

Original Article

Prospective study of insulin-like growth factor-I, insulin-like growth factor-binding protein 3, genetic variants in the *IGF1* and *IGFBP3* genes and risk of coronary artery disease

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Abstract: Although experimental studies have suggested that insulin-like growth factor I (IGF-I) and its binding protein IGFBP-3 might have a role in the aetiology of coronary artery disease (CAD), the relevance of circulating IGFs and their binding proteins in the development of CAD in human populations is unclear. We conducted a nested case-control study, with a mean follow-up of six years, within the EPIC-Norfolk cohort to assess the association between circulating levels of IGF-I and IGFBP-3 and risk of CAD in up to 1,013 cases and 2,055 controls matched for age, sex and study enrolment date. After adjustment for cardiovascular risk factors, we found no association between circulating levels of IGF-I or IGFBP-3 and risk of CAD (odds ratio: 0.98 (95% CI 0.90-1.06) per 1 SD increase in circulating IGF-I; odds ratio: 1.02 (95% CI 0.94-1.12) for IGFBP-3). We examined associations between tagging single nucleotide polymorphisms (tSNPs) at the *IGF1* and *IGFBP3* loci and circulating IGF-I and IGFBP-3 levels in up to 1,133 cases and 2,223 controls and identified three tSNPs (rs1520220, rs3730204, rs2132571) that showed independent association with either circulating IGF-I or IGFBP-3 levels. In an assessment of 31 SNPs spanning the *IGF1* or *IGFBP3* loci, none were associated with risk of CAD in a meta-analysis that included EPIC-Norfolk and eight additional studies comprising up to 9,319 cases and 19,964 controls. Our results indicate that IGF-I and IGFBP-3 are unlikely to be importantly involved in the aetiology of CAD in human populations.

Keywords: Epidemiology, Genetics of cardiovascular disease, Risk factors, IGF1, IGFBP3

Introduction

Insulin-like growth factor I (IGF-I) is a peptide hormone that shares sequence homology with

insulin and has a fundamental role in somatic growth and cellular differentiation, metabolism and survival [1]. The majority of IGFs in the circulation are bound to IGF binding proteins, of

which IGF binding protein 3 (IGFBP-3) is the most predominant, carrying > 80% of circulating IGF-I. But, although IGFBP-3 is an important regulator of the bioactivity of IGF-I, there is evidence to suggest that IGFBP-3 possesses functions independent of its role as an IGF-I carrier protein [2]. It has been suggested that circulating IGF-I is involved in the pathophysiological processes underlying coronary artery disease (CAD). Experimental data in animals have shown that overexpression or administration of IGF-I after myocardial infarction (MI) prevents cardiomyocyte death and improves cardiac function [3,4,5]. Evidence for improvement of cardiac function and increased ventricular mass has been shown in individuals with growth hormone deficiency after short-term IGF-I therapy [6]. Improved cardiac performance has also been demonstrated following administration of recombinant IGF-I to individuals with chronic heart failure [7].

Cross sectional observational studies have reported that circulating IGF-I concentrations are lower in individuals with CAD and MI [8,9,10], but the opposite has been reported as well [11]. Furthermore, increments in IGF-I levels are associated with reduced prevalence of atherosclerotic plaques, as measured by arterial ultrasound [12]. The prospective association between circulating levels of IGF-I and IGFBP-3 and subsequent risk of future CAD, however, remains uncertain [13,14,15,16,17,18,19,20]. Several studies have identified reproducible associations among genetic variants at the loci encoding IGF-I and IGFBP-3 and circulating levels of IGFs. To assess a possible link between circulating IGF-I and IGFBP-3 levels and CAD, there is a need for a comprehensive analysis of the relationship between circulating IGF-I and IGFBP-3 levels, genetic variation at the loci encoding these proteins, and risk of CAD. Therefore we sought to reproduce previously reported associations between tagging single nucleotide polymorphisms (tSNPs) spanning the *IGF1* and *IGFBP3* genes and circulating concentrations of IGF-I and IGFBP-3, and to use these SNPs as genetic tools to assess the possible relation between IGF-I and IGFBP-3 and risk of CAD in a prospective population-based study.

Materials and methods

Study design

We conducted a nested CAD case-control study

among participants of the European Prospective Investigation into Cancer and Nutrition (EPIC)-Norfolk cohort, a prospective population-based study of 25,663 men and women resident in Norfolk, United Kingdom, aged between 45 and 79 years [21]. Participants were recruited from age-sex registers of general practices. At the baseline survey between 1993 and 1997, participants completed a detailed health and lifestyle questionnaire, and additional data were collected by trained nurses at a clinic visit as previously described [21]. Participants were identified as incident CAD cases if they had a hospital admission and/or died with CAD as the underlying cause during follow-up. CAD was defined as ICD9 codes 410 to 414 or ICD10 codes I20-I25 (including acute and old myocardial infarction, angina, aneurysm and dissection of the heart, and other ischaemic heart disease). We report results with follow-up to January 2003, an average of about 6 years. The study was approved by the Norwich District Health Authority Ethics Committee, and all participants gave written informed consent.

Participants

We selected study participants who did not report a history of heart attack or stroke at the baseline clinic visit. During follow-up, 1,138 apparently healthy individuals developed fatal or non-fatal CAD and were classified as cases. Controls were study participants who remained free of any cardiovascular disease during follow-up. Two controls were matched to each case by age (within 5 years), sex and date of inclusion visit (within a range of 3 months). Due to an insufficient number of available controls, because of this stringent matching, only 2,237 controls were identified, i.e. 39 cases were matched to one control and 1,099 cases were matched to two controls. For analyses of the associations among IGF1 and IGFBP3 tSNPs and risk of CAD, complete data were available for 1,133 cases and 2,223 controls. For analyses that also involved circulating IGF-I and IGFBP-3 levels, complete data were available for 1,013 cases and 2,055 controls.

Additional CAD case-control studies

We also combined data from the EPIC-Norfolk study with summary data from eight studies from existing collaborations [22] where genotypes were available *in silico* for 31 SNPs spanning the *IGF1* and *IGFBP3* genes (**Table 1**).

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Table 1. Study characteristics and details of analysis metrics and methods for individual studies in CAD risk meta-analysis

STUDY	Ottawa Heart Study-I	Ottawa Heart Study-II	WTCCC CAD study	PENN CATH	MEDSTAR	CoLaus	GEMS	Rotterdam
SAMPLES								
Description	CAD case-control study of participants recruited from the Champlain region including Ottawa	CAD case-control study of participants recruited from Ottawa and the Cleveland Clinic	CAD case-control study of participants recruited nationally from the UK	CAD case-control study nested within cohort of participants attending University of Pennsylvania Medical Center, PA, USA, for angiography	CAD case-control study nested within cross sectional cohort of participants attending Washington Hospital Center, Washington DC, USA, for angiography	CAD case-cohort set nested within CoLaus study	CAD case-cohort set nested within GEMS study	CAD case-cohort set nested within Rotterdam study
N cases / N controls	1,542 / 1,455	1,912 / 933	1,926 / 2,938	933 / 468	874 / 447	192 / 5,127	262 / 1,580	612 / 4,892
Case/control definition	Cases: individuals exhibited symptomatic CAD before the age of 55 years in men and 65 in women. CAD defined as greater than 50% stenosis of a coronary artery was confirmed by coronary angiography. Cases with a history of diabetes were excluded. Controls: asymptomatic elderly individuals were selected as controls (aged ≥ 65 for men, aged ≥ 70 for women).	Cases: individuals exhibited symptomatic CAD before the age of 55 years in men and 65 in women. CAD defined as greater than 50% stenosis of a coronary artery was confirmed by coronary angiography. Cases with a history of diabetes were excluded. Controls: asymptomatic elderly individuals were selected as controls (aged ≥ 65 for men, aged ≥ 70 for women).	Cases: individuals with premature CAD (a validated history of either MI or coronary revascularisation before age 66 years) and one or more first degree relatives with CAD. Controls: unrelated individuals from 1958 British birth cohort and UK Blood Services.	Cases: individuals who had one or more coronary vessels with ≥ 50% stenosis equally selected among stable CAD cases without history of MI and CAD cases with a history of MI. Controls: men aged > 40 and women aged > 45 showing no evidence of CAD.	Cases: individuals who had one or more coronary vessels with ≥ 50% stenosis equally selected among stable CAD cases without history of MI and CAD cases with a history of MI. Controls: women aged > 55 years and men aged > 60 years with angiographically documented coronaries of ≤ 20% stenosis. If there is 0% coronary narrowing, then men and women aged ≥ 45 years are included.	Cases: individuals who reported previous acute MI, cardiac catheterisation, coronary artery bypass or angina. Controls: individuals without any of above case definitions.	Cases: individuals who reported previous CAD. Controls: individuals who reported no current or previous CAD.	Cases: fatal or non-fatal MI, fatal CHD or sudden death. Controls: individuals without previous or current CAD or MI at follow-up.
Sex (%men / %women)	Cases: 76/24 Controls: 52/48	Cases: 76/24 Controls: 50/50	Cases: 79 / 21 Controls: 49 / 51	Cases: 76 / 24 Controls: 48 / 52	Cases: 72 / 28 Controls: 51 / 49	Cases: 72 / 28 Controls: 46 / 54	Cases: 74 / 26 Controls: 57 / 43	Cases: 51 / 49 Controls: 36 / 63
Age (mean (sd), years)	Cases: 48.7 (7.3) Controls: 75.0 (5.0)	Cases: 48.9 (7.3) Controls: 74.3 (5.8)	Cases: 45.7 (9.7) Controls: 49.8 (7.7)	Cases: 56.8 (9.2) Controls: 61.7 (9.6)	Cases: 54.6 (7.3) Controls: 60.0 (8.9)	Cases: 62.6 (8.4) Controls: 62.5 (8.4)	Cases: 55.0 (7.5) Controls: 52.0 (9.7)	Cases: 72.5 (9.5) Controls: 68.7 (9.0)

