-0.17 (0.18)	0.33 (0.36)
TC (mmol/L)	6.18 ± 0.03	6.16 ± 0.03	6.17 ± 0.03	-0.007 (0.02)	0.67 (0.68)
LDLC (mmol/L)	4.18 ± 0.03	4.16 ± 0.03	4.18 ± 0.03	-0.001 (0.019)	0.84 (0.86)
HDLC (mmol/L)	1.39 ± 0.01	1.40 ± 0.01	1.39 ± 0.01	0.002 (0.007)	0.74 (0.80)
TG (mmol/L)	1.35 ± 0.02	1.33 ± 0.02	1.32 ± 0.02	-0.01 (0.01)	0.19 (0.20)
HOMA-IR	1.67 ± 0.03	1.62 ± 0.03	1.49 ± 0.03	-0.09 (0.03)	2×10^{-6} (8×10^{-8})
Total energy intake (kcal)	2483 ± 15	2332 ± 15	2173 ± 15	-156 (11)	1×10^{-44} (1×10^{-44})
Fiber intake (g/1000 kcal)	6.60 ± 0.02	9.02 ± 0.02	12.49 ± 0.06		

All data is presented as mean ± SEM
*Adjusted for age, sex, total energy intake, season, method, physical activity, alcohol intake, smoking status, and education as needed
†Additional adjustment for BMI
MetS, Metabolic Syndrome; BMI, Body Mass Index; WC, Waist Circumference; FPG, Fasting Plasma Glucose; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; TC, Total Cholesterol; LDLC, Low Density Lipoprotein Cholesterol; HDLC, High Density Lipoprotein Cholesterol; TG, Triglycerides; FPI, Fasting Plasma Insulin; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance

TABLE 3
Mean values of metabolic syndrome components in tertiles of fiber intake by *TCF7L2* rs7903146 genotype in the Malmö Diet and Cancer Study-Cardiovascular Cohort

	TCF7L2 rs7903146 genotype					
Fiber intake	CC	CT	TT	Effect Size (SE)*	P_{trend}*	$P_{interaction}$†(‡)
WC (cm)						
Low	83.9 ± 0.34	83.1 ± 0.42	80.3 ± 1.15	-1.12 (0.42)	0.008	0.008 (0.001)
Medium	83.7 ± 0.34	83.3 ± 0.40	84.3 ± 0.98	0.31 (0.39)	0.42	
High	82.5 ± 0.36	82.9 ± 0.40	81.9 ± 1.14	0.44 (0.39)	0.26	
Effect size (SE)†	-0.71 (0.26)	-0.09 (0.30)	0.88 (0.87)			
P_{trend}†	0.006	0.77	0.31			
HDLC (mmol/L)						
Low	1.39 ± 0.01	1.39 ± 0.01	1.42 ± 0.04	0.001 (0.01)	0.77	0.76 (0.99)
Medium	1.39 ± 0.01	1.42 ± 0.01	1.39 ± 0.03	0.008 (0.01)	0.49	
High	1.39 ± 0.01	1.38 ± 0.01	1.41 ± 0.04	-0.001 (0.01)	0.71	
Effect size (SE)†	0.004 (0.009)	-0.003 (0.01)	-0.007 (0.03)			
P_{trend}†	0.53	0.81	0.62			
TG (mmol/L)						
Low	1.37 ± 0.03	1.32 ± 0.03	1.28 ± 0.08	-0.03 (0.03)	0.56	0.15 (0.24)
Medium	1.34 ± 0.02	1.30 ± 0.03	1.41 ± 0.06	0.006 (0.03)	0.88	
High	1.29 ± 0.03	1.37 ± 0.03	1.29 ± 0.07	0.04 (0.03)	0.10	
Effect size (SE)†	-0.04 (0.02)	0.03 (0.02)	0.004 (0.06)			
P_{trend}†	0.02	0.49	0.80			
FPG (mmol/L)						
Low	5.67 ± 0.03	5.69 ± 0.04	5.78 ± 0.08	0.03 (0.04)	0.40	0.43 (0.61)
Medium	5.59 ± 0.03	5.64 ± 0.04	5.73 ± 0.06	0.07 (0.03)	0.02	
High	5.59 ± 0.03	5.65 ± 0.04	5.63 ± 0.07	0.06 (0.03)	0.055	
Effect size (SE)†	-0.04 (0.02)	-0.02 (0.03)	-0.07 (0.06)			
P_{trend}†	0.07	0.45	0.22			
SBP (mmHg)						
Low	141.3 ± 0.6	139.6 ± 0.8	141.6 ± 1.8	-0.61 (0.73)	0.41	0.47 (0.59)
Medium	140.4 ± 0.6	141.6 ± 0.7	141.4 ± 1.5	1.01 (0.70)	0.15	
High	141.1 ± 0.6	141.4 ± 0.8	140.3 ± 1.7	0.26 (0.73)	0.72	
Effect size (SE)†	-0.12 (0.46)	0.88 (0.57)	-0.58 (1.33)			
P_{trend}†	0.79	0.12	0.66			

DBP (mmHg)						
Low	87.3 ± 0.3	86.7 ± 0.4	86.4 ± 0.9	-0.49 (0.39)	0.22	0.59 (0.76)
Medium	86.8 ± 0.3	86.8 ± 0.4	86.9 ± 0.8	0.14 (0.37)	0.71	
High	86.8 ± 0.3	86.5 ± 0.4	86.3 ± 0.9	-0.11 (0.36)	0.76	
Effect size (SE)†	-0.27 (0.24)	-0.05 (0.29)	-0.07 (0.69)			
P_{trend}†	0.26	0.86	0.92			

All data is presented as mean ± SEM
*Adjusted for age and sex
† Adjusted for age, sex, method, season, total energy, leisure time physical activity, alcohol intake, smoking and education level
‡Additional adjustment for BMI
WC, Waist Circumference; HDLC, High Density Lipoprotein Cholesterol; TG, Triglycerides; FPG, Fasting Plasma Glucose; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure

TABLE 4
Mean values of other metabolic syndrome related traits in tertiles of fiber intake by *TCF7L2* rs7903146 genotype in the Malmö Diet and Cancer Study-Cardiovascular Cohort

	TCF7L2 rs7903146 genotype					
Fiber (g/1000 kcal)	CC	CT	TT	Effect Size*	P_{trend}*	$P_{interaction}$†(‡)
BMI (kg/m^2)						
Low	25.6 ± 0.1	25.4 ± 0.2	24.9 ± 0.4	-0.32 (0.16)	0.04	0.29
Medium	25.8 ± 0.1	25.7 ± 0.2	26.0 ± 0.4	0.04 (0.15)	0.82	
High	25.5 ± 0.1	25.5 ± 0.2	25.2 ± 0.4	-0.02 (0.16)	0.88	
Effect size (SE)†	-0.07 (0.10)	0.03 (0.12)	0.14 (0.33)			
P_{trend}†	0.48	0.82	0.67			
Body Fat (%)						
Low	27.3 ± 0.2	27.1 ± 0.2	26.6 ± 0.5	-0.52 (0.20)	0.01	0.03 (0.049)
Medium	27.0 ± 0.2	27.0 ± 0.2	28.1 ± 0.4	0.05 (0.19)	0.79	
High	26.6 ± 0.2	27.0 ± 0.2	26.8 ± 0.5	0.17 (0.19)	0.37	
Effect size (SE)†	-0.34 (0.13)	-0.06 (0.14)	0.14 (0.39)			
P_{trend}†	0.007	0.69	0.73			
TC (mmol/L)						
Low	6.20 ± 0.04	6.19 ± 0.05	5.99 ± 0.11	-0.06 (0.04)	0.21	0.007 (0.009)
Medium	6.20 ± 0.04	6.14 ± 0.04	6.03 ± 0.10	-0.08 (0.04)	0.05	
High	6.12 ± 0.04	6.20 ± 0.04	6.38 ± 0.11	0.10 (0.04)	0.01	
Effect size (SE)†	-0.04 (0.03)	0.007 (0.03)	0.20 (0.04)			
P_{trend}†	0.14	0.85	0.01			
LDLC (mmol/L)						
Low	4.19 ± 0.04	4.20 ± 0.04	3.99 ± 0.10	-0.05 (0.04)	0.30	0.018 (0.023)
Medium	4.20 ± 0.03	4.12 ± 0.04	3.99 ± 0.09	-0.09 (0.04)	0.018	
High	4.14 ± 0.04	4.19 ± 0.04	4.38 ± 0.10	0.09 (0.04)	0.016	
Effect size (SE)†	-0.02 (0.03)	-0.002 (0.030)	0.20 (0.08)			
P_{trend}†	0.34	0.86	0.007			
FPI						
Low	47.8 ± 1.5	47.4 ± 2.3	42.9 ± 3.0	-1.80 (1.58)	0.16	0.09 (0.21)
Medium	46.1 ± 1.5	48.7 ± 2.2	47.0 ± 2.5	1.66 (2.28)	0.45	

High	44.4 ± 1.6	44.9 ± 2.2	42.6 ± 2.8	0.45 (1.70)	0.23	
Effect size (SE)†	-1.66 (1.13)	-1.31 (1.67)	-0.09 (2.20)			
P_{trend}†	0.00005	0.14	0.88			
HOMA-IR						
Low	1.72 ± 0.04	1.64 ± 0.06	1.54 ± 0.12	-0.10 (0.05)	0.09	0.06 (0.19)
Medium	1.58 ± 0.04	1.65 ± 0.06	1.73 ± 0.10	0.08 (0.06)	0.24	
High	1.48 ± 0.04	1.52 ± 0.06	1.46 ± 0.11	0.03 (0.04)	0.26	
Effect size (SE)†	-0.12 (0.03)	-0.06 (0.05)	-0.04 (0.09)			
P_{trend}†	2×10^{-06}	0.10	0.48			

All data is presented as mean ± SEM
*Adjusted for age and sex
† Adjusted for age, sex, method, season, total energy, leisure time physical activity, alcohol intake, smoking and education level
‡Additional adjustment for BMI
BMI, Body Mass Index; TC, Total Cholesterol; LDLC, Low Density Lipoprotein Cholesterol; FPI, Fasting Plasma Insulin; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance

FIG. 1. (A) Odds ratio (OR) for prevalent metabolic syndrome per tertile of fiber intake according to *TCF7L2* rs7903146 genotype in the Malmö Diet and Cancer Study-Cardiovascular Cohort at baseline. High tertiles of fiber intake associated with lower metabolic syndrome prevalence only among CC genotype carriers (OR 0.87, 0.78-0.98, P = 0.03) but not among CT or TT genotype carriers. (B) OR for prevalent metabolic syndrome per *TCF7L2* risk allele in tertiles of fiber intake. *TCF7L2* rs7903146 risk T-allele associated with lower metabolic syndrome prevalence only in the lowest tertile of fiber intake (OR 0.82, 0.68-0.98, P = 0.03) but not among individuals in the higher fiber intake tertiles. Significant interactions were observed between *TCF7L2* genotype and fiber intake on metabolic syndrome at baseline (*P*=0.02; adjusted for age, sex, method, season, total energy intake leisure time physical activity, alcohol intake, smoking, and education level).

FIG. 2. (A) Odds ratio (OR) for incident metabolic syndrome per tertile of fiber intake according to *TCF7L2* rs7903146 genotype in the Malmö Diet and Cancer Study-Cardiovascular Cohort at follow up. High tertiles of fiber intake associated with lower metabolic syndrome incidence only among CC genotype carriers (OR 0.84, 0.71-0.98, P = 0.03) but not among CT or TT genotype carriers. (B) OR for incident metabolic syndrome per *TCF7L2* risk allele in tertiles of fiber intake. *TCF7L2* rs7903146 risk T-allele did not associate with metabolic syndrome incidence in tertiles of fiber intake. No significant interactions were observed between *TCF7L2* genotype and fiber intake on the risk of metabolic syndrome at follow up using the basic model (*P*=0.20; adjusted for age, sex, method, season, and total energy intake, leisure time physical activity, alcohol intake, smoking, and education level).

FIG. 1.

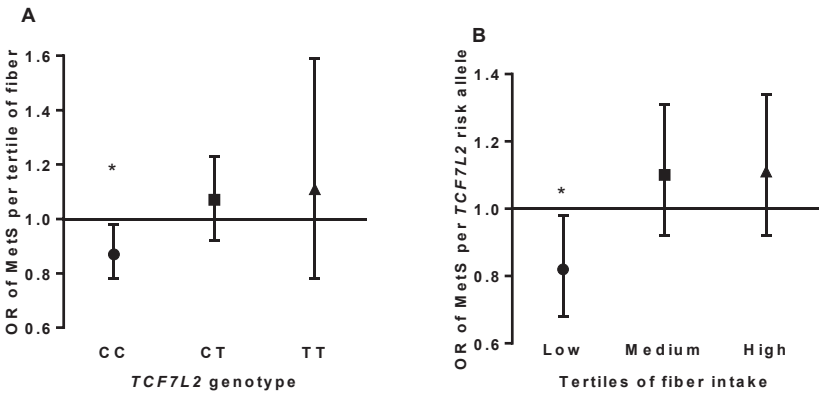
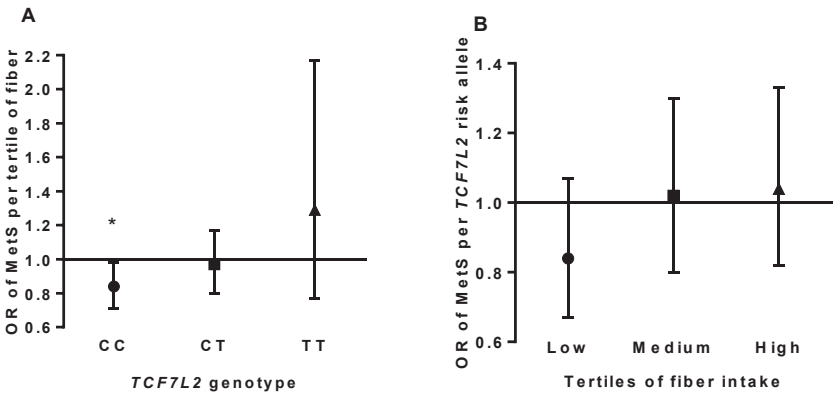

A
OR of MetS per tertile of fiber
1.6
1.4
1.2
1.0
0.8
0.6
*
CC
CT
TT
TCF7L2 genotype
B
OR of MetS per TCF7L2 risk allele
1.4
1.2
1.0
0.8
0.6
*
Low
Medium
High
Tertiles of fiber intake

FIG. 2.


A
OR of MetS per tertile of fiber
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
*
CC
CT
TT
TCF7L2 genotype
B
OR of MetS per TCF7L2 risk allele
1.4
1.2
1.0
0.8
0.6
Low
Medium
High
Tertiles of fiber intake

Paper IV

The chromosome 9p21 variant interacts with vegetable and wine intake to influence the risk of cardiovascular disease: a population based cohort study

George Hindy[1]
Email: george.hindy@med.lu.se

Ulrika Ericson[1]
Email: ulrika.ericson@med.lu.se

Viktor Hamrefors[2]
Email: viktor.hamrefors@med.lu.se

Isabel Drake[3]
Email: isabel.drake@med.lu.se

Elisabet Wirf lt[3]
Email: elisabet.wirfalt@med.lu.se

Olle Melander[2]
Email: olle.melander@med.lu.se

Marju Orho-Melander[1*]
[*] Corresponding author
Email: marju.orho-melander@med.lu.se

[1] Diabetes and Cardiovascular Disease-Genetic Epidemiology, Lund, Sweden

[2] Hypertension and Cardiovascular Disease, Lund, Sweden

[3] Nutrition Epidemiology, Department of Clinical Sciences in Malm , Lund University, Lund, Sweden

Abstract

Background

Chromosome 9p21 variants are associated with cardiovascular disease (CVD) but not with any of its known risk markers. However, recent studies have suggested that the risk associated with 9p21 variation is modified by a prudent dietary pattern and smoking. We tested if the increased risk of CVD by the 9p21 single nucleotide polymorphism rs4977574 is modified by intakes of vegetables, fruits, alcohol, or wine, and if rs4977574 interacts with environmental factors on known CVD risk markers.

Methods

Multivariable Cox regression analyses were performed in 23,949 individuals from the population-based prospective Malm Diet and Cancer Study (MDCS), of whom 3,164 developed CVD during 15 years of follow-up. The rs4977574 variant (major allele: A; minor allele: G) was genotyped using TaqMan Assay Design probes. Dietary data were collected at baseline using a modified diet history method. Cross-sectional analyses were performed in 4,828 MDCS participants with fasting blood levels of circulating risk factors measured at baseline.

Results

Each rs4977574 G allele was associated with a 16% increased incidence of CVD (95% confidence interval (CI), 1.10 1.22). Higher vegetable intake (hazard ratio (HR), 0.95 [CI: 0.91 0.996]), wine intake (HR, 0.91 [CI: 0.86 0.96]), and total alcohol consumption (HR, 0.92 [CI: 0.86 0.98]) were associated with lower CVD incidence. The increased CVD incidence by the G allele was restricted to individuals with medium or high vegetable intake ($P_{interaction}$ = 0.043), and to non- and low consumers of wine ($P_{interaction}$ = 0.029). Although rs4977574 did not associate with any known risk markers, stratification by vegetable intake and smoking suggested an interaction with rs4977574 on glycated hemoglobin and high-density lipoprotein cholesterol ($P_{interaction}$ = 0.015 and 0.049, respectively).

Conclusions

Our results indicate that rs4977574 interacts with vegetable and wine intake to affect the incidence of CVD, and suggest that an interaction may exist between environmental risk factors and rs4977574 on known risk markers of CVD.

Keywords

Cardiovascular disease, Chromosome 9p21, Diet, Gene, Gene diet interactions

Background

The single nucleotide polymorphisms (SNPs) on chromosome 9p21 identified by genome-wide association studies are the strongest known to date to be associated with coronary artery

disease (CAD) [1-3] and myocardial infarction (MI) [4,5]. Variants at this locus have also been associated with abdominal aortic aneurysms, intracranial aneurysms [6], and periodontitis [7]. Approximately 25% of the Caucasian population is homozygous for an allele at this locus that confers a 30 40% increased risk of developing CAD [2]. However, because this risk increase is independent of all conventional risk factors such as plasma lipoprotein levels, diabetes, hypertension, or markers of inflammation, the mechanism by which the 9p21 locus confers risk remains poorly understood [2,4].

Several dietary factors have been shown to associate with cardiovascular disease (CVD). For instance, a dietary intake of vegetables and nuts, and dietary patterns such as the Mediterranean-like diet are associated with protection against CVD, while an intake of *trans*-fatty acids, a high glycemic load and glycemic index diets have shown harmful effects [8]. Previous observations from the Malm Diet and Cancer Study (MDCS) found that a high quality diet (i.e., in line with current dietary recommendations) is associated with lower CVD risk [9]. Strong evidence from the Nurses Health study and the Health Professionals Follow-up Study suggests that diets rich in vegetables and fruits lower the risk of CVD [10]. Moreover, two large meta-analyses combining prospective studies from the United States and Europe confirmed the protective association of higher fruit and vegetable intake with incident CAD and stroke [11,12]. Additionally, moderate alcohol and wine consumption has been recognized as protective against CVD. Moderate alcohol intake has been associated with a lower risk of multiple cardiovascular outcomes in a systemic review and meta-analysis of 84 studies, while high alcohol consumption was associated with increased cardiovascular mortality and stroke incidence, consistent with U- or J-shaped curves [13]. Compelling evidence also exists for wine consumption providing a more pronounced cardio-protective effect compared with other alcoholic beverages [14,15].

A recent multi-ethnic study reported that the risk of MI and CVD determined by 9p21 variants was modified by a prudent diet score mainly driven by the raw vegetable component [16]. Another MDCS finding was of the reported interaction between 9p21 rs4977574 variant and smoking on the CAD incidence: a strong association was only observed among nonsmokers, while this association was significantly attenuated among smokers [17]. In the present study, we tested if the associated effect of the chromosome 9p21 rs4977574 variant on the risk of CVD was modified by vegetable, fruit, wine, or total alcohol intake. We further investigated whether these environmental factors interacted with the 9p21 variant on known risk markers of CVD.

Methods

Study population

Prospective study cohort

The MDCS is a population-based prospective cohort in the city of Malm , Sweden. Individuals born between 1923 and 1950 were recruited from a source population of 74,138 residing in Malm with Swedish reading and writing skills. The baseline examination period extended between March 1991 and October 1996. Written informed consent was obtained from all participants on their first visit to the hospital and after receiving instructions about the study procedures and questionnaires. Anthropometrics, body composition, and blood pressure were directly measured. Study participants were instructed on how to fill in a diet

questionnaire, a diet diary, and an extensive questionnaire covering socioeconomic and lifestyle factors including medical history, smoking habits, education, and physical activity. Participants returned approximately 2 weeks later for a diet history interview. By the end of the baseline examination period, complete dietary, anthropometric, and lifestyle data had been collected on 28,098 participants. Details of the recruitment procedures are described elsewhere [18].

We excluded individuals with prevalent CVD and diabetes. Prevalent CVD cases were identified as individuals with a history of MI or stroke. Prevalent diabetes cases were identified as individuals with a self-reported diabetes diagnosis or those on a self-reported anti-diabetic regimen. Our study population included 23,949 individuals free from CVD or diabetes with available DNA samples and successfully genotyped for the rs4977574 SNP at the chromosome 9p21 locus.

Cross-sectional study

At baseline, 6,103 individuals were randomly selected from the whole MDCS (N = 28,098) to participate in the Malm Diet and Cancer Cardiovascular Cohort (MDC-CC). These individuals underwent additional baseline measurements from fasting blood samples that included low-density lipoprotein cholesterol, high-density lipoprotein cholesterol (HDLC), triglycerides (TG), fasting blood glucose, and high sensitivity C-reactive protein (hsCRP). From this population we excluded individuals with prevalent diabetes, and prevalent CVD (identified as before). Our cross-sectional study included 4,828 individuals with complete data on rs4977574, genotype, diet, fasting blood tests, and blood pressure. The MDCS was approved by the Ethical Committee at Lund University (LU 51 90). All participants provided written informed consent.

Incident cardiovascular disease assessment

Incident CVD cases were identified as individuals with CAD (defined as fatal or nonfatal MI or death due to ischemic heart disease) or individuals with fatal or non-fatal stroke using three registers: the Swedish Hospital Discharge Register, the Swedish Cause of Death Register, and the Stroke Register of Malm . The identification of these register end-points has been described and validated elsewhere [19-21]. The follow-up period for the present study extended to December 31, 2010. MI cases were defined using codes 410 and I21 of the 9th and 10th revisions of the International Classification of Diseases (ICD9 and ICD10), and the stroke cases were defined using codes 430, 431, 434, and 436 of the ICD9, and codes I60, I61, I63, and I64 of the ICD10. A total of 3,164 individuals developed CVD, 1,844 developed CAD, and 1,556 developed stroke over a mean follow-up period of 15 years.

