Identification of a novel proinsulin-associated SNP and demonstration that proinsulin is unlikely to be a causal factor in subclinical vascular remodelling using Mendelian randomisation

Citation

Published Version

Permanent link

Terms of Use

Share Your Story

Identification of a novel proinsulin-associated SNP and demonstration that proinsulin is unlikely to be a causal factor in subclinical vascular remodelling using Mendelian randomisation

Rona J. Strawbridge [a, b, *, 2], Angela Silveira [a], Marcel den Hoed [c], Stefan Gustafsson [d], Jian'an Luan [e], Denis Rybin [f], Josée Dupuis [g, h], Ruifang Li-Gao [i], Maryam Kavousi [j], Abbas Dehghan [j, k], Kadri Haljas [l], Jari Lahti [l, m], Jesper R. Gådin [a], Alexandra Bäcklund [a], Ulf de Faire [n, o], Karl Gertow [a], Phillipe Giral [p], Anuj Goel [q, r], Steve E. Humphries [s], Sudhir Kurl [t], Claudia Langenberg [e], Lars L. Lannfelt [u], Lars Lind [e, v], Cecilia C.M. Lindgren [q], Elmo Mannarino [w], Dennis O. Mook-Kanamori [i, x], Andrew P. Morris [q, y], Renée de Mutsert [i], Rainer Rauramaa [z, aa], Peter Saliba-Gustafsson [a], Bengt Sennblad [a, ab], Andries J. Smit [ac, ad], Ann-Christine Syvänen [ae], Elena Tremoli [af, ag], Fabrizio Veglia [ag], Björn Zethelius [ah], Hanna M. Björck [a], Johan G. Eriksson [ai, aj, ak], Albert Hofman [j, al], Oscar H. Franco [j], Hugh Watkins [q, r], J. Wouter Jukema [am, an], Jose C. Florez [ao, ap, aq], Nicholas J. Wareham [e], James B. Meigs [ap, aq, ar], Erik Ingelsson [d, as], Damiano Baldassarre [af, at], Anders Hamsten [a], on behalf of the IMPROVE study group[1]

[a] Cardiovascular Medicine Unit, Department of Medicine Solna, Karolinska Institutet, Stockholm, Sweden
[b] Mental Health and Wellbeing, Institute of Mental Health and Wellbeing, University of Glasgow, Glasgow, UK
[c] Department of Immunology, Genetics and Pathology and Science for Life Laboratory, Uppsala University, Uppsala, Sweden
[d] Department of Medical Sciences, Molecular Epidemiology and Science for Life Laboratory, Uppsala University, Uppsala, Sweden
[e] MRC Epidemiology Unit, University of Cambridge School of Clinical Medicine, Cambridge, UK
[f] Boston University Data Coordinating Center, Boston, MA, USA
[g] Department of Biostatistics, Boston University School of Public Health, Boston, MA, USA
[h] National Heart, Lung, and Blood Institute's Framingham Heart Study, Framingham, MA, USA
[i] Department of Clinical Epidemiology, Leiden University Medical Center, Leiden, The Netherlands
[j] Department of Epidemiology, Erasmus MC, University Medical Center Rotterdam, The Netherlands
[k] MRC-PHE Centre for Environment and Health, School of Public Health, Imperial College London, UK
[l] Department of Psychology and Logopedics, Faculty of Medicine, University of Helsinki, Helsinki, Finland
[m] Folkhälsan Research Centre, Helsinki, Finland
[n] Division of Cardiovascular Epidemiology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden
[o] Department of Cardiology, Karolinska University Hospital, Stockholm, Sweden
[p] Assistance Publique—Hôpitaux de Paris, Service Endocrinologie-Métabolisme, Groupe Hospitalier Pitié-Salpétrière, Unités de Prévention Cardiovasculaire, Paris, France
[q] Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, UK
[r] Division of Cardiovascular Medicine, Radcliffe Department of Medicine, University of Oxford, Oxford, UK
[s] Centre for Cardiovascular Genetics, Institute Cardiovascular Science, University College London, UK
[t] Institute of Public Health and Clinical Nutrition, University of Eastern Finland, Kuopio Campus, Kuopio, Finland
[u] Department of Public Health and Caring Sciences, Geriatrics, Molecular Geriatrics, Uppsala University, Uppsala, Sweden
[v] Department of Medical Sciences, Uppsala University, Uppsala, Sweden
[w] Department of Clinical and Experimental Medicine, Internal Medicine, Angiology and Arteriosclerosis Diseases, University of Perugia, Perugia, Italy
[x] Department of Public Health and Primary Care, Leiden University Medical Center, Leiden, The Netherlands
[y] Department of Biostatistics, University of Liverpool, Liverpool, UK
[z] Foundation for Research in Health Exercise and Nutrition, Kuopio Research Institute of Exercise Medicine, Kuopio, Finland
[aa] Department of Clinical Physiology and Nuclear Medicine, Kuopio University Hospital, Kuopio, Finland
[ab] Dept of Cell and Molecular Biology, National Bioinformatics Infrastructure Sweden, Science for Life Laboratory, Uppsala University, Sweden
[ac] Department of Medicine, University Medical Center Groningen, Groningen, The Netherlands
[ad] Department of Medicine, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

[1] Additional members of the IMPROVE study group are listed in the Supplementary Data.
[2] Present address: Department of Health and Wellbeing, University of Glasgow, 1 Lilybank Gardens, G12 8RZ.
* Corresponding author. L8:03, Centre for Molecular Medicine, Karolinska Universitetssjukhuset Solna, Stockholm, 171 76, Sweden.