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STUDY	Ottawa Heart Study-I	Ottawa Heart Study-II	WTCCC CAD study	PENN CATH	MEDSTAR	CoLaus	GEMS	Rotterdam
GENOTYPING								
Genotyping platform & SNP panel	Affymetrix 500K & 6.0 Array set	Affymetrix 6.0 Array set	Affymetrix 500K Array Set	Affymetrix Array 6.0	Affymetrix Array 6.0	Affymetrix 500K Array Set	Affymetrix 500K Array Set	Illumina Infinium-II HumanHap550 SNP array (v3)
Genotyping centre	Canadian Cardiovascular Genetic Centre	Canadian Cardiovascular Genetic Centre	Affymetrix	Broad Institute	Broad Institute	Affymetrix Expression Analysis	Affymetrix	Rotterdam
Genotyping calling algorithm	BRLMM & BIRDSEED	BIRDSEED	CHIAMO	BIRDSEED	BIRDSEED	BRLMM	BRLMM	Beadstudio
SAMPLE QC								
Call rate	> 98%	> 98%	≥ 97%	≥ 90%	≥ 90%	≥ 90%	≥ 90%	≥ 97.5
Heterozygosity	NA	NA	<23% or >30%	NA	NA	NA	NA	FDR ≥ 1%
Other exclusions	Ethnic outliers	Ethnic outliers	1) Ethnic outliers 2) External discordance with genotype or phenotype data 3) Related individuals (>86% concordance with another DNA) 4) Duplicate (>99% concordance with another DNA)	1) Sex inconsistency with genetic data from X-linked SNPs 2) Inconsistent genotypes when compared with duplicate samples	1) Sex inconsistency with genetic data from X-linked SNPs 2) Inconsistent genotypes when compared with duplicate samples	1) Sex inconsistency with genetic data from X-linked SNPs 2) Inconsistent genotypes when compared with duplicate samples	1) Sex inconsistency with genetic data from X-linked SNPs 2) Inconsistent genotypes when compared with duplicate samples	1) Ethnic outliers 2) Related individuals 3) Duplicate (>95% concordance with another DNA)
SNP QC filters (prior to imputation)								
MAF	≥ 5%	≥ 1%	≥ 1%	≥ 1%	≥ 1%	≥ 1%	≥ 1%	≥ 1%
HWE	p>10 ⁻³ controls	p>10 ⁻⁴ controls	P > 10 ⁻⁴	P > 10 ⁻⁴	P > 10 ⁻⁴	P > 10 ⁻⁷	P > 10 ⁻⁷	P ≥ 10 ⁻⁶
Call rate	≥ 95%	≥ 95%	≥ 98%	≥ 90%	≥ 90%	≥ 90%	≥ 90%	≥ 98%
Other								
IMPUTATION STATS								
Imputation software	IMPUTE	IMPUTE	IMPUTE	MACH	MACH	IMPUTE	IMPUTE	MACH
Imputation quality metrics	proper_info ≥ 0.40	proper_info ≥ 0.40	proper_info ≥ 0.40	r2hat ≥ 0.3	r2hat ≥ 0.3	proper_info ≥ 0.40	proper_info ≥ 0.40	r2hat ≥ 0.3
Other SNP QC filters applied?	MAF ≥ 1% call rate ≥ 80%	MAF ≥ 1% call rate ≥ 80%	MAF ≥ 1% Call rate > 90%	MAF ≥ 1%	MAF ≥ 1%	MAF ≥ 1%	MAF ≥ 1%	MAF ≥ 1%

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STUDY	Ottawa Heart Study-I	Ottawa Heart Study-II	WTCCC CAD study	PENN CATH	MEDSTAR	CoLaus	GEMS	Rotterdam
DATA ANALYSIS								
Adjustments	sex	sex	age, sex	age, sex	age, sex	age, sex	age, sex	age, sex
Analysis method	additive test	additive test	additive test	logistic regression	logistic regression	logistic regression	logistic regression	Cox regression
Software for analysis	SNPTEST	SNPTEST	PLINK	PLINK	PLINK	PLINK	PLINK	ProbABEL
REFERENCES								
Reference study	22	22, 23	24	26	27	28	30, 31	32
Reference genotyping	22	22	25	27	27	29	29	33

These comprised 9,319 cases and 19,964 controls in a meta-analysis of *IGF1/IGFBP3* SNPs and risk of CAD. In addition to EPIC-Norfolk, the studies included were the Ottawa Heart Studies I and II [23,24], WTCCC CAD study [25,26], PENN CATH and MEDSTAR studies [27,28], and nested case-control studies derived from the CoLaus [29,30], GEMS [30,31,32] and Rotterdam [33,34] studies. Details of these studies and their genotyping and analytical approaches have been published previously and are summarised briefly in **Table 1**.

Biochemical analyses

IGF-I and IGFBP-3 were measured in EPIC-Norfolk using non-fasting serum samples collected at baseline and stored frozen at -80°C for approximately 11 years before measurement. Serum levels of IGF-I were measured by radioimmunoassay using a monoclonal antibody (Blood Products, Elstree, Hertfordshire, UK) and recombinant peptide (Gropep, Adelaide, Australia) for standard and tracer (following iodination using the chloramine-T method). Samples were analysed following acid-acetone extraction to deplete IGF binding proteins (IGFBPs) with an excess of IGF-II added to the resulting extract in order to saturate any residual IGFBPs. Intra-assay coefficient of variation (CV) was 8% and inter-assay CV was 11.1%. Serum levels of IGFBP-3 were measured by radioimmunoassay using an in-house polyclonal antibody raised against recombinant IGFBP-3. The assay was calibrated against recombinant glycosylated IGFBP-3 (Dr C Maack, Celltrix, Santa Clara, CA, USA). Samples were analysed following a 1:100 dilution with antibody used at a final dilution of 1:20,000. Intra-assay coefficient of variation (CV) was 4% and inter-assay CV was 10.1%.

Tagging SNPs for *IGF1* and *IGFBP3* genes

We generated a set of tSNPs for the *IGF1* and *IGFBP3* genes using resequencing data from the University of Washington SeattleSNPs Programs for Genomic Applications (<http://pga.gs.washington.edu/>) and NIEHS resequencing program (<http://egp.gs.washington.edu/>), respectively. From these databases, genotypes were available for SNPs spanning 2 kb upstream and downstream of each gene. The aim of our approach was to identify a set of tSNPs that capture all known common SNPs at these loci with a minor allele frequency (MAF) ≥ 0.05

in individuals of European ancestry. We identified 39 SNPs at the *IGF1* locus and 26 SNPs at the *IGFBP3* locus with a MAF ≥ 0.05 in Coriell individuals with European ethnicity (**Figure 1A** and **B**). We used the Tagger program embedded in Haploview [35,36] to generate a set of 13 tSNPs for the *IGF1* gene, forcing in three SNPs (rs1520220, rs2946834, rs6220) that had previously been found to correlate with circulating IGF-I levels. [37] For *IGFBP3*, we generated a set of 15 tSNPs, forcing in three SNPs (rs2132571, rs2132572, rs2854744) previously found to correlate with circulating IGFBP-3 levels. [37] We subsequently discarded six tSNPs for which we were unable to design an assay due to the repetitive nature of the surrounding sequence (**Figure 1A** and **B**). These SNPs were not correlated with any other SNP and so alternative tSNPs could not be selected. This left a set of 22 tSNPs that captured 59 of the 65 common variants at a mean r^2 of 0.97 (r represents the pairwise correlation coefficient between SNPs). A list of the tSNPs and details of the SNPs captured is given in **Table 2**.

Genotyping

Genomic DNA for the EPIC-Norfolk case-control study was whole genome amplified (WGA) prior to genotyping using a GenomiPhi V2 DNA amplification kit (GE Healthcare, Amersham, UK) following the manufacturer's instructions. WGA DNA was normalised using a Quant-iT PicoGreen dsDNA kit (Invitrogen, Paisley, UK). We conducted concordance checks with genomic DNA using a panel of three SNPs in separate genomic locations and obtained > 95% concordance for these SNPs between WGA and genomic DNA. Nineteen tSNPs were genotyped by KBioscience (<http://www.kbioscience.co.uk>) using KASPar technology, and three (rs6220, rs35767, rs12821878) using a custom Taqman® assay (Applied Biosystems, Warrington, UK) at Strangeways Research Laboratory (University of Cambridge, Cambridge, UK) according to the manufacturer's instructions. Allele calling was done using the ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, Warrington, UK). Cases and controls were randomly allocated across DNA study plates with two duplicate samples and two water controls in each 96-well plate. Concordance between duplicate samples was > 97% for all SNPs using both methods.