Clinical measurements

A balance-beam scale was used to measure weight (in kg) with subjects wearing light clothing and no shoes. A fixed stadiometer was used to measure height (in cm). Body mass index (BMI) was measured as weight (kg) divided by height (m^2). Waist circumference (in cm) was measured midway between the lowest rib margin and iliac crest. Fasting serum lipids, whole blood glucose, glycated hemoglobin (HbA_{1C}), and inflammatory markers were measured from blood samples drawn after an overnight fast. Fasting blood glucose was converted to fasting plasma glucose (FPG) by multiplying the values by 1.13. HbA_{1C} values were converted from the Swedish Mono-S standardization system to the International

Federation of Clinical Chemistry and Laboratory Medicine (IFCC) units using the following formula: IFCC = (10.11 Mono-S) − 8.94. Samples were analyzed by routine standard methods at the Department of Clinical Chemistry, Malm University Hospital. Fasting blood measurements were only available in MDC-CC.

Dietary assessment

The MDCS had a specially designed modified diet history method that consisted of a 7-day menu book, a 168-item questionnaire, and a 45 60 min diet history interview. All cooked meals, cold beverages including alcohol, medicinal drugs, natural remedies, and nutrient supplements consumed were recorded in the menu book. Participants recorded meal patterns, consumption frequencies, and portion sizes of regularly consumed foods not covered by the menu book in the questionnaire. A 48-page booklet was used to help participants estimate portion sizes. Diet history interviews were performed by trained interviewers, during which portion sizes and dishes were estimated using a more extensive book containing photographs. Participants were also asked about their meal patterns, cooking methods, and food choices.

The average daily intake of foods was calculated using data from the menu book and diet questionnaire, then converted into energy and nutrient intakes using the MDC Food and Nutrient Database. This was designed for the MDCS and was derived from the PC KOST2-93 database of the Swedish National Food Administration [22,23].

In September 1994, the coding routines for dietary data were modified slightly to shorten the interview time (from 60 to 45 minutes). This resulted in two slightly different method versions (before and after September 1994) without any major influence on the ranking of individuals [23]. A method variable, classifying data collected before and after September 1994, and a four-category season variable (i.e. winter, spring, summer, and autumn) were created and used as covariates to adjust for variation in data collection over time. The relative validity of the dietary assessment method in MDCS was previously evaluated in a sample of 206 (105 women and 101 men) Malm residents, aged 50 69 years. The reference method was based on weighed food records taken for 3 days every second month over the course of 1 year. Crude Pearson correlation coefficients for vegetables, fruit, wine, and total alcohol intake were 0.68, 0.60, 0.50, and 0.78, respectively, for men, and 0.58, 0.77, 0.65, and 0.83, respectively, for women [24,25].

The dietary exposure variables used were vegetables, fruit, wine, and total alcohol intake. Vegetable intake included all raw, dried, and cooked vegetables. Fruit intake included all citrus and non-citrus fruits and berries, in addition to dried fruits. The weights of dried fruits were corrected for their lower water content. Fruit and vegetable intakes were estimated as g per day and ranked into tertiles. The wine variable included red, white, and fortified wines. Non-consumers of wine were defined as those reporting no consumption of wine in the menu book, and indicating no consumption of wine during the previous month in the questionnaire. Wine non-consumers were categorized into a separate group and the rest were stratified into two groups using the median split. Total alcohol intake was classified into the following four categories based on g of alcohol consumed per day: abstainers (those reporting zero consumption of alcohol in the menu book, and indicating no consumption of alcohol in the previous year in the questionnaire), low (<15 g/d in women or <20 g/d in men), medium (15 30 g/d in women or 20 40 g/d in men), and high (>30 g/d in women or >40 g/d in men). This categorization was based on an assumption of biological risk [26]. For sensitivity analysis, we excluded abstainers and created tertiles of total alcohol consumption.

Other variables used as potential confounders

Leisure time physical activity was assessed by an extensive lifestyle questionnaire adapted from the Minnesota Leisure Time Physical Activity Questionnaire. Participants estimated the amount of time (in min) they spent performing each of 17 different physical activities per week for each season. The duration was multiplied by an intensity factor to create a physical activity score that was divided into tertiles. Participants were also classified as current smokers, former smokers, and never-smokers. The education variable was created by classifying participants according to their highest educational level (≤8 years, 9 10 years, and 11 13 years at school, and university degree).

Genotyping

The chromosome 9p21 rs4977574 SNP (major allele: A; minor allele: G) was genotyped using TaqMan Assay Design probes with a real-time PCR assay using ABI-7900HT equipment (Applied Biosystems, Foster City, CA) according to the manufacturer s instructions. The concordance rate was more than 99.9% in randomly selected samples (20%) and the genotypes were in Hardy Weinberg equilibrium (P = 0.18). We selected the rs4977574 for our study as this SNP provided the strongest evidence for association with early-onset MI in a large genome-wide association study (GWAS) by Kathiresan et al. [5] and as the same SNP has earlier been used in two other interaction studies [16,17]. This SNP is in high linkage disequilibrium (r^2 = 0.91-0.97) with the four SNPs (rs10757278, rs1333049, rs10757274, and rs2383206) originally identified in the discovery GWAS studies [1,2,4].

Statistical analysis

PASW statistics version 21 software (SPSS Inc., Chicago, IL) was used for statistical analyses. The prospective additive genetic association of the chromosome 9p21 rs4977574 G allele with incident CVD was assessed using a multivariable Cox proportional hazards model adjusting for age and sex. In cross-sectional analyses, cardio-metabolic traits and dietary intakes (dependent variables) were examined according to rs4977574 genotype (independent variable) using linear regression analyses with adjustments for age and sex. Prospective associations between tertiles of vegetable and fruit intakes, categories of alcohol or wine intake, and incidence of CVD were assessed in a multivariable Cox proportional hazards model with adjustments for age, sex, total energy intake, season, dietary assessment method, BMI, systolic blood pressure (SBP), use of lipid-lowering medication, use of anti-hypertensive medication, tertiles of leisure time physical activity, smoking status, level of education, and total alcohol intake categories when applicable. In cross-sectional analyses, cardio-metabolic traits and dietary intakes (dependent variables) were examined according to intake categories of vegetables, fruits, wine and alcohol (independent variables), using a multivariable linear regression model with the same adjustments as listed above.

Interactions between rs4977574 and tertiles of vegetable and fruit intakes, and the categories of alcohol or wine intake on incidence of CVD were tested by including both the rs4977574 variable and the environmental variables and their multiplicative factor in the multivariable models, using the same adjustments described above. The tertiles of vegetable and fruit intakes and the categories of wine and total alcohol intakes were treated as continuous variables. Significant prospective interactions between environmental factors and rs4977574 on the incidence of CVD were further explored by cross-sectional interaction analyses

between rs4977574 and quantitative glycemic, lipid, and inflammatory traits measured at baseline with the same adjustments described above.

Because it may be argued that BMI, SBP, and the use of lipid-lowering or anti-hypertensive medication could be on the causal pathway and should not be included in the adjustment models, we additionally performed all of the above analyses excluding these variables as possible confounders. Additional analyses were performed by mutually adjusting the analyses for correlated environmental factors such as vegetable and wine intake, and wine consumption and total alcohol consumption. The *P* values of the study were not corrected for multiple comparisons; those < 0.05 were considered nominally statistically significant. All reported *P* values are two-sided.

Results

The rs4977574 G allele was associated with an increased incidence of CVD (hazard ratio [HR]: 1.16 per G allele; 95% confidence interval [CI]: 1.10 1.22) in MDCS (Table 1), while higher vegetable, wine, and total alcohol intakes were associated with a decreased incidence of CVD (HR, 0.95 [CI: 0.91 0.996], 0.91 [0.86 0.96], and 0.92 [0.86 0.98], respectively). Cross-sectional associations of intakes of vegetables, fruits, wine and alcohol with baseline levels of different cardio-metabolic biomarkers are shown in Additional file 1: Table S1 S4.

Table 1 Characteristics of the Malm Diet and Cancer Study population by rs4977574 genotype

	rs4977574 genotype			HR (95%CI) [a]	P_{trend}
	AA	AG	GG	or β (SE) [b]	
Total Number	7325	11777	4847		
Sex (%women)	62.6	62.3	62.3		
Incident CVD *N* (%)	863 (11.8)	1562 (13.3)	739 (15.3)	1.16 (1.10-1.22)	4 10^{-09}
Age (years)	57.9 7.7	57.9 7.6	57.8 7.7	−0.04 (0.07)	0.60
BMI (kg/m^2)	25.7 3.9	25.6 3.9	25.7 3.9	0.001 (0.035)	0. 98
Waist (cm)	83.6 15.6	83.4 12.8	83.7 16.7	0.02 (0.11)	0.87
SBP (mmHg)	141 20	141 20	141 20	0.21 (0.17)	0 .21
DBP (mmHg)	85 10	85 10	86 10	0.03 (0.09)	0.76
FPG (mmol/L) [c]	5.63 0.84	5.62 0.72	5.66 0.91	0.009 (0.016)	0.59
HbA_{1C} (mmol/mol) [c]	39.6 4.94	39.6 4.82	39.9 5.32	0.13 (0.10)	0. 19
LDLC (mmol/L) [c]	4.15 1.00	4.19 0.98	4.19 0.97	0.02 (0.02)	0. 33
HDLC (mmol/L) [c]	1.39 0.37	1.40 0.38	1.39 0.36	0.001 (0.007)	0.85
Triglycerides (mmol/L) [c]	1.35 0.73	1.32 0.71	1.37 0.90	0.002 (0.015)	0.48
hsCRP [c]	0.26 0.43	0.24 0.39	0.27 0.48	0.003 (0.009)	0.67
Total energy intake (kcal/day)	2280 652	2274 65 3	2276 653	−3.90 (5.26)	0.46
Vegetables (g/day)	180 98	182 100	180 99	−0.1 8 (0.90)	0.85
Fruits (g/day)	196 127	193 125	197 127	0.29 (1.14)	0.80
Wine (g/day)	41.2 58.2	42.8 70.0	42.5 60.6	0. 70 (0.55)	0.20
Alcohol (g/day)	10.6 12.3	10.9 12.7	10.8 12.8	0.13 (0.11)	0.22

Data represented as mean standard deviation.

[a] Multivariable Cox proportional hazards model for incidence of cardiovascular disease (CVD) during follow-up adjusting for age and sex with HR referring to hazard ratio per risk G allele using an additive genetic model.

[b] Linear regression analyses of rs4977574 G allele using an additive genetic model with quantitative traits or characteristics at baseline adjusting for age and sex when appropriate with β referring to associated effect estimate per risk G allele.

[c] In MDC-CC only, N = 4,828 (AA N = 1,460; AG N = 2,381; GG N = 987).

CI, Confidence Interval; CVD, cardiovascular disease; SE, standard error; BMI, Body Mass Index; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; FPG, Fasting Plasma Glucose; HbA_{1C}, Hemoglobin A_{1C}; LDLC, Low-Density Lipoprotein Cholesterol; HDLC, High-Density Lipoprotein Cholesterol; hsCRP, High-Sensitivity C-Reactive Protein.

The CVD risk increase by the rs4977574 G allele was modified by vegetable and wine intakes ($P_{interaction}$ = 0.043 and 0.029, respectively) but not with fruit or total alcohol intakes ($P_{interaction}$ = 0.69 and 0.73, respectively). The association between the rs4977574 G allele and increased incidence of CVD was restricted to the medium and high tertiles of vegetable intake (P_{trend} = 6 10^{-8} and 0.0004, respectively) and to non- and low consumers of wine (P_{trend} = 4 10^{-7} and 0.0004, respectively). The inverse association between higher vegetable intake and CVD incidence was restricted to individuals with no risk alleles (AA genotype, P_{trend} = 0.001), while the inverse association between higher wine consumption and incidence of CVD was restricted to carriers of rs4977574 G alleles (P_{trend} = 0.00009) (AG and GG genotypes, P_{trend} = 0.01 and 0.001, respectively) (Table 2). Both vegetable and wine intake modified the risk of both CAD and stroke by rs4977574 variant in a similar fashion as observed with total CVD. However, the tests of interaction did not reach statistical significance (Additional file 1: Table S6 and S7).

Table 2 Hazard ratio for incident CVD in the Malm Diet and Cancer Study (n = 23,949) according to rs4977574 genotype and dietary or alcohol intake categories

		Hazard Ratio (95% Confidence Interval)				P_{trend} [b, d]	$P_{interaction}$ [e, f]
		AA	AG	GG	Additive Model [b, d]		
	n	7325	11777	4847			
Vegetables (g/day)							0.043, 0.049
Low	7952	1.29 (1.09 1.52)	1.22 (1.04 1.43)	1.50 (1.26 1.79)	1.06 (0.98 1.14)	0.16	
Medium	8041	0.87 (0.73 1.05)	1.27 (1.09 1.48)	1.41 (1.18 1.70)	1.27 (1.17 1.38)	5.6 10^{-8}	
High	7956	1.00 (ref)[a, c]	1.20 (1.02 1.41)	1.43 (1.18 1.72)	1.19 (1.08 1.30)	0.0004	
Per category [c, e]		0.86 (0.79 0.94)	0.98 (0.92 1.05)	0.99 (0.90 1.09)			
P_{trend} [c, e]		0.001	0.61	0.83			
Fruits (g/day)							0.69, 0.80
Low	7886	1.10 (0.93 1.30)	1.26 (1.09 1.46)	1.51 (1.27 1.80)	1.16 (1.07 1.26)	0.0004	
Medium	8030	1.13 (0.96 1.34)	1.25 (1.08 1.45)	1.46 (1.23 1.74)	1.14 (1.04 1.24)	0.003	
High	8033	1.00 (ref)	1.24 (1.07 1.44)	1.44 (1.21 1.72)	1.18 (1.08 1.29)	0.0002	
Per category		0.97 (0.89 1.06)	0.98 (0.92 1.05)	1.00 (0.90 1.10)			
P_{trend}		0.48	0.52	0.92			
Wine (g/day)							0.029, 0.031
Non-consumers	6843	1.03 (0.86 1.22)	1.27 (1.09 1.49)	1.57 (1.32 1.88)	1.23 (1.14 1.34)	4 10^{-7}	
Low	8472	0.93 (0.78 1.11)	1.14 (0.98 1.33)	1.29 (1.08 1.59)	1.16 (1.07 1.26)	0.0004	
High	8634	1.00 (ref)	1.01 (0.87 1.17)	1.17 (0.98 1.40)	1.08 (0.98 1.18)	0.11	
Per category		0.97 (0.88 1.08)	0.91 (0.85 0.98)	0.84 (0.75 0.93)			
P_{trend}		0.61	0.01	0.001			
Alcohol (g/day)							0.73, 0.86
Abstainers	1448	1.19 (0.78 1.81)	1.63 (1.11 2.39)	2.26 (1.49 3.42)	1.34 (1.13 1.60)	0.001	
Low	17331	1.14 (0.81 1.60)	1.36 (0.97 1.91)	1.47 (1.04 2.08)	1.14 (1.07 1.20)	0.00002	
Medium	4150	1.18 (0.81 1.70)	1.06 (0.74 1.52)	1.64 (1.13 2.37)	1.15 (1.02 1.30)	0.03	
High	1020	1 (ref)	1.23 (0.82 1.85)	1.57 (0.99 2.50)	1.29 (1.02 1.63)	0.04	
Per category		0.98 (0.87 1.10)	0.85 (0.78 0.93)	0.96 (0.85 1.09)			
P_{trend}		0.74	0.0004	0.57			

[a] Multivariable Cox proportional hazards model for incidence of cardiovascular disease (CVD) during follow-up with hazard ratio per each category of rs4977574 genotype and food or beverage intake assuming the highest category and AA genotype as reference.
[b] Multivariable Cox proportional hazards model for incidence of cardiovascular disease (CVD) during follow-up with hazard ratio per risk G allele using an additive genetic model.
[c] Multivariable Cox proportional hazards model for incidence of cardiovascular disease (CVD) during follow-up with hazard ratio per each higher food or beverage intake category with the lowest intake category as reference.
[d] Adjusted for age and sex.
[e] Adjusted for age, sex, BMI, SBP, season, method, total energy intake, leisure time physical activity, alcohol intake, smoking status, education, lipid lowering and antihypertensive treatment.
[f] Excluding BMI, SBP, antihypertensive and lipid lowering treatment from the adjustment model.

Concerning interaction with alcohol consumption, we observed that the HR for CVD incidence was lowest among AG genotype carriers in the medium alcohol intake group; however, this was not significantly different from the other intake groups as the interaction was not significant (Table 2). To determine if the lack of interaction between the total alcohol categories and rs4977574 on CVD risk was caused by an absence of statistical power, and as a sensitivity analysis, we excluded abstainers and performed an interaction analysis between tertiles of alcohol consumption and rs4977574 on CVD risk and observed that only among AG carriers the higher alcohol consumption associated with significantly lower incidence of CVD. However, no significant interaction was revealed (Additional file 1: Table S5).

Consistent with earlier studies, rs4977574 did not associate with any of the known clinical or circulating risk factors of CVD (Table 1). However, stratification by environmental factors indicated that there was an interaction between rs4977574 and vegetable intake on baseline HbA_{1C} levels ($P_{interaction}$ = 0.015), as well as smoking status on baseline HDLC levels ($P_{interaction}$ = 0.049) (Additional file 1: Table S8). The rs4977574 G allele was only associated with elevated baseline HbA_{1C} levels in individuals within the lowest vegetable intake tertile (P_{trend} = 0.009), and with lower HDLC among never-smokers only (P_{trend} = 0.045). Moreover, the higher vegetable intake was associated with lower HbA_{1C} levels among G allele carriers only (P_{trend} = 0.0002) (Figure 1). Smoking was strongly associated with lower HDLC levels in MDC-CC (P_{trend} = 1 10^{-10}), and the magnitude of this association was largest among individuals carrying no risk alleles (AA genotype, P_{trend} = 1 10^{-7}) (Figure 2).

Figure 1 Mean HbA_{1C} levels in tertiles of vegetable intake by rs4977574 genotype in MDC-CC. The G allele was only associated with elevated HbA_{1C} levels among individuals in the lowest tertile of vegetable intake (β = 0.48 mmol/mol, SE = 0.18 per G allele, *P* = 0.009). Higher vegetable intake was only associated with lower HbA_{1C} among individuals with AG (β = −0.28 mmol/mol, SE = 0.12 per risk tertile, *P* = 0.019) and GG genotypes (β = −0.43 mmol/mol, SE = 0.20 per risk tertile, *P* = 0.032).

Figure 2 Mean HDLC levels in smoking status categories by rs4977574 genotype in MDC-CC. The G allele was only associated with lower levels of HDLC among never-smokers (β = −0.02 mmol/L, SE = 0.008 per G allele, *P* = 0.045). Higher risk categories of smoking were associated with lower HDLC for all genotypes. The magnitude of this association was largest among individuals with AA genotypes (β = −0.05 mmol/L, SE = 0.01 per higher risk category, *P* = 1 10^{-7}) compared with AG (β = −0.03 mmol/L, SE = 0.009 per higher risk category, *P* = 0.0004) and GG (β = −0.02 mmol/L, SE = 0.01 per higher risk category, P = 0.024) genotypes.

Discussion

In this prospective study, we observed that dietary vegetable and wine intake modified the association of the chromosome 9p21 rs4977574 variant with CVD incidence. As expected, carriers of the rs4977574 G allele were associated with an increased incidence of CVD. Furthermore, a higher vegetable intake and moderate or high wine and alcohol consumption were associated with a lower incidence of CVD. However, the increased incidence of CVD among G allele carriers was restricted to individuals with a medium or high vegetable intake and to individuals reporting a zero or low consumption of wine. When stratified by rs4977574 genotype, the higher vegetable intake was only associated with a lower incidence

of CVD in individuals with no risk alleles, while wine consumption was only associated with a lower incidence of CVD among risk allele carriers.

In line with earlier studies, the 9p21 variant did not associate with any of the traditional clinical risk factors in the present study. However, stratification by vegetable intake and smoking status suggested that it may associate with some of these risk factors, depending on the environment. We observed that presence of the G allele was associated with elevated HbA_{1C} levels among individuals in the lowest tertile of vegetable intake. Further, the G allele was only associated with lower HDLC levels among never-smokers. These nominally significant interactions indicate that the risk increase by 9p21 genetic variation may at least partially be mediated through deleterious effects on glucose and lipid metabolism, dependent on environmental risk factors of CVD.

Multiple interactions were observed between the rs4977574 variant and vegetable and wine intake with respect to CVD incidence. Concerning vegetable intake, the association with the genetic variant was attenuated and not significant among individuals at higher CVD risk because of low vegetable intake. This result is in line with our earlier observation that the associated effect of the 9p21 rs4977574 G allele was attenuated and not significant among smokers, i.e. among individuals already at higher risk because of an environmental risk factor for CVD [17]. Of further relevance to this are the results of an earlier study that reported an interaction between the 9p21 variant and a prudent diet score [16]. However, in contrast to our results, the associated effect of the risk allele was strongest among individuals in the lowest tertile of the prudent diet score. We also observed that wine intake was associated with a lower incidence of CVD, and that the risk increase caused by presence of the G allele was attenuated among consumers of wine. It therefore appears that the attenuation of the risk by the 9p21 variant may be mediated by both environmental risk factors (such as smoking and low vegetable intake) and protective factors (such as high wine intake) for CVD. Interaction analyses on CAD and stroke indicated similar tendencies with both vegetable and wine intake. However, due to decreased power in these subgroup analyses, statistical significance could not be attained. We did not find interaction with total alcohol intake although we observed the AG carriers to have lowest risk of CVD in the medium alcohol intake group and a strongly significantly decreased incidence of CVD by increased alcohol intake only among the AG genotype carriers.

It is important to recognize that vegetable and wine intakes are positively correlated and could reflect the same underlying interaction. However, when we mutually adjusted the analyses for these factors, the results remained similar. It is also of interest that the interaction between the 9p21 variant and wine intake on CVD risk was independent of total alcohol consumption, because an adjustment for total alcohol intake did not affect this result. Furthermore, adjusting for smoking status did not change the observed interactions between rs4977574 and vegetable or wine intake, indicating that these interactions are independent.