[ae] *Department of Medical Sciences, Molecular Medicine and Science for Life Laboratory, Uppsala University, Uppsala, Sweden*
[af] *Dipartimento di Scienze Farmacologiche e Biomolecolari, Università di Milano, Milan, Italy*
[ag] *Centro Cardiologico Monzino, IRCCS, Milan, Italy*
[ah] *Department of Public Health and Caring Sciences/Geriatrics, Uppsala University, Uppsala, Sweden*
[ai] *Department of General Practice and Primary Health Care, University of Helsinki and Helsinki University Hospital, Helsinki, Finland*
[aj] *National Institute for Health and Welfare, Finland*
[ak] *Folkhälsan Research Center, Helsinki, Finland*
[al] *Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, MA, USA*
[am] *Department of Cardiology, Leiden University Medical Center, Leiden, The Netherlands*
[an] *Interuniversity Cardiology Institute of the Netherlands, Utrecht, The Netherlands*
[ao] *Diabetes Unit and Center for Genomic Medicine, Massachusetts General Hospital, Boston, MA, USA*
[ap] *Programs in Metabolism and Medical & Population Genetics, Broad Institute, Cambridge, MA, USA*
[aq] *Department of Medicine, Harvard Medical School, Cambridge, MA, USA*
[ar] *General Medicine Division, Massachusetts General Hospital, Boston, MA, USA*
[as] *Department of Medicine, Division of Cardiovascular Medicine, Stanford University School of Medicine, Stanford, CA, USA*
[at] *Department of Medical Biotechnology and Translational Medicine, Università degli Studi di Milano, Italy*

ARTICLE INFO

ABSTRACT

Background and aims: Increased proinsulin relative to insulin levels have been associated with sub-clinical atherosclerosis (measured by carotid intima-media thickness (cIMT)) and are predictive of future cardiovascular disease (CVD), independently of established risk factors. The mechanisms linking proinsulin to atherosclerosis and CVD are unclear. A genome-wide meta-analysis has identified nine loci associated with circulating proinsulin levels. Using proinsulin-associated SNPs, we set out to use a Mendelian randomisation approach to test the hypothesis that proinsulin plays a causal role in sub-clinical vascular remodelling.
Methods: We studied the high CVD-risk IMPROVE cohort (n = 3345), which has detailed biochemical phenotyping and repeated, state-of-the-art, high-resolution carotid ultrasound examinations. Geno-typing was performed using Illumina Cardio-Metabo and Immuno arrays, which include reported proinsulin-associated loci. Participants with type 2 diabetes (n = 904) were omitted from the analysis. Linear regression was used to identify proinsulin-associated genetic variants.
Results: We identified a proinsulin locus on chromosome 15 (rs8029765) and replicated it in data from 20,003 additional individuals. An 11-SNP score, including the previously identified and the chromosome 15 proinsulin-associated loci, was significantly and negatively associated with baseline IMT_{mean} and IMT_{max} (the primary cIMT phenotypes) but not with progression measures. However, MR-Eggers refuted any significant effect of the proinsulin-associated 11-SNP score, and a non-pleiotropic SNP score of three variants (including rs8029765) demonstrated no effect on baseline or progression cIMT measures.
Conclusions: We identified a novel proinsulin-associated locus and demonstrated that whilst proinsulin levels are associated with cIMT measures, proinsulin *per se* is unlikely to have a causative effect on cIMT.
© 2017 The Authors. Published by Elsevier Ireland Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Hyperinsulinemia, most often a result of systemic insulin resistance, is associated with increased risk of cardiovascular disease (CVD) and severity of coronary artery disease (CAD) [1]. Under normo-glycemic conditions, proinsulin is fully processed to insulin and C-peptide before secretion, with very low concentrations of proinsulin being detectable in the blood. During hyperinsulinemia, increased demand for insulin production results in pancreatic β-cells failing to correctly process proinsulin to mature insulin [2]. Thus, circulating levels of proinsulin are increased (relative to in-sulin levels) and this might be a link between insulin resistance and CVD. In support of this hypothesis, anti-diabetic agents which modulate glucose levels without increasing insulin secretion (for example SGLT2-inhibitors) have shown cardiovascular benefits, whilst those which stimulate insulin (and potentially proinsulin) secretion, such as sulfonylureas, have shown no CVD benefits and in some studies, increased CVD risk [3]. Furthermore, subclinical changes in the wall of the carotid artery, measured by carotid intima-media thickness (cIMT), have been shown to correlate positively with proinsulin levels in healthy individuals [4] and increased proinsulin levels have been demonstrated to predict future CVD, independently of established risk factors [5–9].

However, it remains to be determined whether increased proin-sulin levels merely reflect CVD risk-related processes, or whether proinsulin has a direct adverse functional effect on the vasculature.

Genetic predisposition to increased proinsulin levels [10] pre-viously demonstrated no effect on CVD events, however we pro-posed that increased proinsulin levels could be important in earlier stages of CVD development, such as those reflected by cIMT.

The aims of this project were firstly to explore the existence of additional proinsulin-associated variants using a large panel of candidate loci for metabolic or inflammatory processes and sec-ondly to use MR to assess causality of proinsulin on early-stage atherosclerosis, represented by cIMT. The IMPROVE cohort is uniquely positioned to address the second aim, having extensive genotyping, circulating proinsulin and insulin levels and detailed cIMT measurements in 2441 participants without type 2 diabetes (T2D). Demonstration that proinsulin has a causal role in cIMT would highlight insulin resistance as a target for CVD prevention. As insulin sensitisation by dietary, lifestyle and pharmaceutical interventions is possible, the role of proinsulin in CVD warrants further investigation.