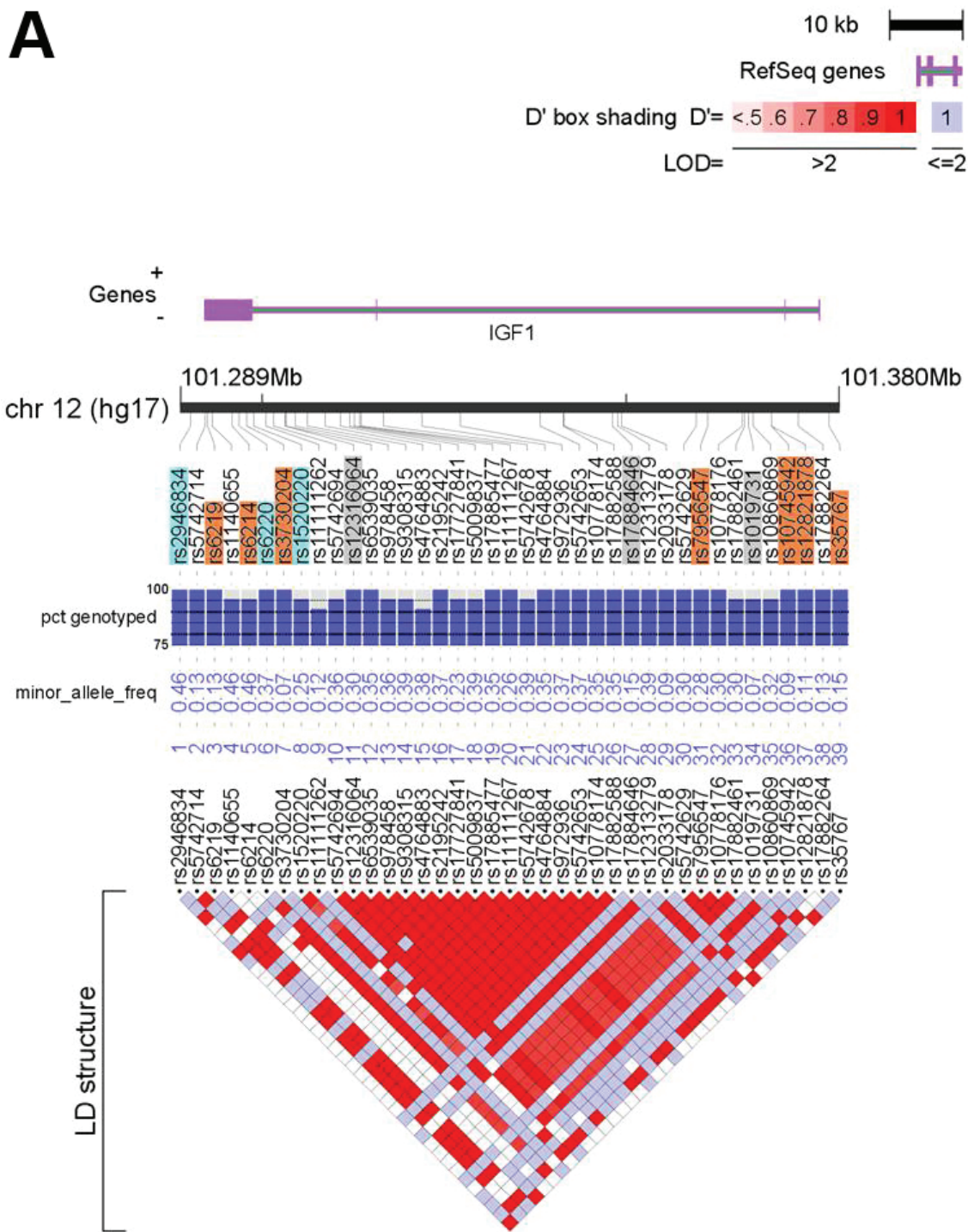


Figure 1 Feature maps of the *IGF1* (A) and *IGFBP3* (B) genes. Positions of the 39 SNPs at the *IGF1* locus and the 26 SNPs at the *IGFBP3* locus with MAF ≥ 0.05 in the resequenced CEPH samples are shown relative to the locus (purple) and chromosome (black bar). SNPs highlighted in orange depict tSNPs and those highlighted in grey are tSNPs that were force included (see text for details). tSNPs highlighted in grey were discarded (see text). Underlying each SNP is QC information and MAF in the CEPH samples. The bottom of each figure depicts an LD plot for the locus with pairwise LD values (D') presented. See the key for details. The figures were generated using LocusView (T. Petryshen, A. Kirby, M. Ainscow, unpublished software, available from the Broad Institute, Cambridge, MA (<http://www.broad.mit.edu/mpg/locusview/>)). (Refer to next page for Figure 1B).

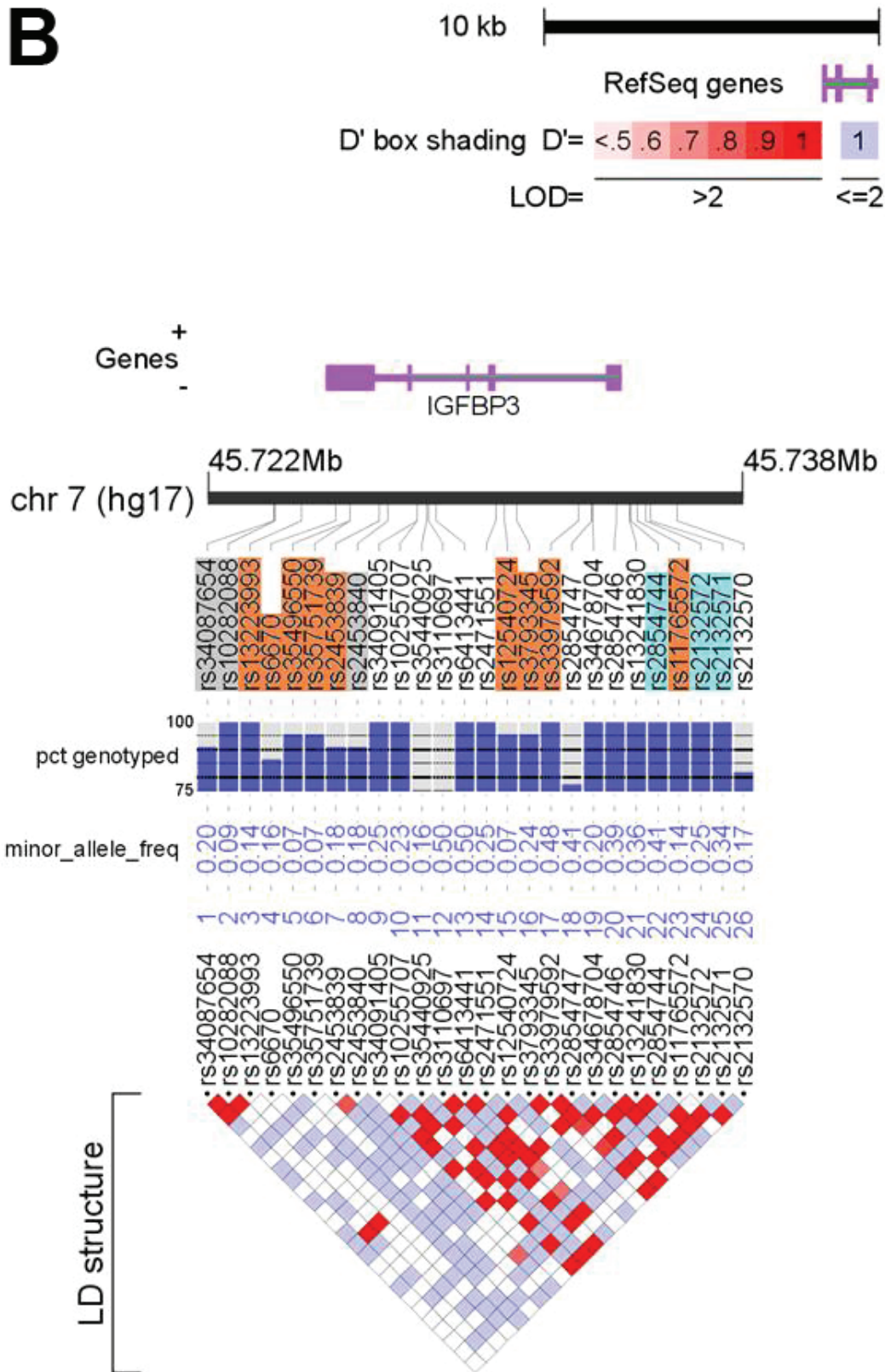


Figure 1. (B) Feature map of the *IGFBP3* gene. (Refer to previous page for figure legend.)

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Table 2. *IGF1* and *IGFBP3* tSNPs genotyped in EPIC-Norfolk

Gene	tSNP	SNPs captured
<i>IGF1</i>	rs35767	rs35767
	rs12821878	rs12821878
	rs10745942	rs2033178, rs10745942
	rs7956547	rs17727841, rs11111267, rs5742629, rs7956547, rs10778176, rs17882461, rs10860869
	rs1520220	rs1520220
	rs3730204	rs3730204
	rs6220	rs6220, rs5742694, rs6539035, rs978458, rs9308315, rs4764883, rs2195242, rs5009837, rs17885477, rs5742678, rs4764884, rs972936, rs5742653, rs10778174, rs10778175, rs12313279
	rs6214	rs6214
	rs6219	rs5742714, rs6219, rs11111262, rs17882264
	rs2946834	rs2946834, rs1140655
	<i>IGFBP3</i>	rs2132571
rs2132572		rs10255707, rs2132572, rs2132570
rs11765572		rs11765572
rs2854744		rs2854746, rs2854744
rs33979592		rs6413441, rs33979592, rs2854747, rs3110697
rs3793345		rs34091405, rs2471551, rs3793345, rs34678704, rs35440925
rs12540724		rs12540724
rs2453839		rs2453839
rs35751739		rs35751739
rs35496550		rs35496550
rs6670		rs6670
rs13223993		rs13223993

Table 3. Baseline characteristics of CAD cases and controls, EPIC-Norfolk, 1993–2003

	Cases n = 1013	Controls n = 2055	P-value *
Age, years	65 ± 8	65 ± 8	Matched
Men (%)	654 (65)	1290 (63)	Matched
Smoking: Never (%)	331 (33)	834 (41)	< 0.0001
Current/Previous (%)	682 (67)	1221 (59)	
Diabetes (%)	63 (6)	33 (2)	< 0.0001
Body mass index, kg/m ²	27.3 ± 3.9	26.3 ± 3.5	< 0.0001
Waist circumference (cm)	94.2 (11.8)	91.1 (11.4)	< 0.0001
Systolic blood pressure, mmHg	144 ± 19	139 ± 18	< 0.0001
Diastolic blood pressure, mmHg	86 ± 12	84 ± 11	< 0.0001
Total cholesterol, mmol/l	6.5 ± 1.2	6.3 ± 1.1	< 0.0001
LDL cholesterol, mmol/l	4.3 ± 1.1	4.1 ± 1.0	< 0.0001
HDL cholesterol, mmol/l	1.26 ± 0.37	1.37 ± 0.40	< 0.0001
Triglycerides, mmol/l †	1.8 (1.3–2.6)	1.6 (1.1–2.2)	< 0.0001
C-reactive protein, mg/l †	2.2 (1.0–4.9)	1.5 (0.7–3.1)	< 0.0001
IGF-I, ng/ml	155.1 ± 52.3	153.5 ± 48.9	0.40
IGFBP-3, mg/l	4.37 ± 1.31	4.30 ± 1.28	0.14

Data are means and standard deviations, unless otherwise stated. *P-values based on linear regression for continuous variables and X² tests for categorical variables. †Data are medians (interquartile range) and P-values based on linear regression using log transformation.