The mechanisms by which the 9p21 locus confers an increased risk of CVD remain incompletely understood. The SNPs in this locus are located in a 53-kb linkage disequilibrium (LD) block that lacks any protein-coding genes [2,4]. However, the large noncoding RNA antisense noncoding RNA in the INK locus (*ANRIL*) (also known as *CDKN2BAS*) has been mapped to the risk interval [27,28]. *ANRIL* expression has been shown to robustly associate with the 9p21 genotype and with the severity of atherosclerosis [29-32]. Although there is no conclusive evidence about the potential connection between *ANRIL* and CVD, several studies suggest a possible role in the epigenetic regulation of gene expression,

and support *ANRIL* as the effector gene in 9p21 [33-36]. Interestingly, several of the bioactive compounds that have been recognized in nutritional epigenetics are found in wine and vegetables such as resveratrol (in red grapes), sulforaphane (in broccoli and sprouts), butyrate (a fermentation product of dietary fiber), and genistein (in fava beans and soya beans) [37-39]. If epigenetic mechanisms contribute to the association between 9p21 and CVD, our results suggest that they could potentially be influenced by nutritional or environmental factors and through gene environment interactions.

It is noteworthy that an independent SNP in the *CDKN2A/B* locus near the 9p21 53-kb LD block has been robustly associated with type 2 diabetes [40,41]. This is particularly intriguing because we observed the rs4977574 risk allele to associate with elevated HbA_{1C} levels among individuals with a lower vegetable intake. Additionally, another interaction was observed between the 9p21 variant and HbA_{1C} on the risk of CAD in individuals with type 2 diabetes [42], such that the risk increase of CAD by the 9p21 risk variant was accentuated in individuals with elevated HbA_{1C} levels.

Our study has a number of limitations, which should be taken into account when analyzing results. First, baseline diet data were projected onto the entire follow-up period in the prospective analyses of CVD risk. Second, the inaccurate reporting of alcohol consumption is a known limitation in epidemiological studies, so could be a potential weakness [25]. Third, the cross-sectional analyses with CVD risk markers suffer from limited causal inference. Fourth, we did not correct the statistical analyses for multiple comparisons because the dietary variables are correlated. However, despite these limitations, the interactions observed between chromosome 9p21 variants and vegetable intake are supported by previous findings in the case/control INTERHEART study and the prospective FINRISK study [16]. Nevertheless, we should keep in mind that the observed significance levels of the interactions in our study were not very robust, so the possibility of false-positive findings cannot be excluded. On the other hand, our study has many important major strengths, including the high relative validity of our dietary assessment method, the combination of a diet diary with a questionnaire, the large sample size, the prospective design, the extensive follow-up of individuals through registers, and the comprehensive ascertainment and verification of CVD cases.

Conclusions

Our results suggest that the chromosome 9p21 SNP rs4977574 interacts with several environmental risk factors to affect the incidence of CVD. Furthermore, the observed modifications of the association between rs4977574 and HbA_{1C} and HDLC levels by vegetable intake and smoking, respectively, provide evidence that the risk increase may include environmental interactions leading to derangements at the level of glucose and lipid metabolism.

Abbreviations

CVD, Cardiovascular disease; CAD, Coronary artery disease; MI, Myocardial infarction; MDCS, Malm diet and cancer study; SNP, Single nucleotide polymorphism; MDC-CC, Malm diet and cancer cardiovascular cohort; HDLC, High-density lipoprotein cholesterol; TG, Triglycerides; hsCRP, High-sensitivity C-reactive protein; ICD, International classification of diseases; BMI, Body mass index; FPG, Fasting plasma glucose; HbA_{1C},

Hemoglobin A_{1C}; IFCC, International federation of clinical chemistry and laboratory medicine; GWAS, Genome-wide association study; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; ANRIL, Antisense noncoding RNA in the INK locus

Competing interests

The authors declare that they have no competing interests.

Authors contributions

GH, UE, VH, OM, and MO-M were involved in the conception and design of the study. ID and EW contributed data. GH performed the statistical analysis. GH and MO-M wrote the manuscript. UE, VH, ID, EW and OM provided critical revision and contributed to the final version of the manuscript. GH and MO-M take primary responsibility for the final content. All authors have read and approved the final version of the manuscript.

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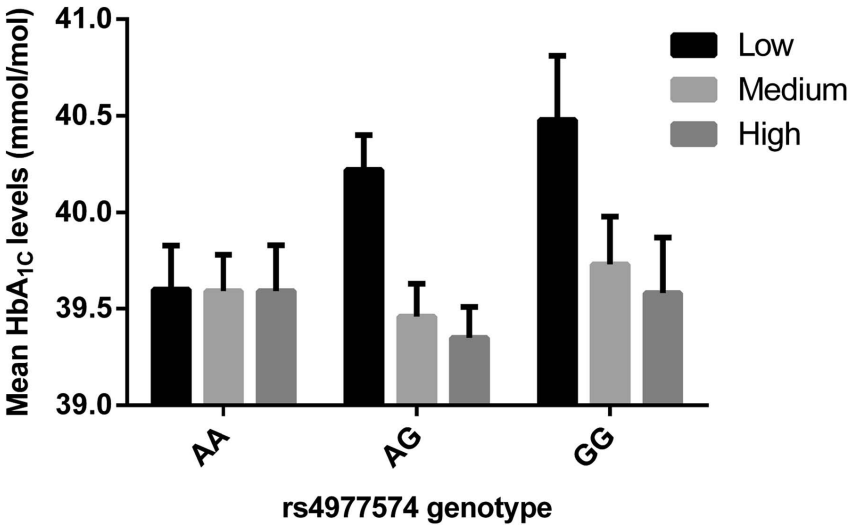
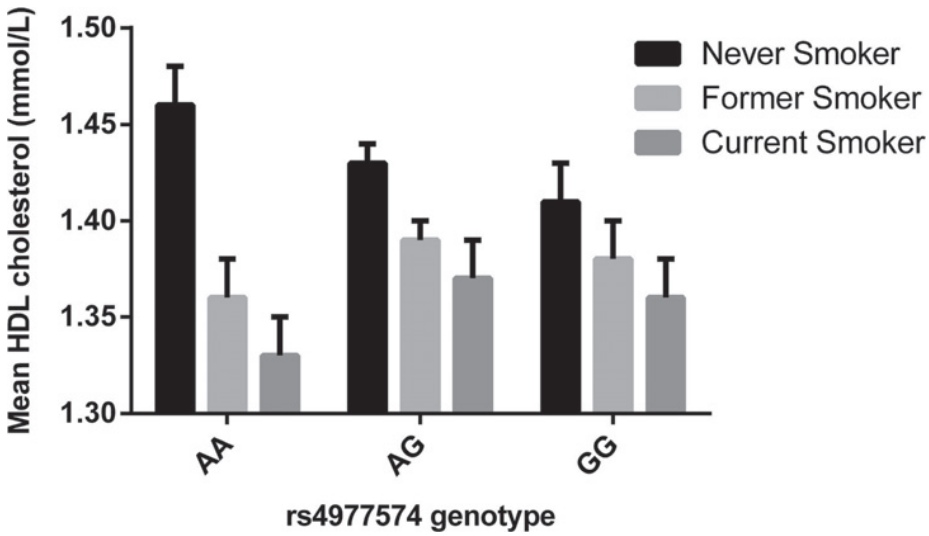

41.0
40.5
40.0
39.5
39.0
Mean HbA1C levels (mmol/mol)
Low
Medium
High
AA
AG
GG
rs4977574 genotype


Mean HDL cholesterol (mmol/L)
1.50
1.45
1.40
1.35
1.30
Never Smoker
Former Smoker
Current Smoker
AA
AG
GG
rs4977574 genotype

Additional files provided with this submission:

ESM 1. Table S1 Characteristics of the Malmö Diet and Cancer Study population according to vegetable intake. Table S2 Characteristics of the Malmö Diet and Cancer Study population according to fruit intake. Table S3 Characteristics of the Malmö Diet and Cancer Study population according to wine consumption. Table S4 Characteristics of the Malmö Diet and Cancer Study population according to alcohol consumption. Table S5 Hazard ratio according to rs4977574 genotype for incident CVD in the Malmö Diet and Cancer study (n = 22,501) according to tertiles of alcohol consumption. Table S6 Hazard ratio for incident CAD in the Malmö Diet and Cancer Study (n = 23,949) according to rs4977574 genotype and dietary or alcohol intake categories. Table S7 Hazard ratio for incident stroke in the Malmö Diet and Cancer Study (n = 23,949) according to rs4977574 genotype and dietary or alcohol intake categories. Table S8 Interaction of rs4977574 variant with vegetable intake, wine intake, and smoking habits on CVD risk markers (66k)
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The chromosome 9p21 variant interacts with vegetable and wine intake to influence the risk of cardiovascular disease

George Hindy[1], Ulrika Ericson[1], Viktor Hamrefors[2], Isabel Drake[3], Elisabet Wirfält[3], Olle Melander[2] and Marju Orho-Melander[1*]

[1]Diabetes and Cardiovascular Disease-Genetic Epidemiology; [2]Hypertension and Cardiovascular Disease; and [3]Nutrition Epidemiology, Department of Clinical Sciences in Malmö, Lund University, Sweden

*Corresponding author

Email addresses:

G. Hindy, george.hindy@med.lu.se

U. Ericson, ulrika.ericson@med.lu.se

V. Hamrefors, viktor.hamrefors@med.lu.se

I. Drake, isabel.drake@med.lu.se

E. Wirfält, elisabet.wirfalt@med.lu.se

O. Melander, olle.melander@med.lu.se

M. Orho-Melander, marju.orho-melander@med.lu.se

Supplementary Table 1 Characteristics of the Malmö Diet and Cancer Study population according to vegetable intake

	Vegetable intake			HR (95%CI) [a]	
	Low	Medium	High	or β (SE) [b]	P_{trend}
Total Number	7952	8041	7956		
Sex (%women)	57.0	63.5	66.6		
Incident CVD (%)	1261 (15.9)	1025 (12.7)	878 (11.0)	0.95 (0.91–0.996)	0.032
Age (years)	59.1 ± 7.8	57.9 ± 7.7	56.6 ± 7.3	−0.57 (0.06)	2×10^{-25}
BMI (kg/m^2)	25.7 ± 4.0	25.6 ± 3.8	25.5 ± 3.9	0.16 (0.03)	2×10^{-07}
Waist (cm)	84.8 ± 15.8	83.2 ± 12.4	82.6 ± 15.1	−0.20 (0.07)	0.006
SBP (mmHg)	143 ± 20	141 ± 20	138 ± 19	−0.42 (0.14)	0.004
DBP (mmHg)	86 ± 10	85 ± 10	85 ± 10	−0.21 (0.08)	0.007
FPG (mmol/L) [c]	5.71 ± 0.89	5.61 ± 0.66	5.59 ± 0.82	−0.04 (0.01)	0.008
HbA_{1C} (mmol/mol) [c]	40.1 ± 5.13	39.6 ± 4.47	39.5 ± 5.21	−0.18 (0.09)	0.03
LDLC (mmol/L) [c]	4.19 ± 0.96	4.16 ± 0.96	4.18 ± 1.02	0.01 (0.02)	0.87
HDLC (mmol/L) [c]	1.36 ± 0.37	1.40 ± 0.37	1.42 ± 0.38	0.02 (0.006)	0.01
Triglycerides (mmol/L) [c]	1.40 ± 0.77	1.33 ± 0.71	1.30 ± 0.78	−0.03 (0.01)	0.006
hsCRP [c]	0.27 ± 0.43	0.25 ± 0.43	0.24 ± 0.41	−0.009 (0.008)	0.03
Total energy intake (kcal/day)	2208 ± 648	2256 ± 625	2365 ± 675	95 (4.6)	2×10^{-93}
Vegetables (g/day)	90 ± 28	164 ± 21	289 ± 90	98 (0.5)	0
Fruits (g/day)	153 ± 109	189 ± 115	242 ± 136	38 (1)	0
Wine (g/)	35.2 ± 56.6	42.9 ± 59.3	48.6 ± 63.3	2.8 (0.4)	3×10^{-13}
Alcohol (g/day)	10.2 ± 13.0	10.8 ± 12.1	11.4 ± 12.6	0.35 (0.10)	0.0002

Data represented as mean ± standard deviation

[a] Multivariable Cox proportional hazards model for incidence of cardiovascular disease (CVD) during follow-up adjusting for age, sex, BMI, SBP, season, method, total energy intake, leisure time physical activity, alcohol intake, smoking status, education, lipid lowering and antihypertensive treatment with HR referring to hazard ratio per tertile of vegetable intake with the lowest tertile as reference

[b] Linear regression analyses of tertiles of vegetable intake with quantitative traits or characteristics at baseline adjusting for age, sex, BMI, SBP, season, method, total energy intake, leisure time physical activity, alcohol intake, smoking status, education, lipid lowering and antihypertensive treatment when appropriate with β referring to associated effect estimate per tertile of vegetable intake with the lowest tertile as reference

[c] In MDC-CC only, N=4,828 (AA N=1,460; AG N=2,381; GG N=987)

CI, Confidence Interval; CVD, cardiovascular disease; SE, standard error; BMI, Body Mass Index; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; FPG, Fasting Plasma Glucose; HbA_{1C}, Hemoglobin A_{1C}; LDLC, Low-Density Lipoprotein Cholesterol; HDLC, High-Density Lipoprotein Cholesterol; hsCRP, High-Sensitivity C-Reactive Protein

Supplementary Table 2 Characteristics of the Malmö Diet and Cancer Study population according to fruit intake

	Fruit intake			HR (95%CI) [a]	
	Low	Medium	High	or β (SE) [b]	P_{trend}
Total Number	7886	8030	8033		
Sex (%women)	51.6	64.7	70.6		
Incident CVD (%)	1126 (14.3)	1069 (13.3)	969 (12.1)	0.98 (0.93–1.02)	0.32
Age (years)	57.2 ± 7.6	58.3 ± 7.8	58.2 ± 7.6	0.61 (0.06)	2×10^{-27}
BMI (kg/m^2)	25.4 ± 3.9	25.6 ± 3.9	25.8 ± 4.0	0.33 (0.03)	6×10^{-26}
Waist (cm)	84.9 ± 13.0	83.3 ± 17.5	82.4 ± 12.4	−0.32 (0.07)	0.00002
SBP (mmHg)	141 ± 20	141 ± 20	140 ± 20	−0.54 (0.15)	0.0003
DBP (mmHg)	86 ± 10	85 ± 10	85 ± 10	−0.19 (0.08)	0.01
FPG (mmol/L) [c]	5.72 ± 0.90	5.60 ± 0.69	5.59 ± 0.79	−0.04 (0.01)	0.01
HbA_{1C} (mmol/mol) [c]	40.2 ± 5.40	39.4 ± 4.54	39.5 ± 4.95	−0.36 (0.09)	0.00006
LDLC (mmol/L) [c]	4.16 ± 0.98	4.17 ± 0.96	4.20 ± 1.01	0.002 (0.018)	0.92
HDLC (mmol/L) [c]	1.35 ± 0.35	1.41 ± 0.39	1.41 ± 0.37	−0.001 (0.006)	0.78
Triglycerides (mmol/L) [c]	1.40 ± 0.84	1.31 ± 0.72	1.31 ± 0.71	−0.01 (0.01)	0.28
hsCRP [c]	0.28 ± 0.44	0.23 ± 0.36	0.25 ± 0.46	−0.004 (0.008)	0.05
Total energy intake (kcal/day)	2249 ± 662	2245 ± 633	2334 ± 660	118 (5)	2×10^{-141}
Vegetables (g/day)	146 ± 79	177 ± 89	219 ± 113	33 (0.8)	0
Fruits (g/day)	76 ± 35	173 ± 29	334 ± 107	126 (0.6)	0
Wine (g/)	45.6 ± 66.9	42.0 ± 57.6	39.2 ± 55.0	1.18 (0.39)	0.003
Alcohol (g/day)	13.1 ± 14.9	10.2 ± 11.7	9.0 ± 10.4	−1.49 (0.10)	2×10^{-53}

Data represented as mean ± standard deviation

[a] Multivariable Cox proportional hazards model for incidence of cardiovascular disease (CVD) during follow-up adjusting for age, sex, BMI, SBP, season, method, total energy intake, leisure time physical activity, alcohol intake, smoking status, education, lipid lowering and antihypertensive treatment with HR referring to hazard ratio per tertile of fruit intake with the lowest tertile as reference

[b] Linear regression analyses of tertiles of vegetable intake with quantitative traits or characteristics at baseline adjusting for age, sex, BMI, SBP, season, method, total energy intake, leisure time physical activity, alcohol intake, smoking status, education, lipid lowering and antihypertensive treatment when appropriate with β referring to associated effect estimate per tertile of fruit intake with the lowest tertile as reference

[c] In MDC-CC only, N=4,828 (AA N=1,460; AG N=2,381; GG N=987)

CI, Confidence Interval; CVD, cardiovascular disease; SE, standard error; BMI, Body Mass Index; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; FPG, Fasting Plasma Glucose; HbA_{1C}, Hemoglobin A_{1C}; LDLC, Low-Density Lipoprotein Cholesterol; HDLC, High-Density Lipoprotein Cholesterol; hsCRP, High-Sensitivity C-Reactive Protein

Supplementary Table 3 Characteristics of the Malmö Diet and Cancer Study population according to wine consumption

	Wine intake			HR (95%CI) [a]	
	Non-consumers	Low	High	or β (SE) [b]	P_{trend}
Total Number	6843	8472	8634		
Sex (%women)	58.6	66.3	61.5		
Incident CVD (%)	1156 (16.9)	1088 (12.8)	920 (10.7)	0.91 (0.86–0.96)	0.0003
Age (years)	59.3 ± 7.6	58.3 ± 7.7	56.3 ± 7.4	−0.51 (0.06)	8×10^{-16}
BMI (kg/m^2)	26.1 ± 4.3	25.6 ± 3.9	25.2 ± 3.5	−0.30 (0.04)	3×10^{-17}
Waist (cm)	85.6 ± 18.6	82.8 ± 12.5	82.5 ± 12.4	−0.39 (0.08)	3×10^{-6}
SBP (mmHg)	143 ± 20	141 ± 20	139 ± 20	−0.25 (0.17)	0.14
DBP (mmHg)	86 ± 10	85 ± 10	85 ± 10	0.11 (0.09)	0.20
FPG (mmol/L) [c]	5.69 ± 0.86	5.58 ± 0.74	5.63 ± 0.70	−0.03 (0.02)	0.08
HbA_{1C} (mmol/mol) [c]	40.5 ± 5.81	39.6 ± 4.73	39.1 ± 4.34	−0.35 (0.10)	0.0005
LDLC (mmol/L) [c]	4.21 ± 1.02	4.19 ± 0.97	4.14 ± 0.97	0.02 (0.02)	0.32
HDLC (mmol/L) [c]	1.30 ± 0.34	1.41 ± 0.37	1.46 ± 0.39	0.05 (0.007)	2×10^{-15}
Triglycerides (mmol/L) [c]	1.41 ± 0.75	1.31 ± 0.69	1.30 ± 0.82	−0.05 (0.02)	0.0004
hsCRP [c]	0.29 ± 0.47	0.23 ± 0.41	0.24 ± 0.40	−0.01 (0.009)	0.05
Total energy intake (kcal/day)	2284 ± 720	2239 ± 624	2306 ± 622	−42.9 (5.42)	3×10^{-15}
Vegetables (g/day)	164 ± 99	181 ± 98	193 ± 100	9.16 (0.91)	1×10^{-23}
Fruits (g/day)	193 ± 133	201 ± 124	190 ± 122	3.62 (1.14)	0.001
Wine (g)	0	18.1 ± 12.0	99 ± 68	36.6 (0.38)	0
Alcohol (g/day)	3.95 ± 8.85	7.24 ± 8.44	19.6 ± 13.4	7.90 (0.08)	0

Data represented as mean ± standard deviation

[a] Multivariable Cox proportional hazards model for incidence of cardiovascular disease (CVD) during follow-up adjusting for age, sex, BMI, SBP, season, method, total energy intake, leisure time physical activity, alcohol intake, smoking status, education, lipid lowering and antihypertensive treatment with HR referring to hazard ratio per wine intake category with non-consumers as reference

[b] Linear regression analyses of tertiles of vegetable intake with quantitative traits or characteristics at baseline adjusting for age, sex, BMI, SBP, season, method, total energy intake, leisure time physical activity, alcohol intake, smoking status, education, lipid lowering and antihypertensive treatment when appropriate with β referring to associated effect estimate per wine intake category with non-consumers as reference

[c] In MDC-CC only, N=4,828 (AA N=1,460; AG N=2,381; GG N=987)

CI, Confidence Interval; CVD, cardiovascular disease; SE, standard error; BMI, Body Mass Index; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; FPG, Fasting Plasma Glucose; HbA_{1C}, Hemoglobin A_{1C}; LDLC, Low-Density Lipoprotein Cholesterol; HDLC, High-Density Lipoprotein Cholesterol; hsCRP, High-Sensitivity C-Reactive Protein

Supplementary Table 4 Characteristics of the Malmö Diet and Cancer Study population according to alcohol consumption

	Alcohol intake				HR (95%CI) [a]	
	Abstainers	Low	Medium	High	or β (SE) [b]	P_{trend}
Total Number	1448	17331	4150	1020		
Sex (%women)	74.1	65.7	51.1	34.9		
Incident CVD (%)	241 (16.6)	2277 (13.1)	508 (12.2)	138 (13.5)	0.92 (0.86–0.98)	0.006
Age (years)	60.3 ± 7.5	58.2 ± 7.7	56.4 ± 7.3	55.9 ± 6.8	−1.10 (0.07)	3×10^{-49}
BMI (kg/m^2)	26.4 ± 4.7	25.6 ± 3.9	25.4 ± 3.6	25.8 ± 3.7	−0.04 (0.04)	0.37
Waist (cm)	83.9 ± 13.0	82.9 ± 15.0	84.6 ± 13.0	89.0 ± 13.2	0.15 (0.10)	0.13
SBP (mmHg)	144 ± 20	141 ± 20	140 ± 20	142 ± 19	1.21 (0.20)	6×10^{-10}
DBP (mmHg)	86 ± 10	85 ± 10	86 ± 10	87 ± 10	0.76 (0.10)	1×10^{-13}
FPG (mmol/L) [c]	5.62 ± 0.70	5.60 ± 0.79	5.68 ± 0.75	6.04 ± 1.12	0.09 (0.02)	6×10^{-6}
HbA_{1C} (mmol/mol) [c]	40.9 ± 4.56	39.7 ± 5.06	39.2 ± 4.42	39.6 ± 5.41	−0.31 (0.13)	0.02
LDLC (mmol/L) [c]	4.36 ± 1.28	4.18 ± 1.00	4.12 ± 0.93	4.12 ± 0.92	−0.01 (0.03)	0.82
HDLC (mmol/L) [c]	1.27 ± 0.31	1.40 ± 0.37	1.42 ± 0.38	1.41 ± 0.39	0.10 (0.009)	2×10^{-31}
Triglycerides (mmol/L) [c]	1.48 ± 0.73	1.31 ± 0.68	1.34 ± 0.80	1.70 ± 1.55	0.03 (0.02)	0.54
hsCRP [b]	0.31 ± 0.49	0.24 ± 0.43	0.24 ± 0.38	0.29 ± 0.51	−0.003 (0.01)	0.58
Total energy intake (kcal/day)	2131 ± 747	2228 ± 630	2416 ± 623	2721 ± 740	96.7 (6.32)	1×10^{-52}
Vegetables (g/day)	161 ± 105	180 ± 99	188 ± 95	188 ± 111	3.32 (1.07)	0.002
Fruits (g/day)	208 ± 142	201 ± 125	174 ± 118	149 ± 119	−20.4 (1.33)	9×10^{-53}
Wine (g)	0 ± 0	25.9 ± 31.8	94.9 ± 62.9	167 ± 133	59.4 (0.52)	0
Alcohol (g/day)	0 ± 0	6.20 ± 5.17	23.8 ± 6.03	50.8 ± 17.8	16.4 (0.08)	0