2. Materials and methods

2.1. Cohort description: IMPROVE

The IMPROVE study recruitment and ultrasound protocol have been described in detail [11,12]. In short, IMPROVE consists of participants without symptoms or history of CVD at enrolment, who presented with at least three classical risk factors for CVD (including family history of CVD, diabetes or impaired fasting glucose, hypoalphalipoproteinemia, hypertension, hyper-triglyceridemia, hypercholesterolemia, current smoking [11, 12]). Between March 2004 and April 2005, 3711 participants were recruited from seven centres in five European countries (Finland, Sweden, the Netherlands, France and Italy). Extensive ultrasound examinations of cIMT were carried out at baseline and after 15 and 30 months, with linear regression of cIMT over time being used to calculate cIMT progression variables [11,12]. Primary cIMT phenotypes used in the present study were the baseline mean and maximum of the common carotid artery intima-media thickness (IMT_{mean} and IMT_{max}, respectively) [11,12], as only these baseline measures would be available in replication cohorts. Secondary analyses were conducted on progression of IMT_{mean} and IMT_{max} as well as baseline and progression measures of additional segments (Supplementary Data) [11,12]. At baseline, a structured medical history and lifestyle questionnaire was completed and blood was sampled. Standard clinical and biochemical phenotyping was performed. Participants with T2D (n = 904, defined as having been diagnosed or treated for diabetes, or fasting glucose ≥ 7 mmol/L) were excluded from all analyses. Fasting intact plasma proinsulin was measured with a sandwich enzyme-linked immunosorbent assay (DRG Instruments GmbH, Marburg, Germany, Supplementary Data). Fasting plasma insulin was analysed by electro-chemiluminescence immunoassay (Meso Scale Discovery, Gaithersburg, MD, USA, Supplementary Data). Cohort characteristics are described in Supplementary Table 1.

2.2. Genotyping in IMPROVE

IMPROVE has been genotyped using both the Illumina CardioMetabo 200k [13] and Immunochip 200k [14] bead array platforms. Single nucleotide polymorphisms (SNPs) were excluded for low call rate (<95%), low minor allele frequency (<0.01) and for deviation from Hardy-Weinberg equilibrium ($p < 1*10-6$). Participants were excluded due to low call rate (<95%), aberrant sex assignment or cryptic relatedness (IBD >0.2). Multi-dimensional scaling (MDS) components 1—3 were calculated in PLINK [15] to enable adjustment for population structure. After quality control 2441 participants without T2D and 254,756 SNPs with minor allele frequency >1% were included in the analysis.

2.3. Identification of proinsulin-associated SNPs in IMPROVE

To identify proinsulin-associated variants [10], 254,756 SNPs were analysed for influence on proinsulin levels. This also enabled assessment of the reported [10] proinsulin-associated loci. The reported lead [10], the analysed proxy or the strongest SNP for each locus are presented in Supplementary Table 2.

Levels of proinsulin, insulin and glucose were natural log normalised prior to analysis. Linear regression, assuming an additive genetic model, was used to analyse the effect of SNPs on proinsulin levels. To enable comparison of SNP effects with the proinsulin genome-wide association study (GWAS) [10], the same three models explored in that paper were tested: insulin model (age, sex, population structure and insulin), glucose model (age, sex, population structure, insulin and glucose) and body-mass index (BMI) model (age, sex, population structure, insulin and BMI). Loci with $p < 1 \times 10^{-5}$ (suggestive evidence of association) in any model were selected for replication. Of note, IMPROVE was not part of the prior proinsulin GWAS [10].

2.4. Replication of proinsulin-associated SNPs

Replication of proinsulin-associated loci was attempted in seven independent cohorts (Fenland (http://www.mrc-epid.cam.ac.uk/Research/Studies/Fenland), FHS [16—18], HBCS [19], PIVUS [20,21], ULSAM [22,23], PROCARDIS cases [24] and PROCARDIS controls [24], n = 18,773—20,003 participants). Replication cohorts are described in the Online Data Supplement and cohort characteristics are presented in Supplementary Table 3. The same analyses and models as the discovery analysis were applied. To improve power and examine consistency of effects, replication results were combined in an inverse variance meta-analysis, using GWAMA [25].

2.5. Expression quantitative trait locus analysis

To further characterize the proinsulin-associated locus on chromosome 15 (rs8029765), genotype-specific gene expression patterns were assessed. This was conducted in liver biopsies from participants undergoing elective surgery for aortic valve and/or ascending aortic disease in ASAP [26] (Supplementary Data) and biopsies from liver or heart and peripheral blood mononuclear cells from organ donation or autopsy specimens in the GTEx [27] database (Supplementary Data).

2.6. In silico analysis of effect on T2D

Proinsulin-increasing alleles of previously identified SNPs rather surprisingly demonstrated a mixture of positive, negative and null effects on T2D risk [10]. Thus to assess the impact of the novel proinsulin-associated locus on chromosome 15 (rs8029765) on risk of T2D, an in silico lookup was performed in the DIAGRAM consortium data. This study of primarily Europeans included 34,840 T2D cases and 114,981 controls with GWAS or metabochip data and the results were downloaded from the DIAGRAM consortium website (http://diagram-consortium.org/downloads.html).

2.7. Effects of proinsulin on cIMT measures in IMPROVE

All phenotypes were assessed for normality and natural logarithmic transformation was applied where necessary, prior to further statistical analysis. The analysis of proinsulin levels for effect on cIMT measures were adjusted in basic (age, sex and population structure) and extended models (age, sex, population structure, BMI, systolic blood pressure (SBP), high density lipoprotein (HDL), triglycerides (TGs) and current smoking) [28]. As the mean cIMT measure encompasses the maximum cIMT measure, these variables were not considered independent and therefore related, multiple testing was not corrected for and $p < 0.05$ was considered significant. Secondary analyses explored the consistency of effects of proinsulin on other cIMT segments and on analysis of progression measures, where the baseline measure of the same segment was also included as a covariate. Because the secondary analyses were exploratory only, the size and direction of effects, rather than significance, is the focus of these analyses. Analyses were performed in STATA (STATAcorp LP, College Station, Texas, USA).