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Statistical analysis

Baseline characteristics were compared between cases and controls (**Table 3**). We natural log transformed triglycerides and C-reactive protein (CRP) before analysis because of their skewed distribution. IGF-I and IGFBP-3 levels were approximately normally distributed. To assess the association between circulating IGF-I and IGFBP-3 levels, cardiovascular risk factors and CAD risk in EPIC-Norfolk (**Tables 4-7**) we constructed quartiles of IGF-I and IGFBP-3 levels in control participants and applied these quartiles to cases. Selection of confounding risk factors was based on the proposed biological mechanisms linking IGFs to the development of CAD [38] and on previous reports examining the

association between IGFs and risk of CAD [14,16,20] and their availability in EPIC-Norfolk. Because of the ambiguity of defining potential confounders and mediators for the association between IGFs and CAD risk, we added potential confounders (see footnote of **Table 6**) in sequential statistical models, as outlined below. We used linear regression to assess the association between IGF-I and IGFBP-3 quartiles and continuous cardiovascular risk factors in control participants only, with adjustment for age and sex. For categorical variables (sex, diabetes status and smoking status) we used logistic regression to test for association with IGF-I and IGFBP-3 quartiles, with adjustment for age and sex, where appropriate. To test for association between circulating levels of IGF-I and IGFBP-3

Table 4. Association between IGF-I levels and cardiovascular risk factors in controls at baseline, EPIC-Norfolk, 1993–2003

Quartiles of IGF-I	1	2	3	4	P-value *
	n = 524	n = 514	n = 506	n = 511	
IGF-I, ng/ml	< 120	120-149	150-184	> 184	
Age, year	67 (66-67)	66 (65-67)	65 (64-66)	63 (63-64)	< 0.0001
Men (%)	278 (53)	307 (60)	337 (67)	374 (73)	< 0.0001
Smoking: Never (%)	208 (40)	206 (40)	206 (41)	200 (39)	0.92
Current/Previous (%)	316 (60)	308 (60)	300 (59)	311 (61)	
Diabetes (%)	12 (2)	3 (1)	5 (1)	7 (1)	0.32
Body mass index, kg/m ²	26.0 (25.7-26.3)	26.3 (26.0-26.6)	26.3 (26.0-26.6)	26.4 (26.1-26.7)	0.15
Waist circumference (cm)	90.5 (89.6-91.3)	91.4 (90.6-92.3)	91.0 (90.2-91.8)	91.8 (90.9-92.6)	0.07
Systolic blood pressure, mmHg	138 (136-139)	139 (138-141)	139 (137-140)	140 (138-141)	0.10
Diastolic blood pressure, mmHg	83 (82-84)	84 (83-85)	84 (83-85)	84 (83-85)	0.04
Total cholesterol, mmol/l	6.2 (6.1-6.3)	6.3 (6.2-6.4)	6.3 (6.2-6.4)	6.3 (6.2-6.3)	0.20
LDL cholesterol, mmol/l	4.0 (3.9-4.1)	4.1 (4.0-4.2)	4.1 (4.0-4.2)	4.1 (4.0-4.1)	0.54
HDL cholesterol, mmol/l	1.38 (1.34-1.41)	1.36 (1.33-1.39)	1.39 (1.36-1.42)	1.36 (1.33-1.39)	0.79
Triglycerides, mmol/l †	1.5 (1.5-1.6)	1.6 (1.6-1.7)	1.6 (1.5-1.6)	1.7 (1.6-1.8)	0.004
C-reactive protein, mg/l †	1.7 (1.5-1.9)	1.6 (1.4-1.8)	1.4 (1.3-1.5)	1.6 (1.4-1.7)	0.07
IGFBP-3, mg/l	3.61 (3.51-3.71)	4.14 (4.04-4.24)	4.51 (4.41-4.61)	4.95 (4.85-5.06)	< 0.0001

Data for continuous traits are sex and age adjusted means and 95% confidence intervals. Data for categorical variables (sex, diabetes and smoking) are sex and age adjusted numbers and proportions. *P-values based on linear regression for continuous variables and logistic regression for categorical variables. †Sex and age adjusted geometric means and 95% confidence intervals. P-values are based on linear regression using log transformation.

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Table 5. Association between IGFBP-3 levels and cardiovascular risk factors in controls at baseline, EPIC-Norfolk, 1993–2003

Quartiles of IGFBP-3	1	2	3	4	P-value
	n = 520	n = 511	n = 515	n = 509	
IGFBP-3, mg/l	< 3.43	3.44-4.18	4.19-5.11	> 5.12	
Age, year	66 (66-67)	65 (65-66)	65 (64-66)	64 (64-65)	< 0.0001
Men (%)	371 (71)	331 (65)	300 (58)	293 (58)	< 0.0001
Smoking: Never (%)	227 (44)	205 (40)	205 (40)	184 (36)	0.02
Current/Previous (%)	293 (56)	306 (60)	310 (60)	325 (64)	
Diabetes (%)	12 (2)	9 (2)	3 (1)	3 (1)	0.002
Body mass index, kg/m ²	25.9 (25.6-26.2)	26.0 (25.7-26.3)	26.4 (26.1-26.7)	26.7 (26.4-27.0)	< 0.0001
Waist circumference (cm)	90.2 (89.3-91.0)	90.6 (89.7-91.4)	91.4 (90.6-92.2)	92.5 (91.7-93.4)	< 0.0001
Systolic blood pressure, mmHg	137 (135-138)	139 (138-141)	140 (138-141)	140 (139-142)	0.002
Diastolic blood pressure, mmHg	82 (82-83)	84 (83-85)	84 (83-85)	84 (83-85)	0.002
Total cholesterol, mmol/l	6.1 (6.0-6.2)	6.2 (6.1-6.3)	6.3 (6.2-6.4)	6.4 (6.3-6.5)	< 0.0001
LDL cholesterol, mmol/l	4.0 (3.9-4.1)	4.0 (3.9-4.1)	4.1 (4.1-4.2)	4.1 (4.0-4.2)	0.05
HDL cholesterol, mmol/l	1.34 (1.31-1.37)	1.40 (1.37-1.43)	1.37 (1.34-1.41)	1.37 (1.34-1.40)	0.39
Triglycerides, mmol/l*	1.5 (1.4-1.5)	1.5 (1.5-1.6)	1.6 (1.6-1.7)	1.8 (1.7-1.9)	< 0.0001
C-reactive protein, mg/l*	1.6 (1.4-1.7)	1.5 (1.4-1.7)	1.5 (1.4-1.7)	1.6 (1.5-1.8)	0.72
IGF-I, ng/ml	129.9 (126.1-133.8)	147.7 (143.8-151.5)	161.8 (157.9-165.6)	175.5 (171.6-179.4)	< 0.0001

Data for continuous traits are sex and age adjusted means and 95% confidence intervals. Data for categorical variables (sex, diabetes and smoking) are sex and age adjusted numbers and proportions. *P-values based on linear regression for continuous variables and logistic regression for categorical variables. †Sex and age adjusted geometric means and 95% confidence intervals. P-values are based on linear regression using log transformation.

and risk of CAD, we used logistic regression to calculate odds ratios and 95% confidence intervals. We analysed these associations using IGF-I and IGFBP-3 levels defined as quartiles based on the distribution among controls and as continuous variables (for a 1 standard deviation (SD) increase). The lowest IGF-I and IGFBP-3 quartiles were used as reference categories and three models were used with increasing degrees of adjustment (Tables 6 and 7). For all three models, we adjusted for study enrolment date to account for differences in storage time of the blood samples. Log likelihood ratio tests were used to test for the presence of effect modification by sex between circulating IGF-I and IGFBP-3 levels and risk of CAD and for pos-

sible interaction between IGF-I and IGFBP-3 on risk of CAD.

Hardy-Weinberg equilibrium was assessed for each tSNP in control participants using the χ^2 statistic (1 df). In our primary tests for association, we used linear per allele regression to test for associations among tSNPs and circulating levels of IGF-I and IGFBP-3 in control participants, adjusted for age and sex. To test the linearity of associations between statistically associated tSNPs and circulating IGF-I or IGFBP-3 levels we used log likelihood ratio tests by comparing a general model with the linear per allele model. We also used log likelihood ratio tests to assess for the presence of sex interac-

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Table 6. Association between IGF-I levels and risk of future CAD, EPIC-Norfolk, 1993–2003

IGF-I levels	Percentile	Cases (%)	Controls (%)	Model 1: adjusted odds ratio (95% CI)*	Model 2: adjusted odds ratio (95% CI)†	Model 3: adjusted odds ratio (95% CI)‡
	< 25th	252 (25)	524 (26)	1.00	1.00	1.00
	25th–49th	262 (26)	514 (25)	1.05 (0.85-1.30)	1.05 (0.84-1.31)	1.03 (0.82-1.29)
	50th–75th	249 (25)	506 (25)	1.01 (0.82-1.26)	1.06 (0.84-1.32)	1.03 (0.82-1.30)
	> 75th	250 (25)	511 (25)	1.00 (0.81-1.25)	0.96 (0.76-1.21)	0.93 (0.72-1.18)
P-trend (1 df)				0.92	0.76	0.56
Per 1 SD increase (48.9 ng/ml)				1.03 (0.95-1.11)	1.01 (0.93-1.09)	0.98 (0.90-1.06)

CI, confidence interval. *Model 1: Adjusted for age (continuous), sex and enrolment date. †Model 2: Adjusted for age (continuous), sex and enrolment date, waist circumference (continuous), smoking (categorical—never smokers vs current/previous smokers), diabetes, HDL-c, LDL-c, triglycerides, CRP, systolic and diastolic blood pressure (continuous). ‡Model 3: Adjusted for age (continuous), sex and enrolment date, waist circumference (continuous), smoking (categorical—never smokers vs current/previous smokers), diabetes, HDL-c, LDL-c, triglycerides, CRP, systolic and diastolic blood pressure (continuous), IGFBP-3 (continuous).