Data represented as mean ± standard deviation

[a] Multivariable Cox proportional hazards model for incidence of cardiovascular disease (CVD) during follow-up adjusting for age, sex, BMI, SBP, season, method, total energy intake, leisure time physical activity, alcohol intake, smoking status, education, lipid lowering and antihypertensive treatment with HR referring to hazard ratio per alcohol intake category with abstainers as reference

[b] Linear regression analyses of tertiles of vegetable intake with quantitative traits or characteristics at baseline adjusting for age, sex, BMI, SBP, season, method, total energy intake, leisure time physical activity, alcohol intake, smoking status, education, lipid lowering and antihypertensive treatment when appropriate with β referring to associated effect estimate per alcohol intake category with abstainers as reference

[c] In MDC-CC only, N=4,828 (AA N=1,460; AG N=2,381; GG N=987)

CI, Confidence Interval; CVD, cardiovascular disease; SE, standard error; BMI, Body Mass Index; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; FPG, Fasting Plasma Glucose; HbA_{1C}, Hemoglobin A_{1C}; LDLC, Low-Density Lipoprotein Cholesterol; HDLC, High-Density Lipoprotein Cholesterol; hsCRP, High-Sensitivity C-Reactive Protein

Supplementary Table 5 Hazard ratio according to rs4977574 genotype for incident CVD in the Malmö Diet and Cancer study (n = 22,501) according to tertiles of alcohol consumption

		Hazard Ratio (95% Confidence Interval)				P_{trend} [b, d]	$P_{interaction}$ [e, f]
		AA	AG	GG	Additive Model [b, d]		
	N	6853	11111	4537			
Alcohol (g/day)							0.24, 0.26
Low	7469	1.01 (0.85–1.19)	1.25 (1.08–1.46)	1.45 (1.22–1.73)	1.18 (1.09–1.29)	0.0001	
Medium	7513	0.91 (0.77–1.09)	1.16 (1.00–1.35)	1.19 (1.00–1.43)	1.16 (1.06–1.27)	0.002	
High	7519	1 (ref)	0.96 (0.83–1.12)	1.26 (1.07–1.50)	1.10 (1.01–1.20)	0.03	
Per category [c, e]		1.00 (0.92–1.10)	0.87 (0.82–0.93)	0.94 (0.85–1.03)			
P_{trend} [c, e]		0.97	0.00009	0.19			

[a] Multivariable Cox proportional hazards model for incidence of cardiovascular disease (CVD) during follow-up with hazard ratio per each category of rs4977574 genotype and alcohol intake assuming the highest category and AA genotype as reference

[b] Multivariable Cox proportional hazards model for incidence of cardiovascular disease (CVD) during follow-up with hazard ratio per risk G allele using an additive genetic model

[c] Multivariable Cox proportional hazards model for incidence of cardiovascular disease (CVD) during follow-up with hazard ratio per each tertile of alcohol intake with the lowest tertile as reference.

[d] Adjusted for age and sex

[e] Adjusted for age, sex, BMI, SBP, season, method, total energy intake, leisure time physical activity, alcohol intake, smoking status, education, lipid lowering and antihypertensive treatment

[f] Excluding BMI, SBP, antihypertensive and lipid lowering treatment from the adjustment model

Supplementary Table 6 Hazard ratio for incident CAD in the Malmö Diet and Cancer Study (n = 23,949) according to rs4977574 genotype and dietary or alcohol intake categories

		HR (95% CI)				P_{trend} [b, d]	$P_{interaction}$ [e, f]
		AA	AG	GG	Additive Model [b, d]		
	n	7325	11777	4847			
Vegetables (g/day)							0.20, 0.25
Low	7952	1.19 (0.96–1.48)	1.16 (0.95–1.42)	1.46 (1.16–1.83)	1.09 (0.98–1.20)	0.11	
Medium	8041	0.79 (0.62–1.00)	1.20 (0.98–1.46)	1.31 (1.03–1.66)	1.29 (1.15–1.44)	0.00002	
High	7956	1.00 (ref)[a, e]	1.12 (0.91–1.38)	1.44 (1.13–1.83)	1.19 (1.05–1.35)	0.005	
Per category [c, e]		0.90 (0.90–1.02)	0.98 (0.90–1.06)	0.99 (0.88–1.12)			
P_{trend} [c, e]		0.09	0.58	0.84			
Fruits (g/day)							1.00, 0.87
Low	7886	1.04 (0.84–1.30)	1.30 (1.07–1.58)	1.59 (1.27–1.99)	1.23 (1.11–1.37)	0.0001	
Medium	8030	1.14 (0.92–1.42)	1.29 (1.06–1.56)	1.33 (1.05–1.68)	1.08 (0.97–1.21)	0.16	
High	8033	1.00 (ref)	1.10 (0.89–1.34)	1.55 (1.24–1.95)	1.23 (1.09–1.38)	0.0002	
Per category		1.02 (0.91–1.14)	0.92 (0.84–1.00)	0.97 (0.86–1.10)			
P_{trend}		0.76	0.044	0.63			
Wine (g/day)							0.13, 0.14
Non-consumers	6843	1.16 (0.92–1.47)	1.50 (1.22–1.85)	1.87 (1.49–2.37)	1.27 (1.14–1.40)	7×10^{-6}	
Low	8472	1.10 (0.88–1.39)	1.26 (1.02–1.55)	1.44 (1.13–1.83)	1.13 (1.01–1.26)	0.03	
High	8634	1.00 (ref)	1.03 (0.84–1.27)	1.28 (1.01–1.63)	1.13 (1.00–1.28)	0.05	
Per category		0.96 (0.84–1.09)	0.83 (0.76–0.92)	0.81 (0.70–0.93)			
P_{trend}		0.50	0.0002	0.002			
Alcohol (g/day)							0.92, 0.78
Abstainers	1448	1.38 (0.82–2.34)	1.32 (0.80–2.18)	2.34 (1.38–3.96)	1.26 (1.00–1.60)	0.06	
Low	17331	1.10 (0.71–1.69)	1.35 (0.88–2.07)	1.48 (0.95–2.28)	1.16 (1.08–1.25)	0.0001	
Medium	4150	1.06 (0.66–1.70)	1.06 (0.68–1.67)	1.58 (0.98–2.52)	1.20 (1.02–1.40)	0.03	

High	1020	1 (ref)	1.04 (0.62–177)	1.52 (0.85–2.73)	1.28 (0.95–1.74)	0.11
Per category		0.92 (0.79–1.07)	0.86 (0.77–0.96)	0.94 (0.80–1.10)		
P_{trend}		0.29	0.01	0.45		

[a] Multivariable Cox proportional hazards model for incidence of coronary artery disease (CAD) during follow-up with hazard ratio per each category of rs4977574 genotype and food or beverage intake assuming the highest category and AA genotype as reference

[b] Multivariable Cox proportional hazards model for incidence of coronary artery disease (CAD) during follow-up with hazard ratio per risk G allele using an additive genetic model

[c] Multivariable Cox proportional hazards model for incidence of coronary artery disease (CAD) during follow-up with hazard ratio per each higher food or beverage intake category with the lowest intake category as reference.

[d] Adjusted for age and sex

[e] Adjusted for age, sex, BMI, SBP, season, method, total energy intake, leisure time physical activity, alcohol intake, smoking status, education, lipid lowering and antihypertensive treatment

[f] Excluding BMI, SBP, antihypertensive and lipid lowering treatment from the adjustment model

Supplementary Table 7 Hazard ratio for incident stroke in the Malmö Diet and Cancer Study (n = 23,949) according to rs4977574 genotype and dietary or alcohol intake categories

		OR (95% CI)				P_{trend} [b, d]	$P_{interaction}$ [e, f]
		AA	AG	GG	Additive Model [b, d]		
	n	7325	11777	4847			
Vegetables (g/day)							0.051, 0.07
Low	7952	1.47 (1.15–1.88)	1.23 (0.98–1.56)	1.55 (1.19–2.02)	1.02 (0.91–1.14)	0.78	
Medium	8041	1.02 (0.79–1.33)	1.31 (1.04–1.65)	1.48 (1.13–1.94)	1.22 (1.08–1.38)	0.002	
High	7956	1.00 (ref)[a, e]	1.29 (1.02–1.65)	1.40 (1.06–1.87)	1.16 (1.02–1.33)	0.03	
Per category [c, e]		0.82 (0.72–0.93)	1.00 (0.91–1.09)	0.97 (0.85–1.12)			
P_{trend} [c, e]		0.002	0.97	0.70			
Fruits (g/day)							0.14, 0.23
Low	7886	1.36 (1.07–1.73)	1.21 (0.97–1.52)	1.48 (1.14–1.93)	1.05 (0.93–1.19)	0.42	
Medium	8030	1.20 (0.94–1.53)	1.22 (0.98–1.53)	1.61 (1.25–2.07)	1.16 (1.03–1.31)	0.02	
High	8033	1.00 (ref)	1.42 (1.14–1.76)	1.37 (1.05–1.78)	1.15 (1.01–1.30)	0.03	
Per category		0.88 (0.78–0.99)	1.08 (0.98–1.18)	1.02 (0.89–1.17)			
P_{trend}		0.04	0.13	0.82			
Wine (g/day)							0.20, 0.19
Non-consumers	6843	0.88 (0.69–1.13)	0.95 (0.76–1.19)	1.14 (0.88–1.48)	1.16 (1.03–1.31)	0.01	
Low	8472	0.75 (0.59–0.97)	0.95 (0.77–1.18)	1.10 (0.86–1.42)	1.20 (1.06–1.35)	0.003	
High	8634	1.00 (ref)	0.92 (0.74–1.13)	1.02 (0.78–1.32)	1.00 (0.88–1.13)	0.94	
Per category		1.02 (0.88–1.18)	1.06 (0.95–1.18)	0.88 (0.75–1.02)			
P_{trend}		0.79	0.34	0.09			
Alcohol (g/day)							0.43, 0.51
Abstainers	1448	0.86 (0.47–1.57)	1.74 (1.03–2.93)	1.70 (0.95–3.07)	1.36 (1.07–1.73)	0.01	
Low	17331	1.02 (0.63–1.64)	1.14 (0.71–1.82)	1.22 (0.76–1.98)	1.09 (1.00–1.18)	0.04	
Medium	4150	1.14 (0.68–1.90)	0.83 (0.50–1.38)	1.50 (0.89–2.53)	1.11 (0.93–1.33)	0.24	

High	1020	1 (ref)	1.24 (0.70–2.20)	1.28 (0.64–2.54)	1.14 (0.81–1.60)	0.45
Per category		1.08 (0.92–1.27)	0.81 (0.71–0.93)	0.99 (0.83–1.19)		
P_{trend}		0.74	0.002	0.94		

[a] Multivariable Cox proportional hazards model for incidence of stroke during follow-up with hazard ratio per each category of rs4977574 genotype and food or beverage intake assuming the highest category and AA genotype as reference

[b] Multivariable Cox proportional hazards model for incidence of stroke during follow-up with hazard ratio per risk G allele using an additive genetic model

[c] Multivariable Cox proportional hazards model for incidence of stroke during follow-up with hazard ratio per each higher food or beverage intake category with the lowest intake category as reference.

[d] Adjusted for age and sex

[e] Adjusted for age, sex, BMI, SBP, season, method, total energy intake, leisure time physical activity, alcohol intake, smoking status, education, lipid lowering and antihypertensive treatment

[f] Excluding BMI, SBP, antihypertensive and lipid lowering treatment from the adjustment model

Supplementary Table 8 Interaction of rs4977574 variant with vegetable intake, wine intake, and smoking habits on CVD risk markers

	Vegetable × 9p21		Wine × 9p21		Smoking × 9p21	
	$P_{interaction}$ [a]	$P_{interaction}$ [c]	$P_{interaction}$ [a]	$P_{interaction}$ [c]	$P_{interaction}$ [b]	$P_{interaction}$ [c]
SBP (mmHg)	0.52	0.41	0.70	0.78	0.94	0.74
DBP (mmHg)	0.93	0.75	0.78	0.70	0.79	0.55
FPG (mmol/L)	0.63	0.55	0.19	0.31	0.88	0.95
HbA_{1C} (mmol/mol)	0.015	0.015	0.53	0.63	0.85	0.96
LDLC (mmol/L)	0.65	0.60	0.75	0.77	0.53	0.62
HDLC (mmol/L)	0.54	0.45	0.24	0.40	0.049	0.024
Triglycerides	0.13	0.10	0.55	0.72	0.15	0.095
hsCRP	0.57	0.49	0.41	0.30	0.40	0.29

[a] Adjusted for age, sex, BMI, SBP, season, method, total energy intake, physical activity leisure time, smoking status, education, lipid lowering and antihypertensive treatment

[b] Adjusted for age, sex, BMI, SBP, total energy intake, physical activity leisure time, education, lipid lowering and antihypertensive treatment

[c] Excluding BMI, SBP, antihypertensive and lipid-lowering treatments from the adjustment model when needed

SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; FPG, Fasting Plasma Glucose; HbA_{1C}, Hemoglobin A_{1C}; LDLC, Low-Density Lipoprotein Cholesterol; HDLC, High-Density Lipoprotein Cholesterol; hsCRP, High-Sensitivity C-Reactive Protein

Paper V

Low LDL cholesterol and increased incidence of type 2 diabetes: Evidence from Mendelian randomization analyses

George Hindy, MD; Gull Rukh, MSc; Peter Almgren, MSc; Christina-Alexandra Schulz, MSc; Ulrika Ericson, PhD; Cristiano Fava, MD, PhD; Gunnar Engström, MD, PhD; Olle Melander, MD, PhD; Marju Orho-Melander, PhD

Author affiliations

Department of Clinical Sciences in Malmö, Skåne University Hospital, Lund University, Malmö, Sweden (Hindy, Rukh, Almgren, Schulz, Ericson, Fava, Engström, Melander, Orho-Melander); Department of Medicine, Verona University, Verona, Italy (Fava)

Corresponding author:

Professor Marju Orho-Melander
Department of Clinical Sciences in Malmö
Lund University
Clinical research center, 91:12, Jan Waldenströms gata 35
SE-205 02 Malmö
Sweden
Phone: +46 40 39 12 10
Fax: +46 40 39 12 22
E-mail: marju.orho-melander@med.lu.se

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ABSTRACT

IMPORTANCE Although several cardio-metabolic traits associate with increased risk of both type 2 diabetes (T2D) and coronary heart disease (CHD), evidence for causality only exists for type 2 diabetes (T2D) by BMI, as well as for CHD by LDL-cholesterol and triglycerides.

OBJECTIVE To investigate the causality of T2D by cardio-metabolic traits and potential differences in causal risk factors between T2D and CHD.

DESIGN Using a Mendelian randomization design, trait-specific weighted genetic risk scores (GRSs), including 31 BMI-, 29 systolic blood pressure-, 41 HDL-cholesterol-, 32 LDL-cholesterol-, 26 triglyceride-, and 15 fasting plasma glucose- (FPG) associated single nucleotide polymorphisms (SNPs), were used as instrumental variables to obtain the causal effects of these traits on T2D and CHD. Multivariable sensitivity analyses were performed using β-coefficients of all SNPs to adjust for pleiotropic effects. The effect of LDL-cholesterol on T2D was further analyzed using β-coefficients from the latest Global Lipids Genetics Consortium (GLGC, n=188577) and Diabetes Genetics Replication and Meta-analysis (DIAGRAM, n=34840 cases and 114981 controls) GWAS data.

PARTICIPANTS We used the population-based Malmö Diet and Cancer Study (MDCS) (n=28589), with a mean follow-up period of 15.4 years.

MAIN OUTCOMES AND MEASURES Analyses were performed using both incident (T2D, n=3257; CHD, n=2428) and combined incident and prevalent (T2D, n=4427; CHD, n=2997) cases.

RESULTS A 1SD increase in BMI and FPG by their respective GRS were associated with increased risk of T2D (HR: 1.904, 95%CI 1.292-2.804 and 2.669; 1.948-3.657, respectively), and a 1SD increase in LDL-cholesterol by its GRS was associated with decreased risk of T2D (HR: 0.876; 0.770-0.996). Multivariable analyses supported the causal association of BMI and FPG with T2D, and the inverse causality of LDL-cholesterol with T2D (P=.027, P=.01 and P=.008), as well as the direct causality of BMI and LDL-cholesterol with CHD (P=.027 and P=.007). Additional multivariable analyses using GWAS data of GLGC and DIAGRAM supported the inverse causality of LDL-cholesterol with T2D ($P=5\times10^{-7}$).

CONCLUSIONS AND RELEVANCE Our results indicate LDL-cholesterol has an opposite role on the causality of T2D and CHD. Further, our results support the beneficial role of weight reduction in the prevention of both T2D and CHD.

Introduction

In observational epidemiology, well validated risk prediction models have been developed for type 2 diabetes (T2D) and coronary heart disease (CHD). These models incorporate several traditional cardio-metabolic biomarkers but fail to address causality even if the biomarkers have independent associations and improve risk discrimination.[1-3] Fasting plasma glucose (FPG), body mass index (BMI), systolic blood pressure (SBP), triglycerides (TG), and high-density lipoprotein cholesterol (HDLC) are known to independently associate with T2D and improve risk discrimination.[3,4]

Several randomized clinical trials have shown that lifestyle interventions that result in weight loss decrease the risk of T2D.[5-7] In contrast, evidence of a beneficial effect of weight reduction for CHD prevention is not well documented.[8,9] However, a recent report from the Da Qing Prevention Study indicated that individuals in the weight loss intervention group experienced lower cardiovascular mortality compared to controls.[10] In addition, any risk reduction observed after weight loss intervention may be attributed not only to weight loss, but also to dietary factors and physical activity that are a part of the intervention. In terms of causality, a few Mendelian randomization studies have indicated that increased BMI is causally related to T2D; however, CHD-related findings have been inconsistent.[11-13]

Blood pressure and blood pressure progression are both independent risk predictors of T2D.[14] A meta-analysis of outcome trials on the treatment of isolated systolic hypertension in the elderly demonstrated a reduced risk of both stroke and coronary events.[15] On the other hand, there is insufficient evidence to suggest that blood pressure lowers T2D incidence; although, some trials have shown an increased risk of new-onset diabetes by thiazides and beta-blockers and a decreased risk by ACE inhibitors and ARBs, mainly due to drug effects.[16]

Low plasma concentrations of HDLC and high TG are both independent risk predictors of T2D.[3,4] An earlier Mendelian randomization study reported no evidence for a causal relationship between TG and T2D.[17] In addition, no direct evidence of causality between HDLC or low-density lipoprotein cholesterol (LDLC) and T2D has been presented to date. However, emerging evidence from statin trials and population-based cohort studies has linked statin use to increased

diabetes incidence.[18-22] Further, a recent Mendelian randomization study reported that LDLC-lowering alleles of single nucleotide polymorphisms (SNPs) in the 3-hydroxy-3-methylglutaryl-CoA (*HMGCR*) gene are associated with increased risk of T2D.[23] These alleles were correlated with lower HMGCR expression, consistent with HMGCR inhibition by statins.[23] LDLC and TG have been confirmed to be causally linked to CHD based on evidence from both randomized controlled trials[24,25] and/or Mendelian randomization studies.[26-28] For HDLC, findings from the CETP inhibitor trial on CHD reduction do not support a causal relationship,[29] which is also in line with several Mendelian randomization studies.[27,28,30] High FPG has also been shown to associate with incident CHD among non-diabetics,[31] but whether this association is causal or not remains unclear.

In this study, we use trait-specific weighted genetic risk scores (GRSs) composed of SNPs associated with BMI, SBP, LDLC, HDLC, TG, and FPG in genome-wide-association studies (GWAS) as measures of genetic predisposition to elevations in cardio-metabolic traits to investigate causal effects on the risk of T2D and CHD in one large middle-aged Swedish population-based study.

Methods

Study population

The Malmö Diet and Cancer Study (MDCS) is a population-based prospective cohort study in the city of Malmö, Sweden. From a source population of 74 138 individuals born between 1923 and 1950 and residing in Malmö, 30 447 individuals were recruited. The baseline examination period ranged from March 1991 to October 1996. Participants were informed about study procedures and questionnaires, and after this written informed consent was obtained from all participants. Anthropometrics, body composition, and blood pressure were directly measured. Study participants were instructed on how to complete an extensive questionnaire covering socio-economic and lifestyle factors, including medical history, smoking habits, education, and physical activity.[32] A total of 6103 randomly selected individuals from the MDCS were included in a cardiovascular sub-cohort (MDC-CC). For these individuals, additional measurements for low-density lipoprotein cholesterol (LDLC), high-density lipoprotein cholesterol (HDLC), triglycerides (TG), and fasting blood glucose (FBG) were taken from fasting blood samples. The MDCS was approved by the Regional Ethical Committee at Lund University (LU 51-90). A total of 28 589 individuals with genotype data were included in our study, and data on fasting lipids and glucose were available for 5432 from the MDC-CC. Details on baseline assessment and ascertainment of baseline and incident T2D and CHD cases are provided in the eMethods in the Supplement.

Genotyping and genetic risk scores

A MALDI-TOF mass spectrometer (Sequenom MassArray, Sequenom, San Diego, CA, USA) was used to genotype DNA samples using Sequenom reagents and protocols. Proxy SNPs were identified using SNAP version 2.2.2 when commercial primers were not available. SNPs that failed Sequenom genotyping were genotyped individually using TaqMan or KASPar allelic discrimination on an ABI 7900HT (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. We excluded individuals with < 60% successfully genotyped SNPs and SNPs with a genotype success rate of < 90% or deviation from Bonferroni-corrected Hardy-Weinberg Equilibrium in each set of SNPs of the specific traits.