2.8. Mendelian randomization (MR) to assess causality of proinsulin on cIMT in IMPROVE

In order to be valid instruments for the MR analyses, the proinsulin-associated loci should demonstrate the expected associations with proinsulin levels. Accordingly, the effect size and direction were examined for all proinsulin-associated loci [10]. Previously published results were considered the gold standard (given the much larger sample size in the GWAS (n~20,000) compared with this study) and were thus referred to in further analyses. Only subjects with complete genotyping for the 11 proinsulin-associated SNPs (10 SNPs in 9 reported loci and the chromosome 15 SNP/locus identified here) were included in this analysis (N = 35 excluded).

As individual SNPs had only small effects on proinsulin levels, we calculated proinsulin-associated SNP scores by summing the number of alleles associated with higher proinsulin levels. SNP scores were calculated unweighted (sum of number of proinsulin-increasing alleles) and weighted (sum of the number of alleles multiplied by the effect size of the allele reported in the GWAS [10]). As there was little difference between the variance of proinsulin explained by the unweighted and weighted SNP scores, for simplicity the unweighted scores were used for further analysis. The majority of proinsulin-associated SNPs have also been associated with other metabolic traits of relevance to CVD. This pleiotropy may introduce confounding or explain some of the proinsulin associations with cIMT. To determine whether this was the case, a further SNP score was constructed, including only the SNPs which had no reported metabolic effects (GWAS catalogue, https://www.ebi.ac.uk/gwas/, Supplementary Data). Analyses were adjusted using the basic model (age, sex and population structure). Instrument variable regression, with the proinsulin SNP scores as the instruments, was used to assess causality of proinsulin on cIMT variables and was conducted in STATA (STATAcorp LP, College Station, Texas, USA). Instrument variable analysis assumes that SNPs have no pleiotropic effects. Therefore, Egger's regression (which can be used to assess associations which include pleiotropic SNPs) was also conducted in R (Mendelian randomization package).

2.9. Replication of MR

Replication of the MR experiment was attempted in 12,113 participants from 5 cohorts (2 of which contributed to the SNP-proinsulin replication analysis) with IMT$_{mean}$ and IMT$_{max}$ phenotypes. The replication cohorts are described in the Online Data Supplement and characteristics are presented in Supplementary Table 4. The cIMT measures were broadly comparable with IMPROVE, with multiple measurements of the carotid artery enabling assessment of both the maximum and mean values, both left and right carotid arteries being measured (except in ULSAM), and the 1st cm proximal to the bifurcation being omitted from common carotid artery measurements (except in PIVUS). Meta-analysis of SNP score associations with IMT measures was conducted in R using the rma function in the METAFOR package (https://cran.r-project.org/web/packages/metafor/index.html).

3. Results

3.1. Identification of proinsulin-associated SNPs

Analysis of the combined chip (adjusting for population structure, age, sex, insulin as well as BMI or glucose [10]) identified a total of 16 SNPs in 3 loci reaching the suggestive level of significance for association with proinsulin ($p < 1*10-5$) (Fig. 1 and Supplementary Tables 5 and 6). Consistent with the published proinsulin GWAS

[10], more proinsulin-associated SNPs were identified using the glucose-adjusted model. Most (n = 13) of these SNPs were located in the *ARAP1* locus. This locus was identified previously [10], so this finding was taken to be a positive control and gave us confidence in the other loci identified. Conditional analysis for the two known signals (rs77756118 and rs11603334) in the *ARAP1* locus rendered these SNPs non-significant (Fig. 2). On chromosome 6, rs2323000 and rs6910151 represent the same signal. On chromosome 15 (rs8029765), the signal is located 29 Mb from the *LARP6* and 20 Mb from the *VPS13C* loci. Conditioning on *LARP6* (strongest SNP) and *VPS13C* (reported lead SNP) signals had negligible effects on chromosome 15 signal (Fig. 2 and Supplementary Table 7), which thus appeared to be a distinct signal. The reason that this SNP was not identified in the previous GWAS meta-analysis was most likely its relatively large effect size in IMPROVE (Beta = 0.09), compared with that in the GWAS discovery analysis (Beta = 0.01)[10]. A stronger effect size in the IMPROVE study compared with the GWAS is plausible: We believe proinsulin levels are important in CVD, and the IMPROVE cohort is enriched for CVD risk factors. In contrast, the GWAS included a much wider spectrum of CVD burden. Hence two proinsulin-associated SNPs, rs2323000 and rs8029765, were taken forward to replication.

3.2. Replication of proinsulin-associated SNPs

Replication results of the two proinsulin-associated SNPs from seven independent cohorts were meta-analysed (n = 18,773−20,004). The meta-analysis refuted the chromosome 6 locus association with proinsulin levels (Table 1). However, nominal significance was observed for the minor allele of rs8029765 (chromosome 15) being associated with higher proinsulin levels in most models (insulin model: Beta ± Se 0.013 ± 0.006, p = 0.04 (combined discovery and replication 0.020 ± 0.006, p = 0.001); BMI model: 0.014 ± 0.006, p = 0.03 (combined discovery and replication 0.020 ± 0.006, p = 0.002)). Additionally adjusting for glucose did not materially alter the effect size (0.012 ± 0.006, p = 0.0709 (combined discovery and replication 0.020 ± 0.006, p = 0.003)). The replication meta-analyses indicated that the effect sizes in women were not significantly different from those in men (p = 0.0659), with no evidence of heterogeneity between the strata (I^2 = 0, sex heterogeneity p = 0.80). These data provide evidence for a novel proinsulin-associated locus on chromosome 15, which did not influence risk of T2D (OR 1.00, confidence interval (CI) 0.95−1.06, p = 0.89, n = 51,411 [29]). rs8029765 (and the SNP in high LD, rs4450375, $R^2 > 0.8$, Supplementary Fig. 1) has not previously been reported to be associated with any of the metabolic or cardiovascular traits we studied.