Table 7. Association between IGFBP-3 levels and risk of future CAD, EPIC-Norfolk, 1993–2003

IGFBP-3 levels	Percentile	Cases (%)	Controls (%)	Model 1: adjusted odds ratio (95% CI)*	Model 2: adjusted odds ratio (95% CI)†	Model 3: adjusted odds ratio (95% CI)‡
	< 25th	253 (25)	520 (25)	1.00	1.00	1.00
	25th–49th	237 (23)	511 (25)	0.97 (0.78-1.21)	0.96 (0.77-1.21)	0.96 (0.76-1.21)
	50th–75th	256 (25)	515 (25)	1.05 (0.85-1.30)	1.00 (0.80-1.26)	1.00 (0.79-1.26)
	> 75th	267 (26)	509 (25)	1.12 (0.90-1.40)	1.01 (0.80-1.27)	1.00 (0.78-1.28)
P-trend (1 df)				0.23	0.86	0.92
Per 1 SD increase (1.28 mg/l)				1.08 (1.00-1.16)	1.03 (0.94-1.11)	1.03 (0.94-1.12)

CI, confidence interval. *Model 1: Adjusted for age (continuous), sex and enrolment date. †Model 2: Adjusted for age (continuous), sex and enrolment date, waist circumference (continuous), smoking (categorical—never smokers vs current/previous smokers), diabetes, HDL-c, LDL-c, triglycerides, CRP, systolic and diastolic blood pressure (continuous). ‡Model 3: Adjusted for age (continuous), sex and enrolment date, waist circumference (continuous), smoking (categorical—never smokers vs current/previous smokers), diabetes, HDL-c, LDL-c, triglycerides, CRP, systolic and diastolic blood pressure (continuous), IGF-I (continuous).

tions with statistically associated tSNPs and circulating IGF-I and IGFBP-3 levels, with the SNP defined as a linear variable.

To assess whether statistically associated tSNPs contributed independently to circulating IGF-I or IGFBP-3 levels we compared the log likelihood of a nested model (2 df) with that of the full model (3 df) by consecutively adding the

other tSNPs (in a linear 1df form) to a model containing the strongest associated tSNP (general inheritance 2df form). We also conducted a reciprocal analysis, adding the strongest associated tSNP (1 df form) to a model with each of the other tSNPs (2 df form). We used pairwise correlation (r^2) to assess the extent of linkage disequilibrium among tSNPs in control participants.

In a secondary analysis for all associated tSNPs, we combined summary estimates (regression coefficients and standard errors) from case and control participants from the above linear regression models using meta-analysis. We used a fixed effects model and inverse-variance weighted averages of regression coefficients to obtain a combined estimate of the overall association between tSNPs and circulating IGF-I and IGFBP-3 levels.

To test for associations among tSNPs and risk of CAD in EPIC-Norfolk we used logistic regression using general and linear models adjusted for age, sex and enrolment date. We combined summary estimates (log odds ratios and standard errors) for each of the eight additional CAD case-control studies using meta-analysis, as above, to obtain a combined estimate of the association between SNPs and risk of CAD for a linear model. Between-study heterogeneity was assessed using the χ^2 test.

To ascertain the presence of haplotype associations at the *IGF1* and *IGFBP3* genes with circulating IGF-I and IGFBP-3 levels and risk of CAD in EPIC-Norfolk, we constructed haplotypes for *IGF1* and *IGFBP3* tSNPs using the program TagSNPs, which uses an expectation-substitution approach to account for the uncertainty caused by unphased genotype data [39]. Individuals with > 50% missing genotype data were excluded. Haplotypes with a frequency of < 1% were considered rare, and were pooled for subsequent analyses. To test for global haplotype association with circulating levels and CAD risk, we used regression to compare a model with additive effects for each common haplotype (using the most common haplotype as referent) to a model with no haplotype effects. We used linear regression and logistic regression with no covariate adjustment to test the association between each haplotype and circulating IGF-I or IGFBP-3 levels and to calculate odds ratios and 95% confidence intervals for the association between each haplotype and risk of CAD, respectively, relative to the most common haplotype that was used as the reference.

All analyses were done using Stata 10.1 (Stata Corporation, College Station, TX, USA).

Results

Association analysis of circulating IGF-I and

IGFBP-3 levels and risk of subsequent CAD

Table 3 shows the distribution of cardiovascular risk factors among cases and controls in the EPIC-Norfolk CAD nested case-control study. As expected and as previously reported [40], cases had more adverse cardiovascular risk profiles than control participants who remained free of disease during follow-up. There was no material difference between circulating IGF-I and IGFBP-3 levels among cases and controls (**Table 3**).

We assessed the association between quartiles of IGF-I and IGFBP-3 and baseline cardiovascular risk factors in control participants (**Tables 4** and **5**). Both IGF-I and IGFBP-3 levels were inversely associated with age, IGF-I levels were higher in men and IGFBP-3 levels higher in women, consistent with previous reports [14,16,17,19,20,41]. Circulating levels of IGF-I were positively associated with IGFBP-3 levels and both circulating IGF-I and IGFBP-3 levels showed positive associations with several cardiovascular risk factors (**Tables 4** and **5**). The latter findings are consistent with a previous report of a cross-sectional study demonstrating an association between higher levels of IGFBP-3 and the metabolic syndrome [42].

We next examined the relationship between circulating IGF-I and IGFBP-3 levels and risk of CAD in EPIC-Norfolk. For IGF-I we found no evidence for association with CAD risk (**Table 6**). For IGFBP-3 we found some evidence for a weak unadjusted association between levels and risk of CAD but this attenuated after adjustment for confounding risk factors (**Table 7**). Previous studies demonstrating an association between circulating IGF-I and IGFBP-3 levels and risk of CAD have comprised a high proportion of cases with fatal CAD or myocardial infarction [14,15,16,20]. Therefore we conducted an additional subgroup analysis of fatal and non-fatal CAD cases and controls. We did not find any evidence for association for either IGF-I or IGFBP-3 with fatal CAD (data not shown). Furthermore there was no evidence for any interaction between sex and IGF-I or IGFBP-3 for risk of CAD or between IGF-I and IGFBP-3 for risk of CAD.

Association analysis of tagging SNPs and circulating IGF-I and IGFBP-3 levels

Of the 22 tSNPs genotyped in the EPIC-Norfolk

CAD nested case-control study (**Figure 1A** and **B** and **Table 2**), three showed deviation from Hardy-Weinberg equilibrium (HWE) in control participants (footnote to **Table 8**) and so were discarded from further analysis. We assessed associations among the remaining 19 tSNPs and circulating levels of IGF-I and IGFBP-3 (**Table 8**). Three tSNPs exhibited a MAF < 0.05 in EPIC-Norfolk compared to that observed in resequenced Coriell individuals (**Table 8** and **Figure 1A** and **B**). Eight tSNPs showed evidence for statistical association with either circulating IGF-I or IGFBP-3 levels at $P \leq 0.05$ (**Table 8**). These include directionally consistent replication of associations between *IGF1* tSNPs rs1520220 and rs6220 and circulating IGF-I levels, and between *IGFBP3* tSNPs rs2132571 and rs2854744 and circulating IGFBP-3 levels, as shown in our previous report [37]. All eight tSNP associations remained statistically associated in a combined analysis of case and control participants (**Table 8**) and demonstrated linear associations with circulating IGF-I or IGFBP-3 levels (**Table 8**). By contrast to our previous report [37], there was no evidence for any interaction between sex and tSNPs with circulating IGF-I or IGFBP-3 levels.

To contextualise the magnitudes of associations observed between tSNPs and circulating levels of IGF-I and IGFBP-3 for ease of interpretation, we present the proportion of a 1 SD change in circulating levels shown by each associated tSNP (**Table 9**). The five tSNPs showing association with IGF-I levels were associated with between 7-30% of a 1 SD change in circulating IGF-I concentrations per allele (**Table 9**). For IGFBP-3, the three statistically associated tSNPs (**Table 8**) were associated with between 7-11% of a 1 SD change in circulating IGFBP-3 concentrations per allele (**Table 9**). We assessed the independence of the individual tSNP associations with circulating IGF-I levels and found that tSNPs rs1520220 and rs3730204 at the *IGF1* locus were sufficient to explain the associations for the other three tSNPs. Similarly at the *IGFBP3* locus, tSNP rs2132571 was sufficient to explain the associations with circulating IGFBP-3 levels observed for the other two tSNPs.

We conducted an analysis to assess for the presence of haplotype effects at the *IGF1* and *IGFBP3* loci that might reflect associations of untyped SNPs (**Tables 10** and **11**). There were

no significant global haplotypic associations for *IGF1* or *IGFBP3* ($P > 0.05$). For *IGF1*, individual haplotypic associations were fully explained by the associations shown by individual SNPs (**Table 9**). However, for *IGFBP3* a strong association with circulating IGFBP-3 levels was shown by a haplotype that contains the minor allele of both tSNPs rs2132571 and rs3793345 (**Table 11**). The magnitude of association of this haplotype with circulating levels is almost twice that shown for either SNP individually (18% of a 1SD change in IGFBP-3 levels compared with 11% for the two SNPs, **Table 9**), suggesting that tSNPs rs2132571 and rs3793345 might capture an untyped variant that has a strong effect on circulating IGFBP-3 levels.