Trait-specific weighted genetic risk scores (GRSs) were created using PLINK software (version 1.07). A total of 31, 29, 32, 41, 26, and 15 SNPs were used to create the BMI, SBP, LDLC, HDLC, TG, and FPG GRSs, respectively (eTables 2-7).[33-38]

Statistical analysis

PASW statistics (version 21) (SPSS Inc., Chicago, IL, USA), PLINK (version 1.07), and R (version 3.1.0) were used in the statistical analyses. First, in order to achieve normality of distribution and comparability between the studied traits, all of the traits studied (LDLC, HDLC, TG, BMI, SBP, and FPG) were natural log-transformed and changed to a z-score. Linear regression was used to study the association between the weighted GRSs and their respective traits, adjusting for age and sex. Cholesterol-lowering therapy was added to the model when the outcome variable was LDLC, HDLC, or TG. Antihypertensive therapy was added when the outcome variable was SBP. Individuals with diabetes at baseline were excluded when the outcome variable was FPG. In addition, the variance explained by the GRS of its respective trait was obtained from the linear regression models without covariates. A Cox proportional hazards model was used to analyze the observed association between the traits and incident cases, adjusting for age and sex. Cholesterol-lowering therapy was added when lipid traits were used as exposure variables and antihypertensive therapy was added when blood pressure was used as exposure variable. Individuals with diabetes at baseline were excluded when the exposure variable was FPG.

To estimate the un-confounded causal effect of the trait on the incident and combined (incident and prevalent combined) cases, we performed Mendelian randomization analyses employing a 2-stage regression approach. In the first stage, predicted values from the linear regression of the traits by their respective GRSs were used as the predictor variables for disease; Cox regression was used in the analysis of incident endpoints, and logistic regression was used in the analysis of overall disease risk, including both incident and prevalent cases. Age and sex were included as covariates in both stages. Cholesterol-lowering therapy was included as a covariate in instrumental variable analyses when the predicted levels of lipid traits were used, antihypertensive therapy was included when predicted levels of SBP were used, and individuals with baseline diabetes were excluded when predicted levels of FPG were used.

The GRS included pleiotropic SNPs, which may have biased our results. Excluding these SNPs would have largely weakened the GRSs as instrumental variables and, therefore, we used a previously described and applied multivariable Mendelian randomization approach.[28] In this approach, the β coefficients obtained from the logistic regression of each of the 153 SNPs on the outcomes (combined incident and prevalent T2D or CHD) (eTable 8) were regressed on the β coefficients obtained from the linear regression of the same SNPs on each cardio-metabolic trait (eTable 9). The β coefficients obtained with the cardio-metabolic traits were included in a single multivariable model as predictor variables to correct for potential pleiotropic effects. When estimating the effects of genetic predisposition, both incident and prevalent cases were included to maximize power; this was done as genotypes are acquired at conception, and for that reason, reverse causality is not an issue in this case.

Finally, we performed additional analyses, previously described by Do *et al*.,[28] using the β coefficients of the 185 LDLC, HDLC, and TG SNPs obtained from the latest Global Lipids Genetics Consortium (GLGC) (188 577 individuals) GWAS,[39] the β coefficients of these 185 SNPs on T2D obtained from the latest Diabetes Genetics Replication and Meta-analysis (DIAGRAM) (34 840 cases and 114 981 controls)[40] consortium GWAS, and the β coefficients of these 185 SNPs on homeostasis model assessment of beta cell function (HOMA-B) and insulin resistance (HOMA-IR) obtained from the Meta-Analyses of Glucose and Insulin-related traits Consortium (MAGIC) (46 186 non-diabetic individuals) GWAS.[38]

Results

Baseline characteristics

eTable 1 shows the baseline characteristics of the MDCS cohort. At the end of the mean follow-up time of 15.4 years, a total of 4427 individuals (15.5%) had T2D and 2997 had CHD (10.5%), and, of these, 3257 individuals had developed T2D and 2428 had developed CHD during the follow-up period. Each GRS was strongly associated with its respective trait. Each standard deviation (SD) of the GRS_{BMI}, GRS_{SBP}, GRS_{LDLC}, GRS_{HDLC}, GRS_{TG}, and GRS_{FPG} was associated 0.090, 0.063, 0.269, 0.245, 0.222, and 0.110 SD increase in BMI ($P = 2 \times 10^{-52}$), SBP ($P = 2 \times 10^{-32}$), LDLC ($P = 8 \times 10^{-90}$), HDLC ($P = 9 \times 10^{-88}$), TG ($P = 2 \times 10^{-66}$), and FPG ($P = 3 \times 10^{-16}$), respectively. These GRSs explained 0.8, 0.5, 7.2, 5.7, 4.3, and 2.2% of the variance of their traits, respectively (Table 1).

Trait and instrumental variable effects on incidence of T2D

Baseline levels of BMI, SBP, DBP, FPG, LDLC, HDLC, TG, ApoB, and ApoA were strongly associated with incidence of T2D (Table 2). In line with trait associations, the instrumental variable (IV) analyses indicated that the predicted elevations of BMI and FPG by their GRSs were directly associated with incidence of T2D (HR: 1.904; 95% CI: 1.292-2.804 and HR: 2.669; 1.948-3.657, per SD of BMI and FPG, respectively) and with overall T2D (OR: 2.455; 1.703-3.539 and OR: 2.716; 2.019-3.652, per SD of BMI and FPG, respectively). In contrast to LDLC trait association, IV analyses suggested that the predicted elevation of LDLC by its GRS was associated inversely with incidence of T2D (HR: 0.876; 0.770-0.996, per SD of LDLC) and overall T2D (OR: 0.879; 0.778-0.992, per SD of LDLC) (Figure 1A). Similar results were obtained using GRS_{LDLC} as a predictor of ApoB, the values of which were available for the entire MDCS cohort. IV analyses indicated that the predicted elevation of ApoB by GRS for LDLC was associated with both incidence of T2D (HR: 0.866; 0.753-0.996, per SD of ApoB) and overall T2D risk (OR: 0.869; 0.762-0.991, per SD of ApoB). IV analyses did not provide evidence for an association between genetic predisposition for elevated SBP, HDLC or TG and T2D (Figure 1A).

Many of the SNPs that were included in the GRSs exhibit pleiotropic associations and thus violate a basic assumption of Mendelian randomization. Therefore, we used a multivariable Mendelian randomization analysis to correct for the possible bias caused by pleiotropic associations of the SNPs in MDCS. Consistent with IV results, this analysis indicated inverse association of SNP β coefficients on LDLC with SNP β coefficients on T2D (β = -0.22; $P = .008$) after adjusting for SNP β coefficients of all the other traits. Furthermore, by using the same analysis we were able to observe direct associations between SNP β coefficients on BMI and FPG with SNP β coefficients on T2D ([β = 0.48; $P = .027$] and [β = 0.45; $P = .012$], respectively) (Table 3).

We also performed similar multivariable Mendelian randomization analyses using the GWAS data from the most recent GLGC and DIAGRAM meta-analyses.[39,40] Using the β coefficients of the 185 LDLC, HDLC, and TG SNPs on these traits from GLGC, and the β coefficients of the same SNPs for T2D from DIAGRAM, we observed an inverse association between SNP β coefficients on LDLC and SNP β coefficients on T2D (β = -0.20; $P = 5 \times 10^{-7}$) after adjustment for the β coefficients of the same SNPs on HDLC and TG. In addition, we observed an inverse association between SNP β coefficients on LDLC with SNP β coefficients on HOMA-IR (β = -0.022; $P = .008$) but not with HOMA-B (Table 4) in similar analyses.

To further rule out that the inverse association between LDLC and T2D could be mediated by HDLC, we repeated the IV analysis in MDCS with further adjustment for ApoA levels at baseline, which revealed an even stronger inverse association between the genetic predisposition for elevated LDLC and incidence of T2D (HR: 0.812; 0.708-0.931, per SD of LDLC; $P = .003$).

Trait and instrumental variable effects on incidence of CHD

The baseline levels of all traits were associated with incident CHD (Table 2). In line with the trait associations, IV analyses showed that the predicted elevations in LDLC and TG by their GRSs were directly associated with incidence of CHD (HR: 1.305; 1.125-1.515 and HR: 1.312; 1.097-1.568, per SD of LDLC and TG, respectively) and with overall risk of CHD (OR: 1.246; 1.074-1.445 and OR: 1.221; 1.022-1.458, per SD, respectively). Similarly, the predicted elevation in baseline ApoB (available in the whole MDCS cohort) by GRS_{LDLC} was associated

with similar direct association with incidence of CHD (HR: 1.336; 1.136-1.571, per SD of ApoB) and overall risk of CHD (OR: 1.270; 1.081-1.492, per SD of ApoB). In contrast with trait associations, IV analyses did not provide significant evidence for association between genetic predisposition for elevated BMI, SBP, HDLC or FPG and CHD (Figure 1B).

The multivariable Mendelian randomization analysis indicated direct associations of SNP β coefficients on LDLC with SNP β coefficients on CHD (β = 0.22; *P* = 0.007) after adjusting for SNP β coefficients of all the other traits. In addition, we observed direct association between SNP β coefficients on BMI with SNP β coefficients on CHD (β = 0.47; *P* = .027) (Table 3) using the same analysis.

Discussion

We performed Mendelian randomization analyses in a prospective study of 28 598 Swedish middle-aged participants of MDCS with >15 years of follow-up to investigate whether genetic predisposition for elevated cardio-metabolic traits is associated with T2D and CHD, and whether there is a difference in the causality of T2D and CHD by these cardio-metabolic traits. Our study found that a genetic predisposition to reduced-plasma LDLC increases the risk of T2D, suggesting an opposite role for LDLC in the development of T2D as compared with CHD. In addition, our results support a causal role for elevated BMI in both T2D and CHD.

We used three different Mendelian randomization analyses to investigate whether the associations between each of the six cardio-metabolic traits with T2D and CHD are causal. First, in MDCS, we used the IV approach to investigate whether genetic predisposition for elevated BMI, SBP, LDLC, HDLC, TG, and FPG translates to increased risk of T2D. Consistent with earlier results from clinical trials and Mendelian randomization studies, we observed significant evidence for a causal relationship between elevated FPG and BMI and higher risk of T2D. Importantly, we found evidence for an inverse causal association between LDLC and T2D, which is in contrast with the observed trait association, but in line with the reported increased risk of T2D by LDLC-lowering observed in statin trials. Second, we used a multivariable Mendelian randomization analysis that adjusted for potential bias by pleiotropic associations of genetic variants with more than one of the different cardio-metabolic traits investigated. This multivariable analysis in MDCS confirmed the direct causal association of BMI and FPG with T2D and the inverse causal association of plasma-LDLC levels with T2D. Third, we performed a second multivariable Mendelian randomization analysis for lipid traits using the GWAS meta-analysis data of GLGC and DIAGRAM[39,40] rather than data from MDCS. We confirmed the causal association of lower LDLC with increased risk of T2D. Using the same approach, we observed an inverse causal association between LDLC and HOMA-IR, suggesting a possible beneficial effect of elevated LDLC on insulin sensitivity. When applying the same Mendelian randomization approaches to CHD, IV analysis indicated direct causal effects of both higher LDLC and TG on CHD, which is consistent with earlier studies.[24-28] Further, a multivariable analysis in MDCS that adjusted for the pleiotropic effects of the SNPs suggested an association

between genetic predisposition to elevated BMI and increased risk of CHD, and confirmed the causal association of elevated LDLC with CHD.

The causal relationship between elevated BMI and T2D has also been observed in other Mendelian randomization studies.[11,12] These observations emphasize the role of increased adiposity in the pathogenesis of T2D; they also emphasizes the role of weight loss, regardless of how it is achieved, as a crucial factor in the prevention of the disease and the improvement of glycemic control in patients with T2D.

The inverse causal relationship between LDLC and T2D observed in our study could provide some explanation for the increased risk of new-onset diabetes associated with statin therapy.[18-23] Rosuvastatin has been found to be associated with increased risk of diabetes and elevated HbA_{1C} levels in the JUPITER (Justification for the Use of Statins in Primary Prevention: an Intervention Trial Evaluating Rosuvastatin) trial.[18] Similar findings have been observed in meta-analyses of clinical trials, suggesting that statins confer elevated risk of diabetes.[20-22] In addition, a recent population-based cohort study indicated increased diabetes risk with statin therapy adherence.[19] However, results from these trials suggest that cardiovascular morbidity and mortality benefits outweigh the elevated risk of developing diabetes.

Although the mechanistic explanation of the association of statins with increased risk for diabetes has been much debated, no clear explanation has yet been suggested. Most of the studies investigating the mechanisms have focused on the putative deleterious effects of statins rather than a potential role of LDLC-lowering *per se*. In a recent large study, genetic variants in the 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*) gene that associate with lower LDLC and lower hepatic HMGCR expression were associated with increased T2D risk and increased body weight.[23] One important conclusion of that study was that genetic inhibition of HMGCR can explain the increased risk of T2D by statin therapy. Our study adds to that conclusion by suggesting that the increased risk of T2D can in fact be a result of the lowering of plasma-LDLC levels, and, as such, may not be restricted to pharmacological or genetic inhibition of HMGCR. In addition, the adjustment for the pleiotropic associations of the SNPs with other cardio-metabolic traits, which may be particularly important for BMI, indicated that the inverse association between LDLC and T2D is unlikely to be mediated by BMI. Furthermore, a recent

study reported lower prevalence of diabetes in patients with familial hypercholesterolemia (n = 14 296) as compared with their unaffected relatives (n = 24 684) (OR, 0.5; 95% CI, 0.43-0.59).[41] This observation is consistent with the inverse causal association between LDLC and T2D observed in our study.

The direct causal effects of LDLC and TG on CHD observed in our IV analyses support previous clinical trial and Mendelian randomization findings.[24-27] The LDLC causal effect on CHD is also consistent with recent evidence from the IMPROVE-IT trial that showed additional benefits of ezetimibe on LDLC-lowering and cardiovascular outcomes.[42] In the multivariable sensitivity analyses, we observed direct causal associations of genetic elevations of both LDLC and BMI with CHD after taking into account other known pleiotropic effects that could affect our results. A previous large Mendelian randomization study using *FTO* SNP as an instrument failed to observe causal associations between BMI and CHD.[11] In this context, it is important to remember that in all Mendelian randomization analyses, including our study, the strength of the instrumental variables is important to consider as only a modest fraction of the variance of most traits can be explained by current available GRSs, and in particular single SNPs. However, although the variance of BMI explained by the GRS used in our study was only 0.8%, our findings support direct causality between BMI and CHD, which is in line with a couple of other Mendelian randomization studies.[12,13] The lack of a significant association between TG and CHD using the multivariable approach is in contrast with the significant association in our IV analysis, and in contrast with the earlier and larger study by Do *et al*.[28] In an attempt to elucidate if the TG result turned non-significant in our multivariable analysis due to the additional incorporation of non-lipid traits in the multivariable model as compared to the Do *et al*. study, we did an additional analysis with only LDLC, HDLC, and TG included in the multivariable analysis. However, TG remained not significantly associated with CHD, probably due to low power, underscoring the weakness of the used instrumental variable. Finally, the lack of an inverse causal association of HDLC with CHD in both the IV and multivariable analyses is consistent with previous clinical trial and Mendelian randomization studies.[27-30]

Our study has some limitations. The GRSs used as IVs included several SNPs that have pleiotropic effects on other cardio-metabolic traits, which violates a basic assumption of

Mendelian randomization. These SNPs were included in order to create GRSs that are able to explain as much as possible of the variation of the predictor traits and thus reduce power limitations. In our analyses, we have tried to correct for such bias by means of including known confounders in the instrumental variable models and by means of performing multivariable Mendelian randomization analyses. For many of the studied traits, the explained variance by their respective GRSs was modest, resulting in low power to detect causal associations. Therefore, in the interpretation of our study results, we have focused on significant outcomes and emphasize that the negative outcomes do not necessarily reflect lack of causation.

In conclusion, genetic mechanisms that raise LDLC seem to have opposite consequences for the risk of T2D and CHD, which is in line with results from randomized clinical trials of statin treatment. Importantly, our results suggest that the increased risk of T2D by decreasing LDLC is not restricted to pharmacological or genetic inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase, and indicate that the lowering of LDLC may uniformly translate into increased risk of T2D. In addition, our study re-emphasizes the importance of weight loss in prevention of both T2D and CHD.

Author contributions: Drs G. Hindy and M. Orho-Melander had full access to all of the data and take full responsibility for the integrity of the data and the accuracy of data analysis. The manuscript has been read and approved by all authors and the ICMJE criteria for authorship have been met.

Study concept and design: Hindy, Orho-Melander.

Acquisition, analysis, or interpretation of data: Hindy, Rukh, Almgren, Schulz, Ericson, Fava, Engström, Melander, Orho-Melander.

Drafting of the manuscript: Hindy, Orho-Melander.

Critical revision of the manuscript for important intellectual content: Hindy, Rukh, Almgren, Schulz, Ericson, Fava, Engström, Melander, Orho-Melander.

Statistical analysis: Hindy, Almgren, Melander, Orho-Melander.

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Study supervision: Hindy, Orho-Melander.

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Figure 1. Observed and causal (IV) association between cardio-metabolic traits and type 2 diabetes (T2D) and coronary heart disease (CHD).
A. Data are presented as hazard ratios (HR) or odds ratios (OR) and confidence intervals (CI) per standard deviation (SD) of the trait. All traits were associated with incident T2D. Predicted elevations in BMI and FPG by their respective genetic risk scores (GRS) were associated with incident (HR: 1.904; 95% CI: 1.292-2.804 and HR: 2.669; 1.948-3.657, per SD, respectively) and overall (OR: 2.455; 1.703-3.539 and OR: 2.716; 2.019-3.652, per SD, respectively) T2D. Predicted elevation of LDLC by its respective GRS was inversely associated with incident (HR: 0.876; 0.770-0.996) and overall (OR: 0.879; 0.778-0.992) T2D. **B.** All traits were associated with incident CHD. Predicted elevations in LDLC and TG by their respective genetic risk scores were associated with incident (HR: 1.305; 95% CI: 1.125-1.515 and HR: 1.312; 1.097-1.568, per SD, respectively) and overall (OR: 1.246; 1.074-1.445 and OR: 1.221; CI: 1.022-1.458 per SD, respectively) CHD. All associations were adjusted for age and sex. Associations with lipid traits were additionally adjusted for lipid lowering treatments. Associations with SBP were additionally adjusted for antihypertensive treatment.

Table 1. Association between genetic risk scores and their respective traits

GRS	β (SE)	*P*	Variance explained (%)
GRS_{BMI}	0.090 (0.006)	2×10^{-52}	0.8
GRS_{SBP}[b]	0.063 (0.005)	2×10^{-32}	0.5
GRS_{LDLC}[a, c]	0.269 (0.013)	8×10^{-90}	7.2
GRS_{HDLC}[a, c]	0.245 (0.012)	9×10^{-88}	5.7
GRS_{TG}[a, c]	0.222 (0.013)	2×10^{-66}	4.3
GRS_{FPG}[a, d]	0.110 (0.013)	3×10^{-16}	2.2

Effect (β) per 1 SD of GRS on 1 SD of the Ln transformed corresponding trait. [a] Data available only in the Malmö Diet and Cancer Study-Cardiovascular Cohort (n=5432). [b] Adjusted for antihypertensive treatment. [c] Adjusted for lipid-lowering treatment. [d] Excluding individuals with diabetes at baseline

Table 2. Baseline characteristics of the Malmö Diet and Cancer Study by incident type 2 diabetes and coronary heart disease

Characteristic	Incident T2D status				Incident CHD status			
	- n = 24 073	+ n = 3257	HR (95% CI)	P	- n = 25 591	+ n = 2428	HR (95% CI)	P
Women (n, %)	15074 (62.6%)	1577 (48.4%)			16207 (63.3%)	915 (37.7%)		
Age (years)	57.8 (7.8)	58.5 (7.0)			57.6 (7.6)	61.8 (6.9)		
BMI (kg/m^2)	25.4 (3.8)	28.4 (4.4)	2.02 (1.96-2.09)	$<1\times 10^{-99}$	25.7 (4.0)	26.8 (4.2)	1.22 (1.17-1.27)	1×10^{-20}
SBP (mmHg)	140 (20)	147 (19)	1.38 (1.33-1.44)	5×10^{-62}	140 (20)	151 (21)	1.39 (1.33-1.45)	7×10^{-47}
DBP (mmHg)	85 (10)	89 (10)	1.35 (1.30-1.40)	7×10^{-56}	85 (10)	89 (10)	1.22 (1.17-1.28)	8×10^{-20}
FPG (mg/dL)[a]	98.7 (9.2)	118.0 (25.4)	4.01 (3.74-4.30)	$<1\times 10^{-99}$	104.1 (25.4)	116.2 (44.2)	1.18 (1.03-1.34)	.015
LDLC (mg/dL)[a]	160.2 (17.4)	166.8 (41.3)	1.15 (1.06-1.24)	.0004	160.6 (38.2)	166.8 (37.5)	1.12 (1.01-1.25)	.027
HDLC (mg/dL)[a]	54.4 (14.3)	48.3 (12.7)	0.63 (0.58-0.68)	2×10^{-29}	54.1 (14.3)	47.9 (13.5)	0.70 (0.63-0.78)	4×10^{-11}
TG (mg/dL)[a]	114.2 (63.7)	152.2 (78.8)	1.68 (1.58-1.80)	3×10^{-55}	119.5 (67.3)	142.5 (89.4)	1.26 (1.15-1.38)	1×10^{-6}
ApoB (mg/dL)	106 (25)	116 (27)	1.40 (1.35-1.46)	3×10^{-64}	106 (26)	117 (26)	1.37 (1.31-1.43)	6×10^{-41}
ApoA (mg/dL)	158 (28)	148 (26)	0.72 (0.70-0.75)	1×10^{-69}	158 (28)	148 (27)	0.76 (0.73-0.80)	1×10^{-32}
Lipid Lowering (n, %)	567 (2.4%)	148 (4.5%)			515 (2.0%)	132 (5.4%)		
Antihypertensives (n, %)	3454 (14.3%)	885 (27.2%)			3817 (14.9%)	650 (26.8%)		

Data presented as means (SD). *Data were available only in the Malmö Diet and Cancer Study-Cardiovascular Cohort (n = 5432) with 842 incident type 2 diabetes cases and 535 incident coronary heart disease cases.
SI conversion factors: To convert FPG to mmol/L, multiply by 0.0556; LDLC and HDLC to mmol/L, multiply by 0.0259; TG to mmol/L, multiply by 0.0113.