3.3. Expression QTL for chromosome 15 locus

The relevance of the chromosome 15 locus was assessed by examining nearby genes for genotype-specific expression. In the GTEx heart (n = 190), liver (n = 97) or PBMC (n = 338) data, generated from relatively healthy individuals, no significant eQTLs were identified. However, in the ASAP cohort, which is significantly burdened with cardiovascular disease, levels of expression were detectable for eight genes in the chromosome 15 locus: *MAN2A2, HDDC3, UNC45A, RCCD1, PRC1, VPS33B, SV2B, SLCO3A1*. In liver samples (n = 209), rs8029765 was significantly associated with expression of *UNC45A* (Supplementary Fig. 2, $p = 5.6*10^{-3}$) with the proinsulin-increasing allele demonstrating lower expression of *UNC45A*. The unc-45 myosin chaperone A encoded by the *UNC45A* gene is a widely expressed chaperone protein [30].
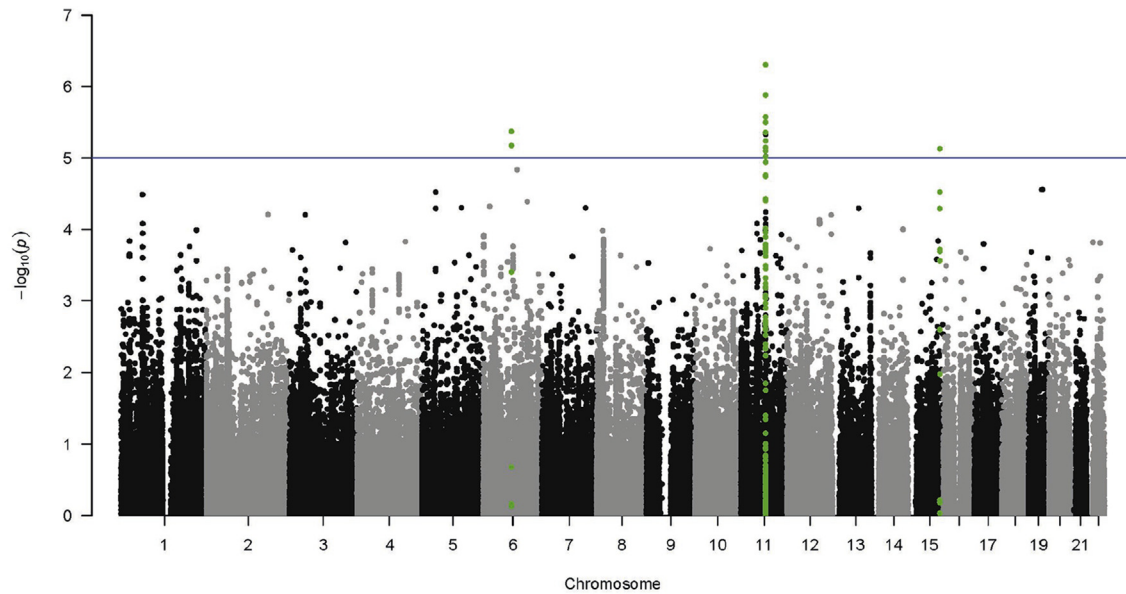
Fig. 1. Manhattan plots of the association between SNPs and proinsulin levels in participants without T2D, adjusted for the glucose model.
SNPs within 250 Kb up or downstream of SNPs with $p < 1*10^{-5}$ are highlighted in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Effects of proinsulin on IMT_{mean} and IMT_{max}

As anticipated, proinsulin levels demonstrated correlations with almost all established risk variables for CVD in the IMPROVE cohort (Supplementary Table 8). For further analysis of IMT measures, models were adjusted for relevant confounders [28]. No statistically significant associations were observed between proinsulin levels and the primary cIMT phenotypes IMT_{mean} and IMT_{max}. Nominal associations ($p < 0.05$) were observed with the IMT_{max} progression, but not baseline measures, independently of established CVD risk factors (Table 2). Secondary analyses indicated that the common carotid (excluding the 1st cm proximal to the bifurcation) demonstrated (non-significant) associations between higher proinsulin levels and larger cIMT measures (Supplementary Table 9). All other segments demonstrated inverse associations.

3.5. Effect of proinsulin-increasing SNP scores on IMT_{mean} and IMT_{max}

Reported proinsulin-associated SNPs demonstrated effects sizes generally comparable with those previously reported [10] (Supplementary Tables 2 and 10), thus were appropriate for use in the MR analysis. A total of 11 proinsulin-associated SNPs were included in the SNP score used for the MR analysis (10 previously reported SNPs in 9 loci and the chromosome 15 SNP, rs8029765). Instrument variable analysis demonstrated that the 11-SNP score was significantly and inversely associated with baseline IMT_{mean} and IMT_{max} but not with progression measures (Table 3). However, MR-Eggers (which is more robust when pleiotropy is present [31]) refuted a significant association between the 11-SNP score and IMT_{mean} (-0.020 ± 0.033 $p = 0.5721$) or IMT_{max} (-0.060 ± 0.054 $p = 0.2738$). As pleiotropic SNPs can bias estimates [31], a further score was constructed, using only non-pleiotropic SNPs (*SNX7* rs1571500, *LARP6* rs7163439 and *UNC45A* rs8029765). This SNP score was not associated with baseline or progression of cIMT measures (Table 3). Replication of the non-pleiotropic SNP score was attempted in 12,113 participants with baseline mean and maximum IMT measures comparable with those in IMPROVE.

Meta-analysis of the effect of the 3-SNP score on baseline IMT_{mean} and IMT_{max} demonstrated no association (Supplementary Fig. 4 and Supplementary Table 11). Analyses of secondary IMT phenotypes demonstrated that no single segment differed from the primary phenotypes analysed (Supplementary Table 12). Proinsulin levels according to SNP score are presented in Supplementary Table 13.