Association analysis of tagging SNPs and risk of CAD

It is possible that variation at the *IGF1* and *IGFBP3* genes could influence risk of CAD via pleiotropic effects on mechanisms independent from circulating levels; for example reflecting tissue-specific variation in IGF-I or IGFBP-3 action. We therefore assessed the association between *IGF1* and *IGFBP3* tSNPs and risk of CAD in the EPIC-Norfolk CAD nested case-control study. **Table 8** shows the association between these 19 SNPs and risk of CAD in up to 1,133 cases and 2,223 controls. We did not find any robust evidence for association with CAD for any of the tSNPs in EPIC-Norfolk. Similarly, global or individual haplotype analyses did not reveal any robust associations with CAD risk for either *IGF1* or *IGFBP3* (**Tables 10** and **11**, global haplotypic P -values > 0.05). To increase statistical power for this analysis, we tested 31 SNPs spanning the *IGF1* and *IGFBP3* genes in eight CAD studies that are part of an established collaborative consortium. SNP genotypes were available *in silico* from genome-wide SNP chips or imputation analyses (**Table 1**). We combined our data from EPIC-Norfolk with these eight studies, in total comprising up to 9,319 cases and 19,964 controls. The results of this additional analysis are shown in **Table 12** and show no evidence for association between common variants at the *IGF1* and *IGFBP3* genes and risk of CAD.

Discussion

This is the first study to conduct a systematic

IGF-1, IGFBP-3, genetic variants and risk of coronary artery disease

Table 8. Statistical associations between *IGF1* and *IGFBP3* tSNPs , circulating IGF-I and IGFBP-3 levels and risk of CAD in EPIC-Norfolk participants

Gene	SNP		Genotype			P-trend *	P-trend pooled cases and controls †
			Common homozygous	Heterozygous	Rare homozygous		
<i>IGF1</i>	rs35767	MAF : 0.15 cases/controls	CC	CT	TT	0.67	-
		OR (95% CI)	776/1559 1.00	266/539 0.99 (0.83-1.17)	21/50 0.84 (0.50-1.42)		
		IGF-I	153.3 (150.9-155.6)	155.1 (151.1-159.1)	159.3 (146.1-172.5)	0.27	0.35
<i>IGF1</i>	rs12821878	MAF : 0.23 cases/controls	GG	GA	AA	0.56	-
		OR (95% CI)	660/1262 1.00	359/773 0.89 (0.76-1.04)	60/101 1.14 (0.81-1.58)		
		IGF-I	155.7 (153.1-158.3)	151.6 (148.2-154.9)	150.9 (141.6-160.2)	0.05	0.02
<i>IGF1</i>	rs7956547	MAF : 0.24 cases/controls	TT	TC	CC	0.29	-
		OR (95% CI)	590/1198 1.00	392/761 1.05 (0.90-1.23)	72/124 1.18 (0.87-1.60)		
		IGF-I	152.0 (149.4-154.7)	155.3 (152.0-158.7)	159.0 (150.6-167.3)	0.05	0.04
<i>IGF1</i>	rs1520220	MAF : 0.17 cases/controls	CC	CG	GG	0.59	-
		OR (95% CI)	731/1478 1.00	288/554 1.05 (0.89-1.24)	36/70 1.04 (0.69-1.57)		
		IGF-I	151.7 (149.2-154.1)	157.5 (153.5-161.5)	162.9 (151.7-174.0)	3.1 x 10⁻³	1.4 x 10⁻³
<i>IGF1</i>	rs3730204	MAF : 0.02 cases/controls	TT	TC	CC	0.07	-
		OR (95% CI)	1046/2072 1.00	18/62 0.58 (0.34-0.98)	1/1 2.00 (0.12-32.0)		
		IGF-I	153.3 (151.2-155.3)	167.1 (155.3-178.9)	214.8 (121.8-307.9)	0.01	0.03
<i>IGF1</i>	rs6220	MAF : 0.27 cases/controls	AA	AG	GG	0.42	-
		OR (95% CI)	562/1157 1.00	419/817 1.05 (0.90-1.23)	83/157 1.09 (0.82-1.45)		
		IGF-I	151.6 (148.9-154.3)	154.9 (151.7-158.2)	163.6 (156.1-171.0)	3.4 x 10⁻³	1.4 x 10⁻³
<i>IGF1</i>	rs6214	MAF : 0.41 cases/controls	GG	GA	AA	0.39	-
		OR (95% CI)	358/722 1.00	509/1053 0.98 (0.83-1.15)	188/335 1.13 (0.91-1.41)		
		IGF-I	153.1 (149.6-156.6)	153.7 (150.8-156.5)	155.1 (150.0-160.2)	0.55	0.94
<i>IGF1</i>	rs6219	MAF : 0.09 cases/controls	GG	GA	AA	0.76	-
		OR (95% CI)	869/1746 1.00	170/319 1.07 (0.88-1.31)	9/23 0.78 (0.36-1.70)		
		IGF-I	152.9 (150.6-155.1)	156.4 (151.2-161.7)	158.5 (139.0-177.9)	0.19	0.04
<i>IGFBP3</i>	rs2132571	MAF : 0.32 cases/controls	GG	GA	AA	0.63	-
		OR (95% CI)	486/980 1.00	441/884 1.01 (0.86-1.18)	119/223 1.08 (0.84-1.38)		
		IGFBP-3	4.42 (4.34-4.50)	4.27 (4.19-4.36)	4.16 (3.99-4.33)	1.3 x 10⁻³	9.8 x 10⁻³
<i>IGFBP3</i>	rs2132572	MAF : 0.23 cases/controls	GG	GA	AA	0.15	-
		OR (95% CI)	655/1264 1.00	357/745 0.92 (0.79-1.08)	52/124 0.81 (0.58-1.13)		
		IGFBP-3	4.32 (4.25-4.40)	4.32 (4.22-4.41)	4.45 (4.23-4.68)	0.56	0.82
<i>IGFBP3</i>	rs11765572	MAF : 0.13 cases/controls	GG	GA	AA	0.63	-
		OR (95% CI)	801/1594 1.00	234/457 1.02 (0.85-1.22)	15/44 0.68 (0.37-1.22)		
		IGFBP-3	4.35 (4.28-4.41)	4.29 (4.17-4.41)	4.04 (3.66-4.43)	0.15	0.80
<i>IGFBP3</i>	rs2854744	MAF : 0.44	CC	CA	AA		

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		cases/controls	308/663	507/977	215/425		
		OR (95% CI)	1.00	1.12 (0.94-1.33)	1.09 (0.88-1.35)	0.36	-
		IGFBP-3	4.28 (4.18-4.38)	4.30 (4.22-4.38)	4.47 (4.34-4.59)	0.03	0.04
<i>IGFBP3</i>	rs3793345	MAF : 0.20	TT	TC	CC		
		cases/controls	662/1351	342/657	48/83		
		OR (95% CI)	1.00	1.06 (0.91-1.25)	1.18 (0.82-1.71)	0.28	-
		IGFBP-3	4.38 (4.31-4.45)	4.26 (4.16-4.36)	4.07 (3.79-4.34)	6.6 x 10⁻³	9.3 x 10⁻⁴
<i>IGFBP3</i>	rs12540724	MAF : 0.03	AA	AG	GG		
		cases/controls	978/1996	78/117	1/2		
		OR (95% CI)	1.00	1.36 (1.01-1.83)	1.00 (0.09-11.1)	0.05	-
		IGFBP-3	4.34 (4.28-4.39)	4.16 (3.92-4.39)	5.51 (3.71-7.31)	0.27	0.07
<i>IGFBP3</i>	rs2453839	MAF : 0.18	TT	TC	CC		
		cases/controls	687/1422	333/624	45/69		
		OR (95% CI)	1.00	1.11 (0.94-1.30)	1.34 (0.91-1.98)	0.08	-
		IGFBP-3	4.34 (4.27-4.41)	4.29 (4.19-4.39)	4.46 (4.15-4.76)	0.84	0.17
<i>IGFBP3</i>	rs35751739	MAF : 0.03	GG	GA	AA		
		cases/controls	1009/1994	47/105	1/2		
		OR (95% CI)	1.00	0.89 (0.62-1.26)	1.01 (0.09-11.2)	0.53	-
		IGFBP-3	4.32 (4.26-4.38)	4.47 (4.22-4.72)	3.87 (2.07-5.66)	0.34	0.96
<i>IGFBP3</i>	rs35496550	MAF : 0.12	AA	A-	-		
		cases/controls	824/1606	203/453	21/27		
		OR (95% CI)	1.00	0.87 (0.73-1.05)	1.52 (0.86-2.71)	0.61	-
		IGFBP-3	4.31 (4.25-4.37)	4.43 (4.31-4.55)	3.99 (3.50-4.48)	0.32	0.43
<i>IGFBP3</i>	rs6670	MAF : 0.22	AA	AT	TT		
		cases/controls	610/1287	381/716	59/103		
		OR (95% CI)	1.00	1.12 (0.96-1.31)	1.21 (0.86-1.69)	0.10	-
		IGFBP-3	4.31 (4.24-4.38)	4.37 (4.28-4.47)	4.41 (4.16-4.66)	0.22	0.36
<i>IGFBP3</i>	rs13223993	MAF : 0.21	GG	GA	AA		
		cases/controls	657/1318	344/679	54/111		
		OR (95% CI)	1.00	1.02 (0.87-1.19)	0.98 (0.70-1.37)	0.96	-
		IGFBP-3	4.34 (4.27-4.41)	4.34 (4.24-4.43)	4.23 (3.99-4.47)	0.57	0.12

All tSNPs above were in Hardy-Weinberg equilibrium (HWE) in control participants ($P \geq 0.05$).

For *IGF1*, tSNPs rs10745942 and rs2946834 were not in HWE and for *IGFBP3* tSNP rs33979592 was not in HWE. These variants were discarded from further analysis in EPIC-Norfolk.

Minor allele frequency (MAF) is based on the EPIC-Norfolk study control participants.

Data for circulating IGF-I and IGFBP-3 levels are sex and age adjusted means and 95% confidence intervals.