Table 3. Multivariable Mendelian randomization analysis of cardio-metabolic traits and T2D and CHD using MDCS data

	β_{T2D}			β_{CHD}		
Predictors	β	SEM	P value	β	SEM	P value
β_{BMI}	0.48	0.22	.027	0.47	0.21	.027
β_{SBP}	-0.15	0.28	.59	0.05	0.27	.85
β_{LDLC}[a]	-0.22	0.08	.008	0.22	0.08	.007
β_{HDLC}[a]	-0.09	0.10	.34	0.005	0.09	.96
β_{TG}[a]	0.05	0.10	.60	-0.007	0.10	.95
β_{FPG}[a]	0.45	0.18	.012	-0.15	0.17	.39

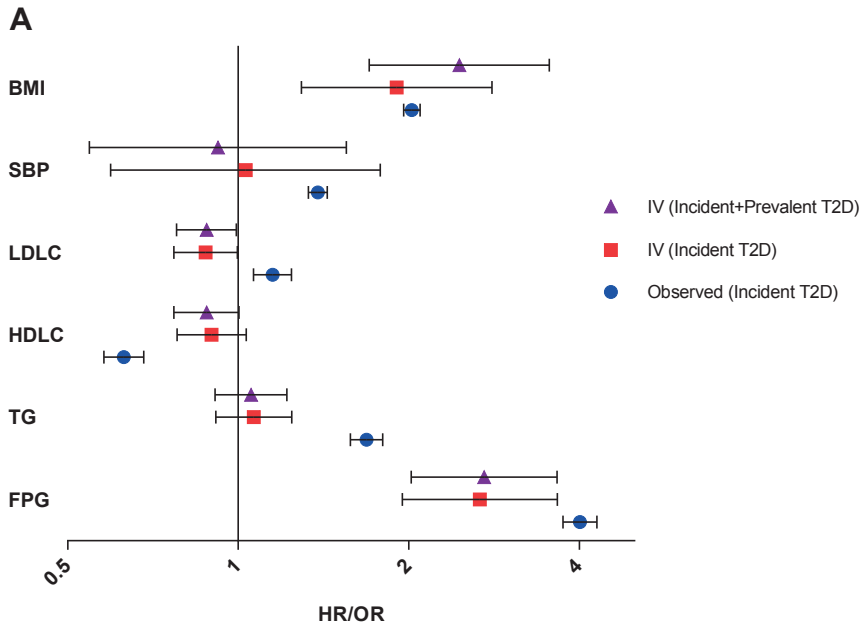
Multivariable linear regression model with β coefficients of 153 SNPs in association with BMI, SBP, LDLC, HDLC, TG, and FPG used as outcome variables and the β coefficients of the same SNPs on T2D and CHD as outcome variables.

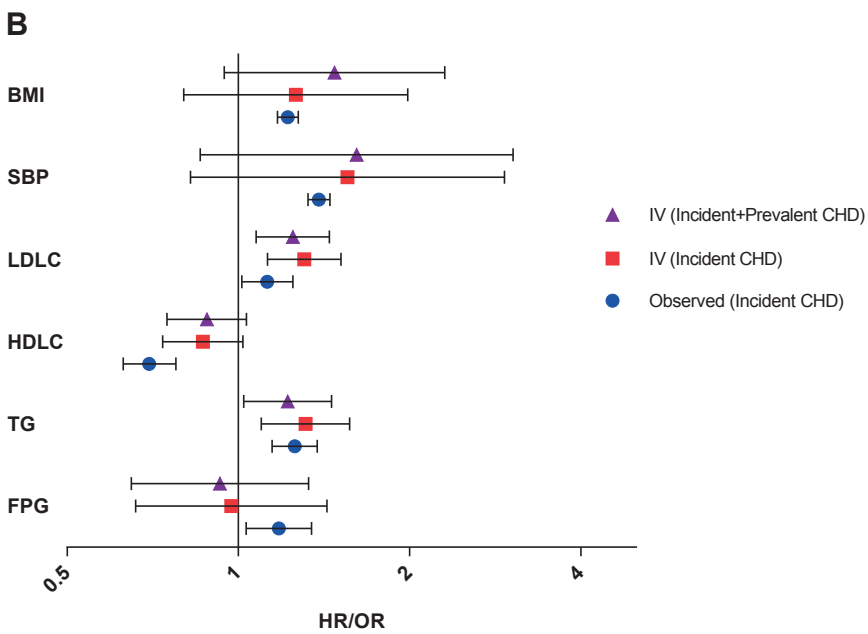
[a] Data were available only in the Malmö Diet and Cancer Study-Cardiovascular Cohort (n = 5432)

Table 4. Multivariable Mendelian randomization analysis of LDLC and T2D, HOMA-B, and HOMA-IR using GWAS data

		β_{T2D}			β_{HOMA-B}			$\beta_{HOMA-IR}$		
Predictor	Adjustment	β	SEM	P value	β	SEM	P value	β	SEM	P value
β_{LDLC}		-0.18	0.04	8×10^{-6}	0.000	0.007	.98	-0.018	0.008	.036
	β_{HDLC}	-0.20	0.04	6×10^{-7}	-0.002	0.007	.73	-0.022	0.008	.007
	β_{TG}	-0.18	0.04	8×10^{-6}	0.000	0.007	1	-0.018	0.008	.030
	β_{HDLC}, β_{TG}	-0.20	0.04	5×10^{-7}	-0.002	0.007	.73	-0.022	0.008	.008

Multivariable linear regression models using 185 lipid associated SNPs from GLGC GWAS.[39] β coefficients of the same SNPs on T2D were obtained from the DIAGRAM GWAS[40] and on HOMA-B and HOMA-IR from the MAGIC GWAS.[38]


A
BMI
SBP
LDLC
HDLC
TG
FPG
0.5
1
2
4
HR/OR
IV (Incident+Prevalent T2D)
IV (Incident T2D)
Observed (Incident T2D)


B
BMI
SBP
LDLC
HDLC
TG
FPG
0.5
1
2
4
HR/OR
IV (Incident+Prevalent CHD)
IV (Incident CHD)
Observed (Incident CHD)

Low LDL cholesterol and increased incidence of type 2 diabetes: Evidence from Mendelian randomization analysis

George Hindy, MD; Gull Rukh, MSc; Peter Almgren, MSc; Christina-Alexandra Schulz, MSc; Ulrika Ericson, PhD; Cristiano Fava, MD, PhD; Gunnar Engström, MD, PhD; Olle Melander, MD, PhD; Marju Orho-Melander, PhD

Author affiliations

Department of Clinical Sciences in Malmö, Skåne University Hospital, Lund University, Malmö, Sweden (Hindy, Rukh, Almgren, Schulz, Ericson, Fava, Engström, Melander, Orho-Melander); Department of Medicine, Verona University, Verona, Italy (Fava)

Corresponding author:

Professor Marju Orho-Melander
Department of Clinical Sciences in Malmö
Lund University
Clinical research centre, 91:12, Jan Waldenströms gata 35
SE-205 02 Malmö
Sweden
Phone: +46 40 39 12 10
Fax: +46 40 39 12 22
E-mail: marju.orho-melander@med.lu.se

eMethods

Baseline Assessment

Blood pressure was measured using a mercury-column sphygmomanometer after ten minutes of rest in the supine position. A balance-beam scale was used to measure weight (kilograms) with subjects wearing light clothing and no shoes. A fixed stadiometer was used to measure height (centimeters). BMI was measured as weight in kilograms divided by height in meters squared. Fasting serum lipids and fasting blood glucose (FBG) were measured from blood samples drawn after an overnight fast. FBG was converted to fasting plasma glucose (FPG) by multiplying the values by 1.13. Samples were analyzed by routine standard methods at the Department of Clinical Chemistry, Malmö University Hospital. Fasting blood measurements were only available in the Malmö Diet and Cancer Cardiovascular Cohort (MDC-CC). ApoB and ApoA1 were measured in non-fasted plasma samples of the entire MDC by Quest Diagnostics (San Juan Capistrano, CA, USA), blinded to case-control status, using an immunonephelometric assay run on the Siemens BNII (Siemens, Newark, DE, USA). The inter-assay variability was < 4.0% for both ApoA1 and ApoB.

Ascertainment of diabetes diagnosis

Diabetes cases (prevalent cases and incident cases) were defined based on six different national and regional diabetes registers: Individuals could be registered as having a diabetes diagnosis in the nationwide Swedish National Diabetes Register[1], the regional Diabetes 2000 register of the Scania region, of which Malmö is the largest city[2], or in the Swedish National Patient Register, which is a principal source of data for numerous research projects and which covers more than 99% of all somatic and psychiatric hospital discharges and Swedish hospital-based outpatient care[3], or they could be classified as diabetes cases if their cause of death was registered as diabetes in the Swedish Cause-of-Death Register, which comprises all deaths in Swedish residents occurring in Sweden or abroad[4], or if they had been prescribed anti-diabetic medication according to the Swedish Prescribed Drug Register[5], or if they had at least two HbA1c recordings ≥ 6.0% using the Swedish Mono-S standardization system (corresponding to 7.0% according to the US National Glycohemoglobin Standardization Program [NGSP]) in the Malmö HbA1c register, which analysed and catalogued all HbA1c samples at the Department of Clinical Chemistry taken in institutional and non-institutional care in the greater Malmö area from 1988 onwards.

In addition, diabetes cases at the baseline examination of MDC-CC were identified based on self-reports of a physician diagnosis or the use of diabetes medication in a baseline questionnaire, or fasting whole blood glucose of ≥ 6.1 mmol/L (corresponding to fasting plasma glucose concentration of ≥ 7.0 mmol/L). Furthermore, a diabetes diagnosis could be determined at the MDC-CC reinvestigation based on self-reports of a physician diagnosis or the use of diabetes medication based on a questionnaire or fasting plasma glucose of ≥ 7.0 mmol/L or a 120-min value post-OGTT plasma glucose of > 11.0 mmol/L.

A total of 4427 cases of T2D were included in our study, 3257 of these were incident cases occurring during the follow-up period.

Ascertainment of coronary heart disease

Individuals who developed fatal or nonfatal MI or those who have died due to ischemic heart disease were defined as individuals with incident coronary heart disease (CHD). The Swedish Hospital Discharge Register and the Swedish Cause of Death Register were used in the ascertainment of CHD cases[6]. MI was defined using codes 410 and I21 of the *International Classification of Diseases* 9th and 10th Revisions (ICD9 and ICD10), respectively. Death due to ischemic heart disease was defined on the basis of codes 412 and 414 of the ICD9 or codes I22-I23 and I25 of the ICD10. The detailed ascertainment procedure has been described elsewhere[7].

A total of 2997 cases of CHD were included in our study, 2428 of these were incident cases recorded during the follow-up period.

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13. Dupuis J, Langenberg C, Prokopenko I, et al. New genetic loci implicated in fasting glucose homeostasis and their impact on type 2 diabetes risk. *Nature genetics.* 2010;42(2):105-116.

eTable 1. Characteristics of the Malmö Diet Cancer Study

Characteristic	MDCS n = 28 589
Women (n, %)	17232 (60.3%)
Age (years)	58.0 (7.7)
BMI (kg/m^2)	25.8 (4.0)
SBP (mmHg)	141 (20)
DBP (mmHg)	86 (10)
FPG (mg/dL)[a]	105.3 (28.3)
LDLC (mg/dL)[a]	161.0 (38.2)
HDLC (mg/dL)[a]	53.3 (14.3)
TG (mg/dL)[a]	122.1 (70.8)
Lipid Lowering (n, %)	833 (2.9%)
Antihypertensives (n, %)	4838 (16.9%)
T2D ($n_{Prevalent}/n_{Incident}$)	1170/3257
CHD ($n_{Prevalent}/n_{Incident}$)	569/2428

Data presented as number (percentage) or means (standard deviation); [a] Data available only in the Malmö Diet and Cancer Study-Cardiovascular Cohort (n = 5432)

SI conversion factors: To convert FPG to mmol/L, multiply by 0.0556; LDLC and HDLC to mmol/L, multiply by 0.0259; TG to mmol/L, multiply by 0.0113.

eTable 2. Components of the body mass index weighted genetic risk score

Locus	SNP	Proxy	Weight	Risk allele	Alleles (major/minor)	MAF
NEGRI	rs2815752		0.13	T	T/C	0.41
SEC16B/RASAL2	rs543874	rs10913469	0.22	C	T/C	0.21
TNNI3K	rs1514175		0.07	A	G/A	0.43
PTBP2	rs1555543		0.06	C	C/A	0.43
TMEM18	rs2867125	rs6548238	0.31	C	C/T	0.17
RBJ	rs713586		0.14	C	T/C	0.48
FANCL	rs887912		0.1	T	C/T	0.27
LRP1B	rs2890652		0.09	C	T/C	0.18
SFRS10/ETV5	rs9816226		0.14	T	T/A	0.16
CADM2	rs13078807		0.1	G	A/G	0.19
GNPDA2	rs10938397		0.18	G	A/G	0.41
SLC39A8	rs13107325		0.19	T	C/T	0.05
FLJ35779	rs2112347		0.1	T	T/G	0.37
TFAP2B	rs987237		0.13	G	A/G	0.18
NUDT3	rs206936		0.06	G	A/G	0.19
LRRN6C	rs10968576		0.11	G	A/G	0.30
BDNF	rs10767664		0.19	A	A/T	0.21
MTCH2	rs3817334		0.06	T	C/T	0.40
RPL27A	rs4929949		0.06	C	C/T	0.498
BCDIN3D/FAIM2	rs7138803		0.12	A	G/A	0.41
MTIF3	rs4771122		0.09	G	A/G	0.26
NRXN3	rs10150332		0.13	C	T/C	0.21
PRKD1	rs11847697		0.17	T	C/T	0.04
MAP2K5	rs2241423		0.13	G	G/A	0.22
FTO	rs1558902		0.39	A	T/A	0.42
SH2B1	rs7359397	rs7498665	0.15	G	A/G	0.40
GPRC5B	rs12444979		0.17	C	C/T	0.13
MC4R	rs571312	rs17782313	0.23	C	T/C	0.24
KCTD15/CHST8	rs29941		0.06	C	C/T	0.33
QPCTL; GIPR	rs2287019		0.15	C	C/T	0.21
TMEM160	rs3810291		0.09	A	A/G	0.32

The weights are based on published effect sizes.[8] MAF: Minor allele frequency

eTable 3. Components of the systolic blood pressure weighted genetic risk score

Locus	SNP	Proxy	Weight	Risk allele	Alleles (major/minor)	MAF
MTHFR/NPPB	rs17367504		0.547	A	A/G	0.15
MOV10	rs2932538		0.388	G	G/A	0.26
SLC4A7	rs13082711		0.315	C	T/C	0.22
ULK4	rs3774372		0.067	C	T/C	0.16
MECOM	rs419076		0.409	T	C/T	0.46
FGF5	rs16998073		0.740	T	A/T	0.35
SLC39A8	rs13107325		0.981	C	C/T	0.05
GUCY1A3/GUCY1B3	rs13139571		0.312	C	C/A	0.22
NPR3/C5orf23	rs1173771		0.504	G	G/A	0.40
EBF1	rs11953630		0.412	C	C/T	0.35
HFE	rs1799945		0.627	G	C/G	0.12
BAT2/BAT5	rs805303		0.376	G	G/A	0.37
CACNB2(5')	rs4373814		0.373	C	G/C	0.44
c10orf107	rs1530440		0.43	C	C/T	0.18
PLCE1	rs932764		0.484	G	A/G	0.45
CYP17A1/NT5C2	rs11191548		0.464	T	T/C	0.11
ADM	rs7129220		0.619	A	G/A	0.10
PLEKHA7	rs381815		0.840	T	C/T	0.28
FLJ32810/TMEM133	rs633185		0.565	C	C/G	0.38
ATP2B1	rs2681492		1.26	T	T/C	0.14
SH2B3	rs3184504		0.448	T	C/T	0.48
TBX5/TBX3	rs10850411		0.354	T	T/C	0.30
CYP1A2/ULK3	rs1378942		0.416	C	A/C	0.32
FES	rs2521501		0.65	T	A/T	0.32
PLCD3	rs12946454		0.210	T	A/T	0.24
GOSR2	rs17608766		0.556	C	T/C	0.14
ZNF652	rs16948048		0.410	G	A/G	0.38
JAG1	rs1327235		0.34	G	A/G	0.45
GNAS/EDN3	rs6015450		0.896	G	A/G	0.14

The weights are based on published effect sizes.[9-11] MAF: Minor allele frequency

eTable 4. Components of the low-density lipoprotein cholesterol weighted genetic risk score

Gene	SNP	Proxy	Weight	Risk allele	Alleles (major/minor)	MAF
IRF2BP2	rs514230		1.13	A	A/T	0.48
LDLRAP1	rs12027135		1.10	T	T/A	0.45
PCSK9	rs2479409		2.01	G	A/G	0.35
SORT1	rs629301		5.65	A	A/C	0.23
ABCG5/8	rs4299376		2.75	G	T/G	0.29
APOB	rs1367117		4.05	A	G/A	0.34
HMGCR	rs12916		2.45	C	T/C	0.41
TIMD4	rs6882076		1.67	C	C/T	0.36
LPA	rs1564348		1.95	G	A/G	0.15
MYLIP	rs3757354		1.43	G	G/A	0.24
FRK	rs11153594	rs9488822	0.89	A	A/T	0.31
HLA	rs3177928		1.83	A	G/A	0.13
HFE	rs1800562		2.22	G	G/A	0.05
DNAH11	rs12670798		1.26	C	T/C	0.25
CYP7A1	rs1030431	rs2081687	0.95	T	C/T	0.34
PPP1R3B	rs2126259	rs9987289	2.22	G	G/A	0.10
TRIB1	rs2954022	rs2954029	1.84	A	A/T	0.47
ABO	rs9411489		2.24	A	G/A	0.21
GPAM	rs1129555	rs2255141	1.08	A	G/A	0.29
APOA1	rs964184		2.85	G	C/G	0.13
FADS1	rs174583	rs174546	1.71	C	C/T	0.33
ST3GAL4	rs11220462		1.95	T	C/T	0.14
BRAP	rs11065987		0.97	A	A/G	0.42
HNF1A	rs1169288		1.42	G	T/G	0.32
NYNRIN	rs8017377		1.14	A	G/A	0.46
CETP	rs7205804	rs3764261	1.45	G	G/T	0.33
HPR	rs2000999		2.00	A	G/A	0.21
APOE	rs4420638		7.14	G	A/G	0.20
CILP2	rs10401969		3.11	T	T/C	0.10
LDLR	rs6511720		6.99	G	G/T	0.10
MAFB	rs2902941	rs2902940	0.98	A	A/G	0.28
TOP1	rs6029526		1.39	A	T/A	0.48

The weights are based on published effect sizes.[12] MAF: Minor allele frequency

eTable 5. Components of the high-density lipoprotein cholesterol genetic risk score

Locus	SNP	Proxy	Weight	Risk allele	Alleles (major/minor)	MAF
GALNT2	rs4846914		-0.61	G	A/G	0.40
PABPC4	rs4660293		-0.48	G	A/G	0.25
ZNF648	rs1689800		-0.47	C	T/C	0.34
APOB	rs1042034		-0.90	A	A/G	0.21
COBLL1	rs12328675		-0.68	T	T/C	0.12
IRS1	rs2972146		-0.46	A	A/C	0.37
SLC39A8	rs13107325		-0.84	T	C/T	0.05
ARL15	rs6450176		-0.49	A	G/A	0.25
C6orf106	rs2814944		-0.49	A	G/A	0.16
CITED2	rs605066		-0.39	C	T/C	0.44
MLXIPL	rs17145738		-0.57	C	C/T	0.12
LPL	rs12678919		-2.25	A	A/G	0.09
PPP1R3B	rs9987289		-1.21	A	G/A	0.10
TRIB1	rs10808546	rs2954029	-0.61	A	A/T	0.47
TRPS1	rs2293889		-0.44	T	G/T	0.43
ABCA1	rs1883025		-0.94	A	G/A	0.24
TTC39B	rs581080		-0.65	G	C/G	0·19
AMPD3	rs2923084		-0.41	G	A/G	0·17
APOA1	rs964184		-1.50	G	C/G	0.13
FADS1#	rs174601	rs174546	-0.73	T	C/T	0.33
LRP4	rs3136441		-0.78	T	T/C	0.14
UBASH3B#	rs7115089	rs7941030	-0.31	T	T/C	0.39
LRP1#	rs3741414	rs11613352	-0.46	C	C/T	0.27
MVK	rs7134594		-0.44	C	T/C	0.46
PDE3A	rs7134375		-0.40	C	C/A	0.43
SBNO1#	rs4759375	rs4759377	-0.86	C	C/T	0.09
ZNF664	rs4765127		-0.44	G	G/T	0.33
LACTB	rs2652834		-0.39	T	C/T	0.21
LIPC	rs1532085		-1.45	G	G/A	0.38
CETP	rs3764261		-3.39	G	G/T	0.33
CMIP	rs2925979		-0.45	A	G/A	0.30
LCAT	rs16942887		-1.27	G	G/A	0.14
PGS1	rs4129767		-0.39	C	T/C	0.48
STARD3	rs11869286		-0.48	G	C/G	0.32
LIPG	rs7241918		-1.31	G	T/G	0.17
MC4R	rs12967135		-0.42	A	G/A	0.23
ANGPTL4	rs7255436		-0.45	C	A/C	0.44
APOE	rs4420638		-1.06	G	A/G	0.20
LOC55908	rs737337		-0.64	C	T/C	0.11
HNF4A	rs1800961		-1.88	T	C/T	0.04
UBE2L3	rs181362		-0.46	A	G/A	0.23

The weights are based on published effect sizes.[12] MAF: Minor allele frequency

eTable 6. Components of the triglyceride weighted genetic risk score

locus	SNP	Proxy	Weight	Risk allele	Alleles (major/minor)	MAF
GALNT2	rs1321257	rs4846914	2.76	G	A/G	0.40
APOB	rs1042034		5.99	A	A/G	0.21
GCKR	rs1260326		8.76	T	C/T	0.37
IRS1	rs2943645	rs2972146	1.89	A	A/C	0.37
MSL2L1	rs645040		2.22	T	T/G	0.22
KLHL8	rs442177		2.25	A	A/C	0.43
MAP3K1	rs9686661		2.57	T	C/T	0.16
TIMD4	rs1553318	rs6882076	2.63	C	C/T	0.36
HLA	rs2247056		2.99	G	G/A	0.27
MLXIPL	rs17145738		9.32	C	C/T	0.12
LPL	rs12678919		13.64	A	A/G	0.09
NAT2	rs1495741		2.85	G	A/G	0.22
PINX1	rs11776767		2.01	C	G/C	0.34
TRIB1	rs2954029		5.64	A	A/T	0.47
CYP26A1	rs2068888		2.28	G	G/A	0.44
APOA1	rs964184		16.95	G	C/G	0.13
FADS1	rs174546		3.82	T	C/T	0.33
LRP1	rs11613352		2.70	C	C/T	0.27
ZNF664	rs12310367	rs4765127	2.42	G	G/T	0.33
CAPN3	rs2412710		7.00	A	G/A	0.02
FRMD5	rs2929282		5.13	T	A/T	0.04
CETP	rs7205804	rs3764261	2.88	G	G/T	0.33
CTF1	rs11649653		2.13	C	C/G	0.41
APOE	rs439401		5.50	C	C/T	0.36
CILP2	rs10401969		7.83	T	T/C	0.10
PLA2G6	rs5756931		1.54	T	T/C	0.36