3.6. Mendelian randomization refutes causality of proinsulin on cIMT

Proinsulin levels vary over time with measurement of levels reflecting the exposure of the vasculature to proinsulin at a single point in time. In contrast, the proinsulin-increasing SNP score reflects a lifetime of exposure to proinsulin. For causality to be demonstrated, the direction of effect of the proinsulin-increasing SNP score on cIMT needs to be consistent with the effect of higher levels of proinsulin on cIMT. A further requirement is that the SNPs only act on cIMT through proinsulin levels.

Here, the direction of effect was consistent between proinsulin levels and proinsulin-increasing SNP scores: higher levels of proinsulin were (non-significantly) associated with smaller cIMT measures in IMPROVE and proinsulin-increasing SNP scores were associated with smaller cIMT measures. However, this finding does not imply causality, because when only the non-pleiotropic SNPs were included in the SNP score, no association with cIMT was observed. Taken together, proinsulin *per se* is unlikely to have a causal effect on cIMT.

4. Discussion

It has previously been reported that circulating levels of proinsulin are predictive of CVD [5–9]. To the best of our knowledge, this is the first study to examine whether proinsulin has a causal effect on early vascular processes, such as increased thickness of the carotid artery wall measured by cIMT. In this study we identified a proinsulin-associated locus and used this, and other previously reported proinsulin-associated SNPs [10], in a MR experiment,
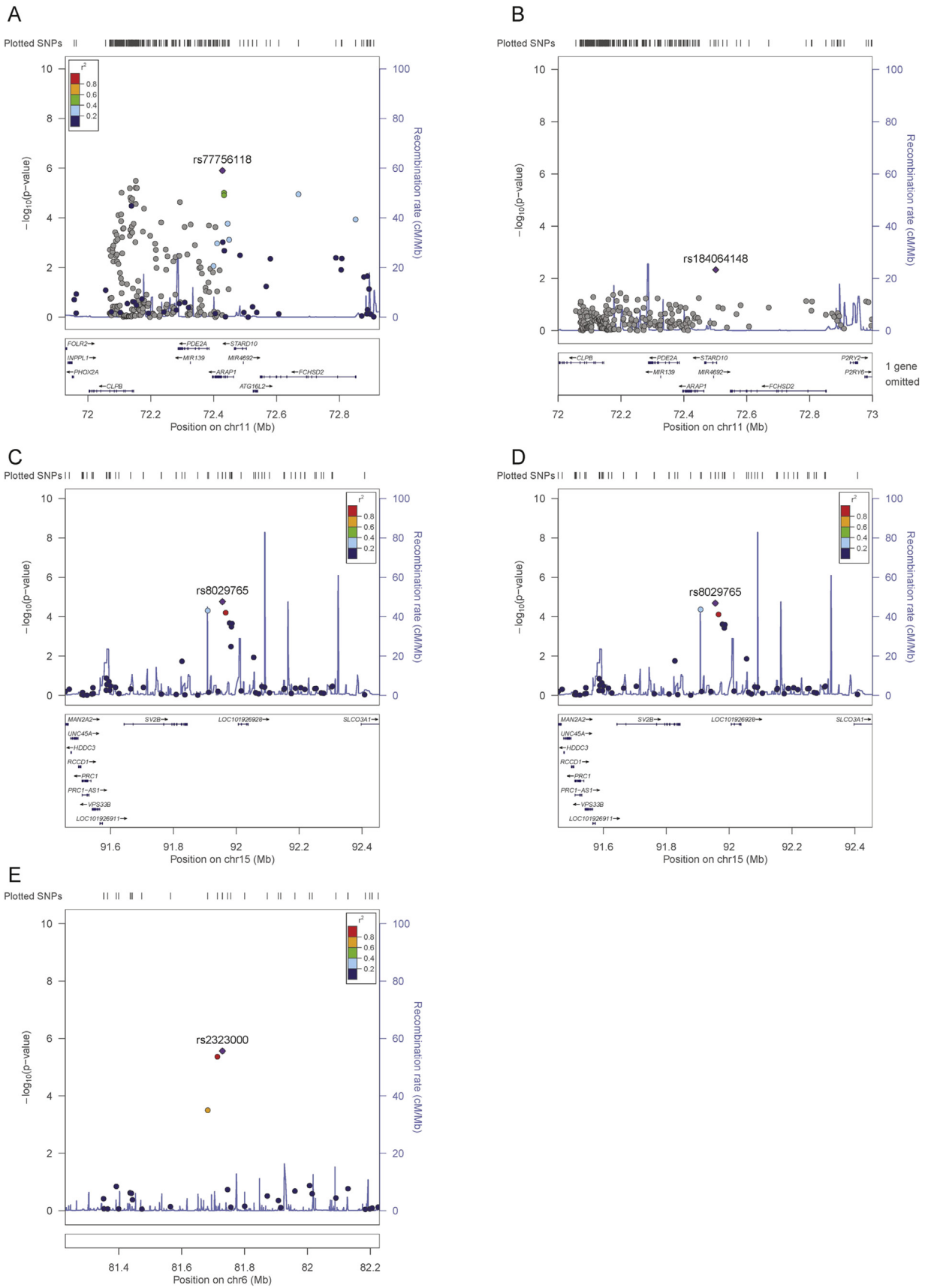
Fig. 2. Regional plots of suggestive loci.
(A) Chromosome 11 locus; (B) chromosome 11 locus after conditioning on the previously reported *ARAP1* loci (rs11603334 and rs77756118); (C) chromosome 15 locus; (D) chromosome 15 locus adjusted for the previously reported, nearby loci (*LARP6* rs7163439 and *VPS13C* rs4502156); (E) chromosome 6 locus.

Table 1
Replication meta-analysis of suggestive novel associations with proinsulin levels.