* Primary tests for association between tSNPs and circulating IGF-I and IGFBP-3 levels were conducted using data from up to 2,223 control participants using linear regression analysis using an additive model adjusted for age and sex.

† Secondary association analyses were conducted by combining summary estimates (beta coefficients and standard errors) from above linear regression analyses in up to 1,133 cases and 2,223 controls using combined analysis.

Tests for association between tSNPs and risk of CAD were conducted using data from up to 1,133 cases and 2,223 controls using logistic regression analysis and general and log additive models with adjustment for age, sex and enrolment date.

OR, odds ratio.

CI, confidence interval.

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Table 9. Summary statistics for *IGF1* and *IGFBP3* tSNPs showing statistical association with circulating IGF-I and IGFBP-3 levels in up to 2,223 EPIC-Norfolk control participants

SNP	Gene	Position in gene	Minor allele	MAF *	β-coefficient (se) †	P-value	Percent of 1SD change in circulating levels ‡
IGF-I							
rs12821878	<i>IGF1</i>	intron 2	A	0.23	-3.406 (1.761)	0.05	7.0%
rs7956547	<i>IGF1</i>	intron 2	C	0.24	3.385 (1.710)	0.05	6.9%
rs1520220	<i>IGF1</i>	intron 3	G	0.17	5.735 (1.936)	3.1 × 10 ⁻³	11.7%
rs3730204	<i>IGF1</i>	3'UTR	C	0.02	14.867 (5.922)	0.01	30.4%
rs6220	<i>IGF1</i>	3'UTR	G	0.27	4.784 (1.635)	3.4 × 10 ⁻³	9.8%
IGFBP-3							
rs2132571	<i>IGFBP3</i>	5'	A	0.32	-0.136 (0.042)	1.3 × 10 ⁻³	10.7%
rs2854744	<i>IGFBP3</i>	5'	A	0.44	0.086 (0.040)	0.03	6.7%
rs3793345	<i>IGFBP3</i>	intron 1	C	0.20	-0.136 (0.050)	6.6 × 10 ⁻³	10.6%

*Based on the EPIC-Norfolk study control participants. †Beta-coefficients represent the change in circulating IGF-I or IGFBP-3 levels per additional minor allele, adjusted for age and sex. ‡Percentage of a 1 SD change in circulating IGF-I or IGFBP-3 levels per additional minor allele, adjusted for age and sex. A 1 SD change in circulating IGF-I was derived from control participants and is 48.9 ng/ml; for IGFBP-3 it is 1.28 mg/l (Table 3).

Table 10. *IGF1* haplotype analysis results for association with circulating IGF-I levels and risk of CAD in EPIC-Norfolk

Haplotype	IGF-I levels			CAD risk	
	Frequency (%) *	β-coefficient (se) †	P-value	OR (95% CI) ‡	P-value
h00000010	0.21	0	-	1.00	-
h01000000	0.20	-3.873 (1.965)	0.05	0.91 (0.79-1.04)	0.18
h00000000	0.18	-0.728 (2.076)	0.73	0.97 (0.84-1.12)	0.68
h00110101	0.09	3.834 (2.595)	0.14	1.04 (0.87-1.24)	0.68
h00100110	0.07	0.158 (2.908)	0.96	1.08 (0.89-1.32)	0.43
h10010100	0.06	4.106 (3.242)	0.21	0.96 (0.76-1.21)	0.73
h10000010	0.05	-1.443 (3.714)	0.70	0.97 (0.75-1.25)	0.80
h00100000	0.04	-2.305 (3.916)	0.56	1.15 (0.88-1.49)	0.30
h01000010	0.03	2.184 (5.348)	0.68	1.37 (0.98-1.92)	0.06
h00101110	0.01	14.573 (6.179)	0.02	0.61 (0.37-1.02)	0.06
h10000000	0.01	-12.969 (8.975)	0.15	0.88 (0.46-1.66)	0.69
Rare	-	8.070 (4.135)	0.05	1.14 (0.87-1.51)	0.34

In the haplotypes, 0 corresponds to the common allele for each SNP and 1 the minor allele (Table 8). SNP order in haplotypes is 5'-3' of the *IGF1* gene, as given in Table 8 as follows: rs35767, rs12821878, rs7956547, rs1520220, rs3730204, rs6220, rs6214, rs6219. *Haplotype frequencies are based on the EPIC-Norfolk study control participants. †Tests for association between haplotypes and circulating IGF-I levels were conducted in up to 2,223 control participants using linear regression with no covariate adjustment. ‡Tests for association between haplotypes and CAD risk were conducted up to 1,133 cases and 2,223 controls using logistic regression analysis with no covariate adjustment. OR, odds ratio. CI, confidence interval.

assessment of the interrelationships among circulating levels of IGF-I and IGFBP-3, common genetic variants at these loci, and risk of CAD. By contrast with previous smaller studies that have assessed the relationship between circu-

lating IGF-I and IGFBP-3 levels and risk of cardiovascular disease, our results—from the largest prospective study to date—suggest that circulating levels of IGF-I and IGFBP-3 and genetic variants at the *IGF1* and *IGFBP3* genes do not

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Table 11. *IGFBP3* haplotype analysis results for association with circulating IGFBP-3 levels and risk of CAD in EPIC-Norfolk

Haplotype	IGFBP-3 levels			CAD risk	
	Frequency (%) *	β -coefficient (se) †	P-value	OR (95% CI) ‡	P-value
h0100000000	0.14	0	-	1.00	-
h0001000000	0.13	0.109 (0.065)	0.10	0.92 (0.78-1.10)	0.36
h10100010000	0.10	-0.122 (0.068)	0.07	1.09 (0.92-1.29)	0.32
h10001000000	0.09	-0.234 (0.075)	1.8 x 10 ⁻³	0.95 (0.78-1.16)	0.60
h00010000010	0.09	0.094 (0.077)	0.22	1.09 (0.90-1.32)	0.38
h10001000010	0.08	-0.015 (0.078)	0.85	1.18 (0.97-1.43)	0.10
h00010000101	0.08	0.033 (0.078)	0.67	0.92 (0.75-1.13)	0.44
h00010010000	0.04	0.141 (0.106)	0.18	1.23 (0.95-1.60)	0.12
h00010000001	0.04	-0.155 (0.113)	0.17	1.13 (0.85-1.48)	0.40
h01000000001	0.04	0.014 (0.111)	0.90	0.90 (0.67-1.21)	0.48
h01000100001	0.02	-0.088 (0.135)	0.51	1.38 (1.00-1.89)	0.05
h00010000100	0.02	0.160 (0.176)	0.36	1.27 (0.83-1.94)	0.27
h00010010010	0.02	0.101 (0.194)	0.60	1.18 (0.73-1.92)	0.50
h01000001000	0.01	0.040 (0.191)	0.83	1.08 (0.67-1.73)	0.75
Rare	-	0.034 (0.077)	0.65	0.87 (0.71-1.07)	0.20

In the haplotypes, 0 corresponds to the common allele for each SNP and 1 the minor allele (Table 8). SNP order in haplotypes is 5'-3' of the *IGFBP3* gene, as given in Table 8 as follows: rs2132571, rs2132572, rs11765572, rs2854744, rs3793345, rs12540724, rs2453839, rs35751739, rs35496550, rs6670, rs13223993. *Haplotype frequencies are based on the EPIC-Norfolk study control participants. †Tests for association between haplotypes and circulating IGFBP-3 levels were conducted in up to 2,223 control participants using linear regression with no covariate adjustment. ‡Tests for association between haplotypes and CAD risk were conducted up to 1,133 cases and 2,223 controls using logistic regression analysis with no covariate adjustment. OR, odds ratio. CI, confidence interval.

materially influence the risk of CAD.

Our findings suggesting a lack of association between circulating IGF-I and IGFBP-3 levels and risk of CAD differ from those in a population-based nested case-control study in Denmark that reported a positive association between circulating IGFBP-3 levels and ischemic heart disease risk [14] and in the Cardiovascular Health Study, where low circulating IGFBP-3 levels were associated with an increased risk of coronary events in elderly individuals [15]. The Danish study and other prospective studies have also reported inverse associations between circulating IGF-I levels and risk of ischemic heart disease [14,16,43] and heart failure [20], although a recent study has found a positive association between IGF-I levels and risk of heart failure, but no association with overall incidence of cardiovascular disease [13]. However, our results are consistent with three recent reports based on prospective cohort studies, two of which were conducted in women

only [17,18,19]. The studies above are small and therefore subject to random variability, with no previous individual study exceeding 550 cases. Furthermore, previous studies have been conducted in relatively disparate populations (e.g. three were conducted in relatively elderly populations [13,16,20]). Such diversity highlights potential limitations in combining the findings of all available studies.