The weights are based on published effect sizes.[12] MAF: Minor allele frequency

eTable 7. Components of the fasting plasma glucose weighted genetic risk score

Locus	SNP	Proxy	Weight	Risk Allele	Alleles (major/minor)	MAF
PROX1	rs340874		0.013	G	G/A	0.46
G6PC2	rs560887		0.075	G	G/A	0.30
GCKR	rs780094		0.029	G	G/A	0.36
SLC2A2	rs11920090		0.02	T	T/A	0.13
ADCY5	rs11708067		0.027	A	A/G	0.23
DGKB/TMEM195	rs2191349		0.03	T	T/G	0.49
SLC30A8	rs13266634		0.027	C	C/T	0.32
GLIS3	rs7034200		0.018	A	C/A	0.48
TCF7L2	rs7903146		0.023	T	C/T	0.26
ADRA2A	rs10885122		0.022	G	G/T	0.12
CRY2	rs11605924		0.015	A	C/A	0.49
FADS1	rs174550		0.017	T	T/C	0.34
MADD	rs7944584		0.021	A	A/T	0.25
MTNR1B	rs10830963		0.067	G	C/G	0.29
FAM148B/C2CD4B	rs11071657		0.008	A	A/G	0.39

The weights are based on published effect sizes.[13] MAF: Minor allele frequency

eTable 8. Association of cardio-metabolic SNPs with type 2 diabetes and coronary heart disease

		Incident T2D		All T2D		Incident CHD		All CHD	
Main Trait	SNP	β	P value	β	P value	β	P value	β	P value
BMI	rs1558902	0.060	0.018	0.052	0.031	0.012	0.683	0.031	0.293
BMI	rs6548238	0.039	0.241	0.059	0.057	0.040	0.304	0.059	0.124
BMI	rs571312	-0.008	0.784	0.011	0.701	0.006	0.855	0.023	0.497
BMI	rs10938397	0.001	0.961	0.021	0.364	0.013	0.650	0.039	0.177
BMI	rs10767664	-0.034	0.274	-0.027	0.356	0.021	0.559	0.046	0.200
BMI	rs2815752	0.075	0.003	0.086	<0.0005	0.014	0.633	0.007	0.808
BMI	rs7359397	0.041	0.114	0.046	0.060	0.040	0.178	0.029	0.321
BMI	rs9816226	0.024	0.481	0.017	0.592	-0.009	0.822	-0.005	0.903
BMI	rs3817334	0.042	0.104	0.040	0.098	0.040	0.183	0.051	0.085
BMI	rs29941	0.010	0.713	0.027	0.263	-0.035	0.245	-0.028	0.346
BMI	rs543874	-0.008	0.793	0.018	0.531	-0.013	0.714	0.003	0.923
BMI	rs987237	0.065	0.044	0.082	0.008	-0.009	0.811	0.002	0.952
BMI	rs7138803	-0.003	0.919	0.009	0.697	0.035	0.226	0.022	0.439
BMI	rs10150332	0.047	0.124	0.044	0.135	-0.016	0.661	-0.025	0.491
BMI	rs713586	-0.012	0.646	-0.013	0.592	0.009	0.768	0.009	0.752
BMI	rs12444979	-0.018	0.640	-0.005	0.899	-0.009	0.841	-0.014	0.752
BMI	rs2241423	0.067	0.028	0.068	0.019	-0.007	0.844	-0.037	0.283
BMI	rs2287019	0.012	0.701	0.005	0.873	0.017	0.635	0.009	0.794
BMI	rs1514175	-0.030	0.244	-0.031	0.195	-0.027	0.361	-0.053	0.071
BMI	rs13107325	-0.028	0.641	0.017	0.759	-0.008	0.904	-0.034	0.628
BMI	rs2112347	0.037	0.163	0.025	0.321	-0.001	0.962	-0.019	0.529
BMI	rs10968576	0.029	0.288	0.057	0.028	0.029	0.365	0.038	0.233
BMI	rs3810291	0.061	0.025	0.075	0.003	-0.016	0.623	-0.013	0.684
BMI	rs887912	0.032	0.257	0.014	0.604	-0.026	0.433	-0.036	0.281
BMI	rs13078807	-0.005	0.888	0.018	0.556	0.043	0.244	0.016	0.662
BMI	rs11847697	0.108	0.078	0.146	0.012	0.067	0.358	0.102	0.154
BMI	rs2890652	-0.046	0.168	-0.055	0.081	-0.032	0.409	-0.046	0.225
BMI	rs1555543	-0.023	0.367	-0.016	0.518	0.016	0.583	0.011	0.703
BMI	rs4771122	-0.027	0.354	0.006	0.832	-0.026	0.436	-0.018	0.584
BMI	rs4929949	-0.047	0.065	-0.045	0.060	0.007	0.811	0.025	0.382
BMI	rs206936	0.046	0.150	0.043	0.151	-0.042	0.261	-0.023	0.532
LDL	rs1564348	0.027	0.447	0.048	0.150	0.018	0.654	0.029	0.467
LDL	rs2479409	-0.028	0.294	-0.032	0.203	0.064	0.036	0.057	0.058
LDL	rs3757354	-0.049	0.106	-0.057	0.046	-0.046	0.190	-0.052	0.137
LDL	rs4299376	0.056	0.041	0.033	0.199	0.016	0.620	0.028	0.379
LDL	rs4420638	-0.089	0.005	-0.076	0.010	0.044	0.214	0.047	0.177
LDL	rs6029526	0.009	0.716	0.015	0.520	-0.039	0.187	-0.022	0.443
LDL	rs629301	-0.005	0.858	-0.016	0.565	0.092	0.009	0.105	0.003
LDL	rs6511720	-0.010	0.812	-0.001	0.972	0.148	0.004	0.159	0.002
LDL	rs8017377	-0.023	0.367	-0.012	0.614	-0.038	0.208	-0.068	0.021
LDL	rs9411489	0.103	0.001	0.092	0.001	0.031	0.381	0.037	0.290
LDL	rs1367117	-0.026	0.337	-0.012	0.650	0.004	0.901	0.006	0.847
LDL	rs11220462	0.005	0.885	-0.005	0.878	0.099	0.015	0.086	0.035

LDL	rs1800562	-0.071	0.205	-0.035	0.512	0.051	0.453	0.075	0.266
LDL	rs12027135	0.013	0.600	0.003	0.911	-0.018	0.550	-0.008	0.778
LDL	rs514230	<0.0005	0.993	-0.012	0.605	0.028	0.341	0.038	0.191
LDL	rs12916	-0.027	0.294	-0.008	0.745	0.029	0.330	0.063	0.030
LDL	rs6882076	0.034	0.191	0.048	0.050	0.070	0.022	0.063	0.036
LDL	rs3177928	-0.023	0.542	-0.021	0.547	-0.104	0.021	-0.096	0.028
LDL	rs9488822	-0.005	0.846	-0.008	0.768	-0.028	0.367	-0.035	0.261
LDL	rs12670798	-0.001	0.972	-0.002	0.942	-0.012	0.719	0.027	0.422
LDL	rs9987289	-0.032	0.453	-0.029	0.471	0.014	0.783	0.015	0.766
LDL	rs2081687	-0.023	0.393	-0.035	0.164	-0.008	0.800	-0.016	0.611
LDL	rs2954029	0.001	0.969	-0.001	0.980	0.039	0.178	0.055	0.054
LDL	rs2255141	0.006	0.843	-0.012	0.635	0.004	0.913	-0.001	0.970
LDL	rs174546	-0.023	0.380	0.014	0.584	0.021	0.492	0.020	0.508
LDL	rs964184	0.018	0.625	0.015	0.662	0.069	0.099	0.064	0.120
LDL	rs11065987	0.009	0.716	0.005	0.836	-0.033	0.265	-0.056	0.052
LDL	rs1169288	0.064	0.017	0.075	0.003	-0.061	0.055	-0.056	0.074
LDL	rs3764261	-0.006	0.818	-0.006	0.808	0.047	0.131	0.060	0.052
LDL	rs2000999	0.060	0.045	0.052	0.070	0.041	0.247	0.050	0.148
LDL	rs10401969	-0.142	<0.0005	-0.143	<0.0005	0.047	0.335	0.020	0.673
LDL	rs2902940	0.005	0.865	0.014	0.583	0.031	0.349	0.014	0.652
HDL	rs11869286	0.039	0.144	0.039	0.116	0.017	0.583	0.016	0.586
HDL	rs12328675	0.059	0.136	0.086	0.020	-0.026	0.556	-0.009	0.839
HDL	rs12967135	-0.002	0.959	0.008	0.768	-0.013	0.719	<0.0005	0.998
HDL	rs13107325	0.034	0.558	0.075	0.164	-0.028	0.679	-0.030	0.658
HDL	rs1532085	0.026	0.315	0.019	0.447	-0.032	0.291	-0.030	0.305
HDL	rs1689800	0.010	0.702	-0.002	0.929	-0.004	0.887	-0.013	0.664
HDL	rs16942887	<0.0005	0.990	0.033	0.351	0.032	0.464	0.044	0.304
HDL	rs1800961	0.056	0.372	0.090	0.124	0.028	0.709	0.019	0.789
HDL	rs181362	-0.013	0.661	-0.016	0.569	-0.014	0.679	-0.029	0.391
HDL	rs1883025	-0.017	0.571	-0.002	0.946	-0.011	0.754	-0.029	0.394
HDL	rs2293889	0.011	0.666	0.029	0.217	-0.008	0.774	-0.007	0.803
HDL	rs2652834	-0.023	0.465	-0.008	0.777	-0.002	0.947	-0.005	0.888
HDL	rs2814944	-0.001	0.973	-0.004	0.901	-0.054	0.186	-0.047	0.238
HDL	rs2923084	0.001	0.974	0.005	0.881	0.048	0.212	0.036	0.353
HDL	rs2925979	0.107	<0.0005	0.076	0.003	0.070	0.027	0.068	0.030
HDL	rs2972146	0.086	0.001	0.099	<0.0005	0.054	0.076	0.051	0.088
HDL	rs3136441	-0.019	0.586	0.001	0.969	0.045	0.286	0.051	0.223
HDL	rs3764261	-0.006	0.818	-0.006	0.808	0.047	0.131	0.060	0.052
HDL	rs4129767	0.001	0.953	-0.008	0.727	-0.061	0.036	-0.051	0.076
HDL	rs4660293	0.008	0.776	0.027	0.314	0.005	0.889	0.011	0.746
HDL	rs4765127	0.038	0.166	0.041	0.110	0.045	0.157	0.047	0.131
HDL	rs4846914	0.013	0.606	0.002	0.946	0.006	0.832	0.014	0.630
HDL	rs581080	0.026	0.416	0.019	0.527	0.021	0.562	0.021	0.566
HDL	rs605066	0.028	0.260	0.043	0.073	0.036	0.224	0.049	0.090
HDL	rs7134375	0.007	0.791	0.004	0.879	-0.014	0.646	-0.002	0.947
HDL	rs7134594	-0.031	0.227	-0.017	0.477	-0.005	0.856	-0.015	0.618
HDL	rs7241918	-0.037	0.280	-0.038	0.228	-0.014	0.711	-0.013	0.728

HDL	rs7255436	0.011	0.677	0.014	0.558	-0.010	0.729	-0.006	0.849
HDL	rs737337	0.071	0.071	0.070	0.058	0.021	0.658	0.033	0.465
HDL	rs9987289	0.032	0.453	0.029	0.471	-0.014	0.783	-0.015	0.766
HDL	rs6450176	0.024	0.403	0.025	0.362	-0.049	0.143	-0.040	0.221
HDL	rs4759377	0.036	0.415	0.061	0.141	-0.050	0.314	-0.067	0.167
HDL	rs1042034	0.020	0.530	0.018	0.564	-0.055	0.135	-0.058	0.110
HDL	rs12678919	0.053	0.248	0.031	0.459	0.090	0.095	0.061	0.239
HDL	rs2954029	0.001	0.969	-0.001	0.980	0.039	0.178	0.055	0.054
HDL	rs17145738	-0.036	0.343	0.007	0.850	0.003	0.950	-0.013	0.773
HDL	rs174546	0.023	0.380	-0.014	0.584	-0.021	0.492	-0.020	0.508
HDL	rs964184	0.018	0.625	0.015	0.662	0.069	0.099	0.064	0.120
HDL	rs7941030	-0.012	0.650	-0.002	0.946	0.008	0.782	0.008	0.794
HDL	rs11613352	0.046	0.117	0.066	0.017	0.103	0.002	0.082	0.014
HDL	rs4420638	-0.089	0.005	-0.076	0.010	0.044	0.214	0.047	0.177
TG	rs1042034	0.020	0.530	0.018	0.564	-0.055	0.135	-0.058	0.110
TG	rs11613352	0.046	0.117	0.066	0.017	0.103	0.002	0.082	0.014
TG	rs11776767	-0.005	0.846	0.009	0.727	-0.036	0.240	-0.013	0.657
TG	rs1260326	-0.024	0.374	-0.033	0.194	-0.002	0.937	0.011	0.708
TG	rs12678919	0.053	0.248	0.031	0.459	0.090	0.095	0.061	0.239
TG	rs1495741	-0.016	0.614	0.026	0.365	0.035	0.333	0.055	0.116
TG	rs17145738	-0.036	0.343	0.007	0.850	0.003	0.950	-0.013	0.773
TG	rs174546	0.023	0.380	-0.014	0.584	-0.021	0.492	-0.020	0.508
TG	rs2068888	0.047	0.067	0.041	0.088	0.080	0.007	0.086	0.003
TG	rs2412710	0.016	0.867	-0.015	0.870	0.002	0.986	-0.077	0.490
TG	rs2929282	-0.013	0.836	-0.041	0.495	-0.082	0.284	-0.102	0.173
TG	rs2954029	0.001	0.969	-0.001	0.980	0.039	0.178	0.055	0.054
TG	rs439401	-0.016	0.545	-0.011	0.660	0.013	0.674	0.022	0.471
TG	rs442177	0.010	0.694	0.005	0.839	0.008	0.797	0.018	0.532
TG	rs645040	0.059	0.056	0.063	0.030	0.039	0.277	0.020	0.565
TG	rs964184	0.018	0.625	0.015	0.662	0.069	0.099	0.064	0.120
TG	rs9686661	0.057	0.096	0.059	0.064	0.035	0.379	0.001	0.973
TG	rs2247056	0.058	0.047	0.061	0.023	0.025	0.447	0.028	0.383
TG	rs5756931	0.062	0.020	0.072	0.004	-0.015	0.625	-0.008	0.792
TG	rs11649653	0.021	0.424	0.022	0.358	0.051	0.086	0.050	0.089
TG	rs4846914	0.013	0.606	0.002	0.946	0.006	0.832	0.014	0.630
TG	rs2972146	0.086	0.001	0.099	<0.0005	0.054	0.076	0.051	0.088
TG	rs6882076	0.034	0.191	0.049	0.048	0.070	0.022	0.063	0.037
TG	rs4765127	0.038	0.166	0.041	0.110	0.045	0.157	0.047	0.131
TG	rs3764261	-0.006	0.818	-0.006	0.808	0.047	0.131	0.060	0.052
TG	rs10401969	-0.142	<0.0005	-0.143	<0.0005	0.047	0.335	0.020	0.673
FPG	rs340874	0.050	0.041	0.057	0.014	-0.038	0.178	-0.032	0.252
FPG	rs560887	0.041	0.127	0.010	0.695	-0.010	0.755	-0.019	0.543
FPG	rs780094	0.035	0.174	0.035	0.139	-0.018	0.547	-0.031	0.285
FPG	rs11920090	0.045	0.214	0.070	0.043	0.010	0.816	0.020	0.634
FPG	rs11708067	0.075	0.014	0.066	0.022	-0.079	0.020	-0.095	0.005
FPG	rs2191349	0.067	0.007	0.074	0.001	0.051	0.074	0.042	0.140
FPG	rs13266634	0.107	<0.0005	0.123	<0.0005	0.045	0.144	0.075	0.013

FPG	rs7034200	0.076	0.003	0.081	0.001	-0.001	0.963	-0.023	0.432
FPG	rs7903146	0.272	<0.0005	0.305	<0.0005	0.016	0.620	-0.004	0.913
FPG	rs10885122	-0.043	0.243	-0.047	0.177	0.034	0.443	0.004	0.926
FPG	rs11605924	0.013	0.584	0.020	0.375	-0.075	0.008	-0.075	0.007
FPG	rs174550	-0.022	0.389	0.010	0.672	0.015	0.627	0.012	0.699
FPG	rs7944584	0.062	0.030	0.041	0.128	-0.057	0.079	-0.057	0.073
FPG	rs10830963	0.011	0.692	0.023	0.356	0.006	0.847	0.011	0.722
FPG	rs11071657	0.018	0.466	0.029	0.217	0.034	0.236	0.036	0.212
SBP	rs17367504	0.034	0.340	0.023	0.495	0.003	0.951	<0.0005	0.999
SBP	rs2932538	0.013	0.648	0.016	0.549	0.020	0.549	0.008	0.812
SBP	rs13082711	0.053	0.073	0.036	0.207	0.018	0.611	0.019	0.572
SBP	rs3774372	0.069	0.043	0.058	0.073	-0.008	0.842	0.001	0.977
SBP	rs419076	-0.005	0.856	-0.005	0.849	-0.003	0.914	-0.016	0.570
SBP	rs16998073	0.010	0.695	-0.005	0.833	0.058	0.055	0.048	0.108
SBP	rs13107325	-0.024	0.672	-0.067	0.213	0.035	0.610	0.027	0.686
SBP	rs13139571	0.017	0.566	0.020	0.492	0.072	0.044	0.074	0.035
SBP	rs1173771	0.032	0.213	0.034	0.166	0.009	0.754	0.023	0.433
SBP	rs11953630	0.021	0.436	0.004	0.877	-0.006	0.844	-0.008	0.788
SBP	rs1799945	0.012	0.750	0.036	0.324	-0.031	0.497	-0.048	0.279
SBP	rs805303	0.086	0.001	0.067	0.006	0.003	0.928	0.008	0.776
SBP	rs4373814	0.032	0.207	0.017	0.481	0.036	0.219	0.051	0.078
SBP	rs1530440	-0.021	0.531	-0.046	0.131	0.052	0.178	0.052	0.166
SBP	rs932764	-0.011	0.665	-0.020	0.403	-0.018	0.538	-0.030	0.301
SBP	rs11191548	0.035	0.400	0.036	0.354	0.022	0.654	0.015	0.751
SBP	rs7129220	-0.051	0.231	-0.061	0.128	0.057	0.230	0.032	0.502
SBP	rs381815	-0.003	0.902	0.010	0.698	0.018	0.574	0.023	0.474
SBP	rs633185	0.038	0.092	0.031	0.135	0.013	0.604	0.012	0.646
SBP	rs17249754	-0.056	0.110	-0.036	0.287	-0.074	0.069	-0.074	0.067
SBP	rs3184504	-0.005	0.839	-0.004	0.865	0.079	0.007	0.103	<0.0005
SBP	rs10850411	0.019	0.479	0.006	0.828	-0.053	0.087	-0.026	0.390
SBP	rs1378942	0.018	0.515	0.017	0.528	0.021	0.510	0.013	0.692
SBP	rs2521501	0.021	0.440	0.042	0.097	0.073	0.019	0.098	0.001
SBP	rs12946454	0.036	0.217	0.027	0.321	-0.005	0.886	-0.005	0.890
SBP	rs17608766	0.029	0.420	0.027	0.426	-0.013	0.766	-0.004	0.918
SBP	rs16948048	<0.0005	0.994	-0.007	0.788	-0.024	0.425	<0.0005	0.995
SBP	rs1327235	0.049	0.053	0.034	0.153	-0.029	0.317	-0.030	0.300
SBP	rs6015450	-0.007	0.858	-0.005	0.891	0.029	0.498	0.050	0.223

eTable 9. Association between cardio-metabolic SNPs and cardio-metabolic traits

		BMI		SBP		LDLC		HDLC		TG		FPG	
Main Trait	SNP	β	P value	β	P value	β	P value	β	P value	β	P value	β	P value
BMI	rs1558902	0.068	<0.0005	0.012	0.118	-0.011	0.566	-0.007	0.724	0.029	0.133	0.012	0.388
BMI	rs6548238	0.047	<0.0005	0.002	0.816	-0.032	0.232	-0.044	0.069	-0.017	0.500	-0.016	0.379
BMI	rs571312	0.034	0.001	-0.003	0.762	-0.050	0.032	0.007	0.733	-0.038	0.095	0.003	0.866
BMI	rs10938397	0.029	0.001	0.025	0.001	-0.008	0.702	0.018	0.316	-0.003	0.893	0.015	0.275
BMI	rs10767664	0.035	0.001	0.002	0.805	0.013	0.590	-0.007	0.753	0.034	0.141	0.012	0.443
BMI	rs2815752	0.036	<0.0005	0.009	0.247	-0.004	0.852	-0.040	0.030	0.053	0.006	0.015	0.270
BMI	rs7359397	0.022	0.010	0.011	0.161	-0.006	0.757	-0.032	0.085	0.020	0.303	0.009	0.527
BMI	rs9816226	0.038	0.001	0.003	0.746	0.022	0.398	0.061	0.012	0.009	0.739	0.008	0.660
BMI	rs3817334	0.013	0.137	-0.020	0.011	-0.016	0.430	0.001	0.952	0.028	0.161	-0.019	0.155
BMI	rs29941	0.016	0.063	0.007	0.380	-0.043	0.034	-0.037	0.053	0.030	0.132	0.019	0.173
BMI	rs543874	0.023	0.027	-0.003	0.757	-0.021	0.379	0.015	0.501	-0.001	0.966	-0.004	0.794
BMI	rs987237	0.046	<0.0005	0.017	0.093	0.027	0.296	0.006	0.793	0.025	0.332	0.004	0.831
BMI	rs7138803	0.020	0.017	0.011	0.158	-0.014	0.493	-0.010	0.586	-0.029	0.130	0.026	0.054
BMI	rs10150332	0.016	0.128	0.012	0.222	-0.049	0.042	-0.022	0.327	-0.039	0.097	-0.023	0.153
BMI	rs713586	0.024	0.005	-0.003	0.676	0.003	0.881	-0.006	0.729	-0.002	0.919	0.019	0.148
BMI	rs12444979	0.025	0.052	0.003	0.778	0.061	0.038	-0.022	0.412	0.030	0.296	0.012	0.538
BMI	rs2241423	0.032	0.001	0.029	0.002	-0.017	0.467	-0.018	0.413	0.027	0.239	0.032	0.048
BMI	rs2287019	0.038	<0.0005	0.016	0.099	0.017	0.473	0.038	0.092	0.012	0.608	0.017	0.289
BMI	rs1514175	0.010	0.260	-0.007	0.345	-0.005	0.819	-0.023	0.215	0.027	0.156	-0.007	0.611
BMI	rs13107325	0.056	0.005	-0.051	0.005	-0.054	0.235	-0.084	0.045	0.049	0.267	-0.047	0.127
BMI	rs2112347	0.020	0.021	<0.0005	0.958	-0.068	0.001	0.013	0.506	-0.027	0.174	-0.004	0.773
BMI	rs10968576	0.018	0.053	0.018	0.036	0.023	0.288	-0.007	0.730	0.038	0.068	-0.003	0.859
BMI	rs3810291	0.009	0.327	0.006	0.433	0.046	0.029	0.001	0.972	0.018	0.371	-0.014	0.318
BMI	rs887912	0.007	0.485	-0.005	0.568	0.032	0.144	-0.002	0.917	-0.016	0.458	-0.009	0.535
BMI	rs13078807	0.034	0.002	-0.014	0.146	-0.011	0.648	0.004	0.863	0.027	0.267	-0.018	0.277
BMI	rs11847697	0.037	0.086	<0.0005	0.989	-0.021	0.674	0.007	0.879	-0.013	0.787	-0.026	0.432