CHR	SNP	BP	A1	A2	MAF	Insulin model (N^a = 20,003)			BMI model (N^a = 19,986)			Glucose model (N^a = 19,684)			Direction	ISq
						Beta	Se	p	Beta	Se	p	Beta	Se	p		
6	rs2323000	81729597	T	C	0.049	−0.001	0.011	0.9022	0.002	0.011	0.8671	0.000	0.011	0.9727	− + -++	0
15	rs8029765	91955915	G	T	0.178	0.013	0.006	**0.0387**	0.014	0.006	**0.0307**	0.012	0.006	0.0709	-++++++	0

Where: A1 is the minor/effect allele; A2, major/non effect allele; MAF, minor allele frequency; Insulin model, adjusted for age, sex, MDS1-3, insulin; BMI model, adjusted for age, sex, MDS 1–3, insulin, BMI; Glucose model, adjusted for age, sex, MDS1-3, insulin, glucose; Full model, adjusted for age, sex, MDS 1–3, insulin, BMI, smoking, LDL, TGs, glucose. N^a, Smallest N; Direction, direction of effect in each cohort; ISq, heterogeneity I square value. Direction and Isq were the same for all models. Cohorts: PROCARDIS cases, PROCARDIS controls, PIVUS, ULSAM, HBCS, FHS, FENLAND.
Bold indicates significant results.

Table 2
Associations between proinsulin levels and IMT measures in IMPROVE.

		Basic + proinsulin			Extended + proinsulin		
		Beta	Se	p	Beta	Se	p
Baseline	IMTmean	−0.004	0.003	0.180	−0.004	0.003	0.124
	IMTmax	−0.002	0.005	0.668	0.000	0.005	0.977
Progression[a]	IMTmean	0.000	0.001	0.864	0.000	0.001	0.689
	IMTmax	−0.011	0.005	**0.032**	−0.011	0.005	**0.047**

Basic model, MDS1-3, age, sex, insulin; extended model, MDS1-3, age, sex, insulin, BMI, SBP, HDL, TGs, current smoking.
[a] Also adjusted for the baseline measure.

Table 3
Effect of the proinsulin-raising SNP score on IMT phenotypes in IMPROVE.

		Proinsulin SNP score (11 SNPs)		Non-metabolic SNP score (3 SNPs)	
		Beta	p	Beta	p
Baseline	IMTmean	−0.002	**0.045**	−0.002	0.263
	IMTmax	−0.004	**0.027**	−0.005	0.206
Progression[a]	IMTmean	0.000	0.816	0.000	0.583
	IMTmax	0.001	0.755	−0.003	0.538

Basic model, MDS1-3, age, sex, insulin.
[a] Also adjusted for the baseline measure.

which demonstrated that proinsulin is unlikely to be a causal factor in determining cIMT.

The robust proinsulin-associated loci generally had sizes and directions of effect in IMPROVE that were comparable with those previously reported [10], which added support to the validity of the proinsulin-associated SNP, and enabled the use of proinsulin SNP scores in an MR analysis. The locus (lead SNP rs8029765) demonstrated a genotype-specific effect on UNC45A expression, with the allele associated with higher levels of proinsulin being associated with lower expression of UNC45A. Low levels of UNC45A have been shown to inhibit cell proliferation and differentiation whereas high levels are associated with a number of cancers [32]. The chaperone protein encoded by UNC45A is also involved in intra-cellular trafficking, via its association with myosin [32,33]. It is possible that genetically low UNC45A levels could influence proinsulin levels, either via aberrant trafficking of vesicles (leading to release of incompletely processed proinsulin) or as a result of a reduced number of β -cells in the pancreas (leaving the pancreas more vulnerable to damaging stress during insulin resistance and hyperinsulinemia). Five genetic variants in the UNC45A genomic region have been reported in association with metabolic traits (GWAS catalogue, dated 20161017). However, these are all ~500 kb from the chromosome 15 locus and are at most in low linkage disequilibrium with rs8029765 (r^2 < 0.11, Supplementary Fig. 3). In addition, they show no association with proinsulin levels in this

study (smallest p > 0.2411), suggesting that these signals are unrelated. The lack of effect of rs8029765 on T2D is consistent with other proinsulin-associated loci, for example PCSK1, which demonstrated strong effects on proinsulin levels but convincingly null effects on T2D[10].

The MR analyses can be used to demonstrate causality if i) proinsulin levels are associated with cIMT; ii) SNPs demonstrate robust associations with proinsulin; and iii) SNPs influence cIMT only through proinsulin, and not via other mechanisms. Proinsulin levels have been shown to influence cIMT in a previous report from a cross-sectional study of healthy middle-aged men [4]. However, in IMPROVE the association was weak and non-significant. Robust associations between SNPs and proinsulin levels have been reported [10] and were consistent in IMPROVE. Whilst some association was observed between the 11-SNP score and cIMT, adjustment for pleiotropy (using MR-Eggers) or omission of the pleiotropic SNPs (3-SNP score) rendered the associations with cIMT non-significant. These results suggest that associations between proinsulin and cIMT are most likely due to confounding and pleiotropic effects of SNPs. Despite these findings, CVD prevention strategies which aim to reduce insulin resistance are still of value, as insulin resistance promotes other CVD risk factors including obesity and leads to T2D. In addition, proinsulin processing to insulin also releases c-peptide, which negatively correlate with HDL levels [34], therefore reducing hyperinsulinemia could enable increased HDL levels.

A limitation of this study is lack of power to detect an association, with much larger sample sizes (n > 350,000) being required to conclusively address this question. MR analyses are frequently weakened by small effect sizes of genetic variants and inability to collect large enough sample sizes to enable drawing of definitive conclusions. In the absence of sufficient sample sizes with cIMT measures, alternative methodologies would be required to confirm these findings. Metabolic phenotypes are particularly challenging because of the complexity of interlinking mechanisms.