In addition to other known limitations of observational studies, such as incomplete control of confounders and bias, these apparently divergent findings may be due to several factors—heterogeneity in phenotype, age and sex of participants, heterogeneity among IGF-I and IGFBP-3 immunoassays, and pleiotropic effects of IGFs and their binding proteins that may have different actions in different contexts. We did not measure IGFBP-1 in EPIC-Norfolk, and it is possible that circulating levels of this binding protein may modify the relationships of other IGFs and clinical biomarkers on disease, which has

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Table 12. Association between *IGF1* and *IGFBP3* SNPs and risk of CAD in a combined analysis of nine studies

SNP	Gene	Position in gene	Minor allele	MAF *	No. cases / controls	OR (95% CI) †	P-value	P-value for heterogeneity
rs35767	<i>IGF1</i>	5'	T	0.15	8,963 / 19,660	0.96 (0.90-1.02)	0.19	0.95
rs12821878	<i>IGF1</i>	intron 2	A	0.23	9,319 / 19,964	1.03 (0.98-1.08)	0.21	0.96
rs10745942	<i>IGF1</i>	intron 2	A	0.04	8,012 / 17,600	0.94 (0.86-1.03)	0.18	0.72
rs10860869	<i>IGF1</i>	intron 2	T	0.26	8,248 / 17,836	0.97 (0.93-1.02)	0.32	0.41
rs1019731	<i>IGF1</i>	intron 2	T	0.17	8,120 / 17,722	1.03 (0.97-1.10)	0.36	0.95
rs10778176	<i>IGF1</i>	intron 2	T	0.26	8,223 / 17,812	0.97 (0.93-1.02)	0.31	0.42
rs7956547	<i>IGF1</i>	intron 2	C	0.24	9,100 / 19,602	1.00 (0.95-1.05)	0.97	0.37
rs5742629	<i>IGF1</i>	intron 2	G	0.27	8,187 / 17,757	0.98 (0.93-1.03)	0.37	0.40
rs2033178	<i>IGF1</i>	intron 2	T	0.05	8,209 / 17,816	0.97 (0.89-1.06)	0.55	0.65
rs972936	<i>IGF1</i>	intron 2	A	0.23	8,245 / 17,839	0.96 (0.91-1.01)	0.11	0.43
rs5742678	<i>IGF1</i>	intron 2	G	0.23	8,251 / 17,839	0.96 (0.91-1.01)	0.13	0.42
rs5009837	<i>IGF1</i>	intron 3	T	0.25	8,249 / 17,833	0.97 (0.92-1.01)	0.17	0.40
rs17727841	<i>IGF1</i>	intron 3	C	0.16	8,227 / 17,828	0.98 (0.92-1.04)	0.45	0.71
rs9308315	<i>IGF1</i>	intron 3	A	0.23	8,181 / 17,797	0.96 (0.91-1.01)	0.15	0.56
rs978458	<i>IGF1</i>	intron 3	A	0.23	8,247 / 17,834	0.96 (0.91-1.01)	0.13	0.53
rs5742694	<i>IGF1</i>	intron 3	G	0.19	7,798 / 11,129	0.96 (0.91-1.02)	0.16	0.28
rs1520220	<i>IGF1</i>	intron 3	G	0.17	9,212 / 19,850	0.98 (0.93-1.04)	0.53	0.10
rs6214	<i>IGF1</i>	3'UTR	A	0.41	6,770 / 16,683	1.01 (0.96-1.06)	0.66	0.78
rs6219	<i>IGF1</i>	3'UTR	A	0.09	9,207 / 19,844	1.01 (0.94-1.08)	0.81	0.36
rs5742714	<i>IGF1</i>	3'UTR	C	0.08	8,160 / 17,758	1.01 (0.93-1.09)	0.90	0.27
rs2946834	<i>IGF1</i>	3'	T	0.31	8,062 / 17,678	0.98 (0.93-1.03)	0.43	0.08
rs2132571	<i>IGFBP3</i>	5'	A	0.32	8,748 / 13,126	1.01 (0.96-1.05)	0.76	0.55
rs2132572	<i>IGFBP3</i>	5'	A	0.23	8,811 / 13,213	0.97 (0.92-1.02)	0.18	0.17
rs2471551	<i>IGFBP3</i>	intron 1	C	0.29	4,741 / 13,432	0.99 (0.91-1.07)	0.71	0.26
rs3110697	<i>IGFBP3</i>	intron 3	A	0.49	4,780 / 13,441	1.00 (0.93-1.06)	0.88	0.54
rs10255707	<i>IGFBP3</i>	intron 3	T	0.17	7,988 / 17,605	0.99 (0.93-1.04)	0.63	0.21
rs2453840	<i>IGFBP3</i>	intron 4	T	0.22	5,848 / 14,603	1.01 (0.94-1.09)	0.70	0.57
rs2453839	<i>IGFBP3</i>	intron 4	C	0.18	9,289 / 19,943	1.01 (0.96-1.07)	0.58	0.35
rs6670	<i>IGFBP3</i>	3'UTR	T	0.22	3,923 / 14,620	1.01 (0.93-1.10)	0.81	7.3 x 10 ⁻³
rs13223993	<i>IGFBP3</i>	3'	A	0.21	9,108 / 19,744	0.96 (0.91-1.00)	0.07	0.03
rs10282088	<i>IGFBP3</i>	3'	A	0.11	8,198 / 17,821	0.98 (0.92-1.03)	0.41	3.2 x 10 ⁻³

*MAFs in italics are based on the EPIC-Norfolk study control participants and all others are based on HapMap CEU samples. †Odds ratios are based on the additive model. OR, odds ratio. CI, confidence interval.

been shown in the context of the metabolic syndrome [42,44]. Although we measured circulating IGFs in samples that were non-fasting and were measured only once at baseline, our study showed the expected associations between age and sex and circulating IGF-I and IGFBP-3 levels and consistent replication of several SNP associations with circulating levels, thus decreasing the likelihood of substantial random measurement error. Furthermore, intra-individual variation in levels of IGFs is low. Indeed, it has been reported in 249 participants with serial measurements (mean interval three years) that circulating levels of IGFs have high levels of within-person correlation ($r > 0.8$) over time [15].

We identified several SNPs at the *IGF1* and *IGFBP3* loci that are associated with circulating IGF-I and IGFBP-3 levels. At the *IGF1* locus, five tSNPs were associated with circulating IGF-I levels. The associations for rs1520220, rs6220 and rs3730204 are consistent with previous reports [37,45,46,47,48,49]. In a multivariate analysis of the *IGF1* tSNPs, we found that rs1520220 and rs3730204 were sufficient to account for the other three tSNP associations with circulating IGF-I levels. tSNP rs3730204 is located in the 3' untranslated region (UTR) of the *IGF1* gene (Figure 1A, thus it is a good functional candidate as it could potentially influence post-transcriptional processing

of *IGF1* mRNA. However, this SNP is a low-frequency variant (MAF 0.02) and, with only two rare homozygotes in our dataset, it is likely that our observed association is inflated. As rs1520220 is located in intronic sequence it is more likely that this SNP is capturing an untyped functional variant elsewhere in the gene.

We found three tSNPs at the *IGFBP3* gene that were associated with circulating IGFBP-3 levels. Again, associations for tSNPs rs2132571 and rs2854744 are consistent with previous reports [37,46,50,51,52,53]. In a multivariate analysis of the three associated *IGFBP3* tSNPs we found that rs2132571 explained the other two tSNP associations with circulating IGFBP-3 levels at this locus in EPIC-Norfolk, although the results from our haplotype analysis suggest that rs2132571 and rs3793345 jointly capture an untyped functional variant with a substantial effect on circulating IGFBP-3 levels (**Table 11**). SNP rs2132571 lies 5' of the *IGFBP3* locus and is moderately correlated with rs2854744 in EPIC-Norfolk (r^2 0.61), thus it may be capturing some residual signal from this SNP that has been shown in cellular expression studies to directly influence promoter activity of the *IGFBP3* gene [52].

Some of the disparities in the associations observed between specific SNPs and IGF-I and IGFBP-3 levels among studies are likely due to sampling variation and subtle differences in LD across these loci among populations. This reflects the fact that we are not directly assessing the causal variant(s) underlying these associations, perhaps with the exception of rs2854744 that shows very reproducible associations across multiple studies and ethnic groups and has evidence to support its functionality. Cellular expression studies will be required to assess the functionality of other associated tSNPs at both of these loci.

To our knowledge, our study is the first to assess the risk between common SNPs at the *IGF1* and *IGFBP3* loci with risk of CAD in a large prospective study. The strengths of our study are its size, and that we used a systematic tagging approach to capture all common SNPs (MAF \geq 0.05) at the *IGF1* and *IGFBP3* loci. However, it is possible that there are rare variants at these loci that we have not captured in our current analysis and we may not have had sufficient statistical power to detect modest SNP

associations with CAD risk. An assessment of the coverage between our SNPs and those in HapMap suggests that, at an r^2 threshold of 0.8, we captured 45% and 66% of the variation above MAF 1% in *IGF1* and *IGFBP3* respectively spanning +/- 10 kb of the loci, although this will be an underestimation because several of our tSNPs are not in HapMap. We may also have missed some additional associations between SNPs and circulating levels due to exclusion of three tSNPs that deviated from HWE in our samples and six SNPs which failed assay design. We did not genotype the microsatellite polymorphism in the promoter region of the *IGF1* gene that has been reported to be associated with circulating IGF-I levels and risk of myocardial infarction, although these associations are inconsistent [53,54,55,56,57]. A recent paper by Dupuis et al has identified an association between SNP rs35767 and glycaemic traits, with the G allele associated with an adverse glycaemic profile [58]. Although the association between this SNP and circulating IGF-I levels was not statistically significant in our study, the corresponding allele (coded as C in our data) does show a trend with lower IGF-I levels (**Table 8**), consistent with these findings. Two recent reports by Palles et al and Schumacher et al have found a similar association between SNP rs35767 and IGF-I levels [59,60], so our lack of statistical association may have been simply due to insufficient sample size or variation among populations as discussed above. We did not find any evidence for association with SNP rs35767 and CAD risk in around 9,000 cases and 20,000 controls (**Table 12**), but given the small magnitude of the associations with glycaemic traits in Dupuis et al, it is possible that there might be a weak association with CAD that could be detected with a larger sample. Palles et al have also identified three SNPs that lie approximately 70 kb 5' to the *IGF1* gene that are associated with circulating IGF-I levels [59]. These SNPs lie near to a putative transcription factor binding site that regulates *IGF1* gene expression [59], thus there are likely to be additional variants both *cis* and *trans* that might affect the expression of IGFs that will need to be characterised by further resequencing and replication efforts. However, the comprehensive SNP tagging approach that we have used in our analysis is likely to have captured the majority of the common variation at the *IGF1* and *IGFBP3* loci that will be relevant to the general population.

In summary, our results suggest that circulating levels of insulin-like growth factor-I (IGF-I), its major binding protein IGF-binding protein 3 (IGFBP-3) and genetic variants at the loci encoding these proteins do not influence the risk of CAD.

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Conflicts of Interest: None

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