BMI	rs2890652	-0.007	0.527	-0.003	0.745	0.026	0.306	-0.002	0.934	-0.061	0.014	-0.005	0.760
BMI	rs1555543	-0.002	0.844	0.006	0.445	-0.042	0.035	0.033	0.069	-0.016	0.403	-0.014	0.307
BMI	rs4771122	-0.004	0.676	-0.004	0.635	0.001	0.961	-0.030	0.157	-0.005	0.839	-0.003	0.857
BMI	rs4929949	0.016	0.059	0.013	0.087	<0.0005	0.999	0.024	0.186	-0.038	0.044	-0.004	0.754
BMI	rs206936	0.018	0.085	-0.004	0.685	-0.025	0.323	-0.055	0.019	0.035	0.158	0.002	0.907
LDL	rs1564348	0.004	0.733	-0.010	0.344	0.064	0.021	-0.022	0.385	0.047	0.081	0.043	0.020
LDL	rs2479409	-0.004	0.656	0.002	0.819	0.053	0.010	0.006	0.770	-0.025	0.213	0.008	0.579
LDL	rs3757354	0.004	0.664	0.013	0.143	0.043	0.065	0.027	0.217	-0.001	0.978	0.007	0.646
LDL	rs4299376	-0.002	0.843	-0.009	0.307	0.129	<0.0005	0.036	0.074	0.008	0.704	0.015	0.290
LDL	rs4420638	-0.019	0.069	-0.009	0.323	0.218	<0.0005	-0.038	0.081	0.046	0.049	-0.007	0.654
LDL	rs6029526	<0.0005	0.965	0.002	0.772	0.029	0.146	0.028	0.120	-0.023	0.230	0.005	0.699
LDL	rs629301	-0.004	0.709	-0.012	0.182	0.145	<0.0005	-0.024	0.252	0.035	0.114	0.006	0.677
LDL	rs6511720	-0.014	0.328	0.010	0.445	0.245	<0.0005	-0.035	0.261	0.027	0.409	-0.002	0.936
LDL	rs8017377	-0.012	0.155	-0.013	0.101	0.040	0.042	<0.0005	0.978	0.007	0.722	0.003	0.838
LDL	rs9411489	0.001	0.933	0.003	0.726	0.104	<0.0005	-0.018	0.410	-0.009	0.692	0.014	0.396
LDL	rs1367117	-0.005	0.614	0.015	0.063	0.109	<0.0005	-0.040	0.035	0.039	0.052	0.015	0.270
LDL	rs11220462	0.019	0.118	-0.003	0.776	0.026	0.360	-0.045	0.095	0.045	0.113	0.015	0.424
LDL	rs1800562	0.004	0.849	-0.020	0.263	0.055	0.224	0.097	0.021	-0.072	0.103	0.013	0.663
LDL	rs12027135	-0.004	0.650	-0.006	0.424	0.064	0.001	0.007	0.706	-0.007	0.697	-0.012	0.348
LDL	rs514230	0.007	0.372	0.012	0.126	0.016	0.411	0.012	0.485	0.008	0.688	0.035	0.006
LDL	rs12916	-0.013	0.124	-0.001	0.903	0.082	<0.0005	-0.018	0.325	0.015	0.448	0.003	0.818
LDL	rs6882076	-0.016	0.072	-0.012	0.120	0.036	0.077	0.027	0.145	0.031	0.110	0.019	0.155
LDL	rs3177928	-0.012	0.329	0.013	0.255	0.048	0.086	-0.057	0.029	0.041	0.135	-0.010	0.614
LDL	rs9488822	-0.002	0.838	<0.0005	0.953	0.027	0.209	-0.006	0.755	-0.006	0.772	0.010	0.489
LDL	rs12670798	-0.013	0.190	<0.0005	0.964	0.045	0.044	0.023	0.257	0.022	0.308	-0.020	0.182
LDL	rs9987289	0.016	0.267	0.001	0.910	0.094	0.004	0.139	<0.0005	-0.050	0.114	-0.056	0.010
LDL	rs2081687	-0.015	0.084	0.013	0.106	0.005	0.799	0.005	0.782	0.024	0.232	-0.023	0.094
LDL	rs2954029	0.004	0.636	0.011	0.136	0.079	<0.0005	-0.049	0.007	0.084	<0.0005	0.008	0.515
LDL	rs2255141	0.007	0.441	-0.003	0.762	0.035	0.101	0.026	0.195	0.015	0.481	-0.014	0.345

LDL	rs174546	0.013	0.134	0.010	0.232	0.050	0.016	0.006	0.765	-0.046	0.022	0.001	0.970
LDL	rs964184	-0.008	0.527	0.012	0.294	0.126	<0.0005	-0.153	<0.0005	0.286	<0.0005	-0.020	0.280
LDL	rs11065987	0.008	0.363	-0.012	0.125	0.021	0.284	0.036	0.046	-0.047	0.014	0.019	0.139
LDL	rs1169288	0.014	0.109	0.002	0.783	0.025	0.229	0.019	0.323	-0.010	0.638	0.030	0.034
LDL	rs3764261	0.002	0.828	-0.006	0.462	0.073	<0.0005	-0.247	<0.0005	0.021	0.302	0.005	0.712
LDL	rs2000999	0.006	0.524	0.013	0.160	0.015	0.519	0.015	0.493	-0.007	0.754	-0.020	0.195
LDL	rs10401969	-0.006	0.666	-0.007	0.558	0.086	0.007	0.067	0.024	0.040	0.209	-0.015	0.506
LDL	rs2902940	<0.0005	0.984	0.012	0.171	0.032	0.132	-0.006	0.749	0.036	0.088	0.006	0.700
HDL	rs11869286	-0.001	0.921	-0.015	0.066	0.025	0.214	-0.024	0.193	0.009	0.640	0.017	0.207
HDL	rs12328675	-0.014	0.277	0.008	0.504	-0.017	0.586	-0.044	0.117	0.020	0.512	0.016	0.421
HDL	rs12967135	0.029	0.004	-0.011	0.244	-0.044	0.055	0.001	0.978	-0.031	0.160	0.004	0.818
HDL	rs13107325	0.059	0.003	-0.060	0.001	-0.043	0.339	-0.090	0.030	0.054	0.217	-0.031	0.302
HDL	rs1532085	0.005	0.596	0.007	0.401	-0.057	0.005	-0.085	<0.0005	-0.057	0.004	0.001	0.955
HDL	rs1689800	-0.001	0.912	0.014	0.090	0.025	0.215	-0.023	0.218	0.045	0.025	0.014	0.316
HDL	rs16942887	0.012	0.340	0.007	0.513	-0.030	0.307	-0.057	0.036	0.020	0.488	-0.017	0.405
HDL	rs1800961	0.008	0.715	-0.005	0.801	0.007	0.888	-0.115	0.010	0.004	0.938	0.045	0.167
HDL	rs181362	-0.006	0.532	-0.002	0.834	-0.013	0.597	-0.041	0.059	0.011	0.631	-0.026	0.095
HDL	rs1883025	-0.007	0.507	-0.021	0.018	-0.030	0.182	-0.060	0.005	-0.048	0.034	-0.037	0.014
HDL	rs2293889	-0.005	0.537	0.004	0.596	0.023	0.242	-0.020	0.280	-0.002	0.905	0.003	0.806
HDL	rs2652834	-0.005	0.649	0.011	0.242	0.018	0.455	-0.002	0.926	0.013	0.593	<0.0005	0.995
HDL	rs2814944	0.004	0.741	0.002	0.834	-0.039	0.146	-0.044	0.075	0.033	0.206	0.006	0.740
HDL	rs2923084	-0.009	0.451	-0.014	0.165	0.002	0.925	0.006	0.804	0.002	0.941	0.013	0.446
HDL	rs2925979	-0.006	0.488	0.005	0.570	-0.062	0.004	-0.042	0.036	0.022	0.295	0.021	0.155
HDL	rs2972146	-0.006	0.478	0.016	0.039	0.009	0.642	-0.047	0.012	0.032	0.106	-0.004	0.766
HDL	rs3136441	-0.013	0.280	-0.002	0.863	-0.027	0.329	-0.030	0.246	0.028	0.307	-0.032	0.092
HDL	rs3764261	0.002	0.828	-0.006	0.462	0.073	<0.0005	-0.247	<0.0005	0.021	0.302	0.005	0.712
HDL	rs4129767	0.001	0.890	-0.005	0.498	-0.032	0.099	-0.011	0.550	-0.009	0.654	-0.015	0.261
HDL	rs4660293	0.009	0.368	-0.007	0.416	-0.030	0.185	-0.032	0.127	0.040	0.070	0.015	0.333
HDL	rs4765127	-0.021	0.020	0.003	0.731	0.022	0.320	-0.008	0.703	0.018	0.388	0.002	0.888

HDL	rs4846914	0.014	0.091	-0.002	0.826	0.036	0.071	-0.095	<0.0005	0.053	0.007	0.012	0.362
HDL	rs581080	0.013	0.235	-0.002	0.796	-0.019	0.421	-0.060	0.007	0.006	0.807	-0.001	0.932
HDL	rs605066	-0.001	0.933	0.011	0.146	-0.007	0.739	-0.011	0.532	0.024	0.205	-0.018	0.173
HDL	rs7134375	-0.001	0.933	<0.0005	0.977	0.009	0.660	-0.037	0.045	0.033	0.094	-0.003	0.845
HDL	rs7134594	-0.010	0.257	0.007	0.372	-0.001	0.966	-0.020	0.280	0.016	0.399	0.022	0.098
HDL	rs7241918	-0.019	0.088	0.014	0.152	-0.035	0.180	-0.042	0.082	-0.012	0.628	0.034	0.045
HDL	rs7255436	-0.013	0.126	-0.002	0.812	-0.022	0.273	-0.028	0.135	0.003	0.891	0.008	0.547
HDL	rs737337	-0.014	0.293	0.003	0.813	0.015	0.636	-0.096	0.001	0.033	0.276	0.017	0.420
HDL	rs9987289	-0.016	0.267	-0.001	0.910	-0.094	0.004	-0.139	<0.0005	0.050	0.114	0.056	0.010
HDL	rs6450176	-0.021	0.025	0.012	0.154	-0.013	0.542	-0.051	0.012	0.003	0.899	-0.015	0.308
HDL	rs4759377	-0.004	0.756	0.013	0.310	-0.025	0.446	-0.048	0.117	-0.017	0.597	-0.003	0.897
HDL	rs1042034	-0.011	0.281	0.007	0.443	0.109	<0.0005	-0.064	0.005	0.104	<0.0005	0.016	0.322
HDL	rs12678919	0.018	0.228	0.008	0.559	0.011	0.742	-0.202	<0.0005	0.193	<0.0005	-0.019	0.403
HDL	rs2954029	0.004	0.636	0.011	0.136	0.079	<0.0005	-0.049	0.007	0.084	<0.0005	0.008	0.515
HDL	rs17145738	0.017	0.183	-0.017	0.143	-0.018	0.536	-0.080	0.004	0.154	<0.0005	-0.015	0.437
HDL	rs174546	-0.013	0.134	-0.010	0.232	-0.050	0.016	-0.006	0.765	0.046	0.022	-0.001	0.970
HDL	rs964184	-0.008	0.527	0.012	0.294	0.126	<0.0005	-0.153	<0.0005	0.286	<0.0005	-0.020	0.280
HDL	rs7941030	-0.006	0.488	-0.001	0.925	-0.014	0.470	-0.018	0.322	-0.004	0.817	-0.014	0.274
HDL	rs11613352	-0.010	0.320	-0.009	0.325	-0.005	0.829	-0.035	0.085	0.039	0.075	0.028	0.059
HDL	rs4420638	-0.019	0.069	-0.009	0.323	0.218	<0.0005	-0.038	0.081	0.046	0.049	-0.007	0.654
TG	rs1042034	-0.011	0.281	0.007	0.443	0.109	<0.0005	-0.064	0.005	0.104	<0.0005	0.016	0.322
TG	rs11613352	-0.010	0.320	-0.009	0.325	-0.005	0.829	-0.035	0.085	0.039	0.075	0.028	0.059
TG	rs11776767	-0.006	0.490	-0.012	0.118	0.019	0.360	-0.003	0.878	0.018	0.360	0.017	0.215
TG	rs1260326	0.002	0.823	-0.010	0.209	-0.006	0.758	-0.008	0.672	0.105	<0.0005	-0.038	0.006
TG	rs12678919	0.018	0.228	0.008	0.559	0.011	0.742	-0.202	<0.0005	0.193	<0.0005	-0.019	0.403
TG	rs1495741	0.005	0.598	0.009	0.362	0.022	0.354	0.044	0.045	0.019	0.412	0.032	0.048
TG	rs17145738	0.017	0.183	-0.017	0.143	-0.018	0.536	-0.080	0.004	0.154	<0.0005	-0.015	0.437
TG	rs174546	-0.013	0.134	-0.010	0.232	-0.050	0.016	-0.006	0.765	0.046	0.022	-0.001	0.970
TG	rs2068888	-0.010	0.218	-0.008	0.323	0.041	0.037	-0.018	0.323	0.051	0.009	0.006	0.663

TG	rs2412710	-0.073	0.020	0.024	0.398	0.085	0.266	-0.201	0.005	0.226	0.003	-0.004	0.943
TG	rs2929282	-0.019	0.352	0.026	0.162	0.102	0.041	-0.148	0.001	0.119	0.015	0.028	0.397
TG	rs2954029	0.004	0.636	0.011	0.136	0.079	<0.0005	-0.049	0.007	0.084	<0.0005	0.008	0.515
TG	rs439401	-0.002	0.815	-0.001	0.870	0.041	0.045	-0.006	0.772	0.096	<0.0005	-0.001	0.953
TG	rs442177	0.016	0.052	-0.017	0.025	0.022	0.277	0.002	0.927	0.029	0.138	0.017	0.213
TG	rs645040	0.025	0.013	0.002	0.801	0.021	0.374	-0.052	0.017	0.027	0.243	0.051	0.001
TG	rs964184	-0.008	0.527	0.012	0.294	0.126	<0.0005	-0.153	<0.0005	0.286	<0.0005	-0.020	0.280
TG	rs9686661	0.029	0.011	0.016	0.131	0.001	0.974	-0.037	0.129	0.056	0.034	-0.002	0.896
TG	rs2247056	0.004	0.676	-0.006	0.504	0.046	0.032	-0.015	0.464	0.047	0.027	-0.025	0.090
TG	rs5756931	0.001	0.901	0.004	0.574	-0.018	0.371	-0.007	0.707	0.034	0.091	0.018	0.177
TG	rs11649653	<0.0005	0.964	0.005	0.487	0.007	0.745	-0.012	0.511	-0.014	0.460	-0.012	0.359
TG	rs4846914	0.014	0.091	-0.002	0.826	0.036	0.071	-0.095	<0.0005	0.053	0.007	0.012	0.362
TG	rs2972146	-0.006	0.478	0.016	0.039	0.009	0.642	-0.047	0.012	0.032	0.106	-0.004	0.766
TG	rs6882076	-0.016	0.071	-0.012	0.121	0.036	0.077	0.027	0.145	0.031	0.110	0.019	0.155
TG	rs4765127	-0.021	0.020	0.003	0.731	0.022	0.320	-0.008	0.703	0.018	0.388	0.002	0.888
TG	rs3764261	0.002	0.828	-0.006	0.462	0.073	<0.0005	-0.247	<0.0005	0.021	0.302	0.005	0.712
TG	rs10401969	-0.006	0.666	-0.007	0.558	0.086	0.007	0.067	0.024	0.040	0.209	-0.015	0.506
FPG	rs340874	-0.011	0.184	<0.0005	0.995	0.026	0.177	-0.038	0.038	0.032	0.093	0.019	0.140
FPG	rs560887	-0.008	0.358	0.016	0.047	0.001	0.963	-0.012	0.549	-0.035	0.094	0.117	<0.0005
FPG	rs780094	-0.010	0.232	0.007	0.372	-0.005	0.815	0.003	0.868	-0.099	<0.0005	0.038	0.006
FPG	rs11920090	-0.013	0.270	0.004	0.680	-0.003	0.917	-0.012	0.653	-0.003	0.908	0.035	0.068
FPG	rs11708067	-0.005	0.611	-0.003	0.769	0.025	0.281	-0.004	0.843	0.038	0.093	0.030	0.057
FPG	rs2191349	-0.001	0.886	0.003	0.659	0.007	0.734	-0.021	0.254	-0.007	0.716	0.014	0.287
FPG	rs13266634	-0.004	0.666	0.004	0.592	-0.008	0.694	-0.024	0.220	0.017	0.398	0.026	0.063
FPG	rs7034200	0.017	0.050	-0.012	0.130	-0.022	0.275	0.036	0.053	-0.006	0.745	-0.003	0.814
FPG	rs7903146	-0.013	0.184	0.002	0.802	-0.008	0.703	0.003	0.898	0.018	0.400	0.041	0.005
FPG	rs10885122	-0.014	0.264	0.018	0.103	-0.025	0.406	-0.020	0.472	0.004	0.894	0.007	0.748
FPG	rs11605924	-0.002	0.843	0.002	0.833	0.038	0.054	0.015	0.422	-0.014	0.483	0.010	0.433
FPG	rs174550	0.013	0.125	0.005	0.488	0.051	0.014	0.004	0.824	-0.050	0.014	0.001	0.956

FPG	rs7944584	0.012	0.196	-0.015	0.077	-0.016	0.488	0.015	0.460	0.026	0.244	0.022	0.156
FPG	rs10830963	0.004	0.683	0.016	0.046	0.015	0.491	-0.018	0.373	-0.003	0.899	0.074	<0.0005
FPG	rs11071657	0.007	0.372	0.001	0.942	-0.016	0.429	0.040	0.030	-0.025	0.204	-0.005	0.723
SBP	rs17367504	-0.014	0.240	0.026	0.015	-0.029	0.286	-0.001	0.973	0.033	0.220	0.008	0.652
SBP	rs2932538	-0.002	0.864	0.018	0.040	0.005	0.829	-0.027	0.192	0.020	0.365	-0.002	0.885
SBP	rs13082711	-0.007	0.475	-0.002	0.849	-0.013	0.562	0.032	0.140	-0.043	0.057	-0.005	0.733
SBP	rs3774372	0.032	0.005	-0.007	0.483	0.002	0.936	-0.033	0.186	0.056	0.035	0.006	0.724
SBP	rs419076	0.002	0.836	0.021	0.005	0.016	0.411	-0.003	0.865	-0.004	0.833	-0.001	0.926
SBP	rs16998073	-0.016	0.068	0.035	<0.0005	0.001	0.979	0.006	0.747	0.028	0.179	0.001	0.925
SBP	rs13107325	-0.059	0.003	0.061	<0.0005	0.042	0.351	0.090	0.029	-0.054	0.218	0.032	0.287
SBP	rs13139571	0.015	0.147	0.013	0.155	-0.004	0.883	-0.033	0.137	-0.005	0.828	0.016	0.305
SBP	rs1173771	0.012	0.173	0.039	<0.0005	-0.010	0.639	-0.046	0.015	-0.002	0.920	0.015	0.285
SBP	rs11953630	-0.004	0.628	0.018	0.023	0.014	0.494	-0.016	0.398	0.009	0.672	0.022	0.116
SBP	rs1799945	-0.007	0.584	0.032	0.007	-0.050	0.094	0.025	0.370	0.022	0.454	-0.032	0.106
SBP	rs805303	0.002	0.803	0.011	0.161	-0.003	0.898	0.005	0.800	-0.010	0.617	0.012	0.350
SBP	rs4373814	0.015	0.088	0.011	0.172	-0.005	0.807	-0.022	0.246	0.017	0.404	0.007	0.596
SBP	rs1530440	0.008	0.476	0.022	0.028	-0.030	0.226	0.003	0.908	0.007	0.777	0.014	0.407
SBP	rs932764	0.007	0.379	0.029	<0.0005	-0.020	0.306	0.007	0.709	-0.010	0.605	0.002	0.866
SBP	rs11191548	-0.023	0.101	0.041	0.001	0.055	0.081	-0.042	0.148	0.071	0.021	0.012	0.584
SBP	rs7129220	-0.001	0.958	0.007	0.561	0.042	0.191	0.013	0.654	0.031	0.317	0.009	0.663
SBP	rs381815	0.005	0.602	0.028	0.001	0.048	0.029	0.013	0.509	0.009	0.681	0.031	0.038
SBP	rs633185	0.007	0.323	0.022	0.001	-0.018	0.291	0.017	0.272	-0.005	0.762	<0.0005	0.993
SBP	rs17249754	-0.023	0.058	0.024	0.026	0.063	0.024	0.002	0.935	0.040	0.142	0.019	0.315
SBP	rs3184504	-0.011	0.171	0.013	0.097	-0.016	0.390	-0.026	0.137	0.033	0.078	-0.015	0.236
SBP	rs10850411	-0.005	0.573	0.022	0.007	0.026	0.214	-0.019	0.321	0.026	0.206	0.013	0.341
SBP	rs1378942	0.003	0.776	0.014	0.094	-0.005	0.828	-0.016	0.407	<0.0005	0.997	-0.008	0.583
SBP	rs2521501	<0.0005	0.989	0.031	<0.0005	-0.010	0.657	0.014	0.472	0.002	0.924	0.002	0.862
SBP	rs12946454	0.008	0.433	0.011	0.208	-0.010	0.662	0.002	0.921	0.021	0.350	0.018	0.229
SBP	rs17608766	0.005	0.701	0.030	0.006	-0.001	0.965	0.038	0.141	-0.036	0.188	-0.032	0.085

SBP	rs16948048	0.015	0.083	0.003	0.714	-0.007	0.719	-0.004	0.836	-0.001	0.969	-0.008	0.578
SBP	rs1327235	-0.003	0.745	0.011	0.156	-0.001	0.969	0.010	0.563	-0.011	0.547	0.008	0.548
SBP	rs6015450	-0.013	0.279	0.022	0.043	<0.0005	0.987	-0.056	0.034	0.026	0.349	0.002	0.923