A unique strength of this study is the detailed measurements of the cIMT. The secondary analyses in the present study demonstrated that a positive association between proinsulin and cIMT was limited to only one segment in the carotid tree, which is an interesting and novel observation. However the uniqueness of IMPROVE is also a limiting factor in that there are few available cohorts with comparable cIMT measures, which precludes replication of the cIMT segments considered in the secondary analysis. IMT measures are often rather variable, especially when considering progression over a relatively short time frame. We recognise this limitation, but note that the standard deviations for the studies included in this analysis are not excessive, that there is consistency across the studies and we have focused on the baseline measures and merely comment upon progression data. The weak association between proinsulin and cIMT is a weakness of this study, however there is prior independent evidence for the association between

proinsulin and cIMT [4]. Biomarker measurements reflect a snapshot in time, whereas genetic variants reflect the lifetime exposure to biomarker levels. Thus by using genetic variants robustly associated with proinsulin levels, MR analyses give a more stable lifetime risk assessment. For a genetic discovery analysis, the IMPROVE cohort is relatively small. However, the high CVD-risk profile of all subjects renders it a relevant cohort for association studies of CVD-associated biomarkers. In addition, all participants had at least 3 of 9 established CVD risk factors [12], meaning that the repertoire of potential confounders was very varied across the cohort and thereby limiting the potential for selection bias. Replication of the chromosome 15 proinsulin-associated SNP was conducted in a population of similar size to that utilised in the proinsulin GWAS [10], thus giving confidence to this finding.

In conclusion, we have identified a proinsulin-associated locus and used MR to demonstrate that proinsulin *per se* is unlikely to have a causal effect on cIMT, a proxy measurement for early atherosclerosis.

Conflict of interest

A.D has received consultancy and research support from Metagenics Inc. (outside the scope of submitted work). Metagenics Inc. had no role in design and conduct of the study; collection, management, analysis, and interpretation of the data; or preparation, review, or approval of the manuscript. E.I is an advisor and consultant for Precision Wellness, Inc., and advisor for Cellink for work unrelated to the present project. The other authors have nothing to disclose.

Financial support

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.atherosclerosis.2017.09.031.

References

[1] P. Bavenholm, A. Proudler, P. Tornvall, et al., Insulin, intact and split proinsulin, and coronary artery disease in young men, Circulation 92 (1995) 1422—1429.

[2] M.E. Roder, D. Porte Jr., R.S. Schwartz, et al., Disproportionately elevated proinsulin levels reflect the degree of impaired B cell secretory capacity in patients with noninsulin-dependent diabetes mellitus, J. Clin. Endocrinol. Metab. 83 (1998) 604—608.

[3] F. Paneni, T.F. Luscher, Cardiovascular protection in the treatment of type 2 diabetes: a review of clinical trial results across drug classes, Am. J. Cardiol. 120 (2017) S17—S27.

[4] S. Boquist, G. Ruotolo, R. Tang, et al., Alimentary lipemia, postprandial triglyceride-rich lipoproteins, and common carotid intima-media thickness in healthy, middle-aged men, Circulation 100 (1999) 723—728.

[5] S.E. Kahn, D.L. Leonetti, R.L. Prigeon, et al., Relationship of proinsulin and insulin with noninsulin-dependent diabetes mellitus and coronary heart disease in Japanese-American men: impact of obesity—clinical research center study, J. Clin. Endocrinol. Metab. 80 (1995) 1399—1406.

[6] J.S. Yudkin, M. May, P. Elwood, et al., Concentrations of proinsulin like molecules predict coronary heart disease risk independently of insulin: prospective data from the Caerphilly Study, Diabetologia 45 (2002) 327—336.

[7] J.S. Yudkin, D.I.W. Phillips, S. Stanner, Proteinuria and progressive renal disease: birth weight and microalbuminuria, Nephrol. Dial. Transpl. 12 (1997) 10—13.

[8] B. Lindahl, B. Dinesen, M. Eliasson, et al., High proinsulin concentration precedes acute myocardial infarction in a nondiabetic population, Metabolism 48 (1999) 1197—1202.

[9] B. Zethelius, L. Byberg, C.N. Hales, et al., Proinsulin is an independent predictor of coronary heart disease: report from a 27-year follow-up study, Circulation 105 (2002) 2153—2158.

[10] R.J. Strawbridge, J. Dupuis, I. Prokopenko, et al., Genome-wide association identifies nine common variants associated with fasting proinsulin levels and provides new insights into the pathophysiology of type 2 diabetes, Diabetes 60 (2011) 2624—2634.

[11] D. Baldassarre, A. Hamsten, F. Veglia, et al., Measurements of carotid intima-media thickness and of interadventitia common carotid diameter improve prediction of cardiovascular events: results of the IMPROVE (Carotid Intima Media Thickness [IMT] and IMT-Progression as Predictors of Vascular Events in a High Risk European Population) study, J. Am. Coll. Cardiol. 60 (2012) 1489—1499.

[12] D. Baldassarre, K. Nyyssonen, R. Rauramaa, et al., Cross-sectional analysis of baseline data to identify the major determinants of carotid intima-media thickness in a European population: the IMPROVE study, Eur. Heart J. 31 (2010) 614—622.

[13] B.F. Voight, H.M. Kang, J. Ding, et al., The metabochip, a custom genotyping array for genetic studies of metabolic, cardiovascular, and anthropometric traits, PLoS Genet. 8 (2012) e1002793.

[14] G. Trynka, K.A. Hunt, N.A. Bockett, et al., Dense genotyping identifies and localizes multiple common and rare variant association signals in celiac disease, Nat. Genet. 43 (2011) 1193—1201.

[15] S. Purcell, B. Neale, K. Todd-Brown, et al., PLINK: a tool set for whole-genome association and population-based linkage analyses, Am. J. Hum. Genet. 81 (2007) 559—575.

[16] G.L. Splansky, D. Corey, Q. Yang, et al., The third generation cohort of the national heart, Lung, and blood Institute's Framingham heart study: design, recruitment, and initial examination, Am. J. Epidemiol. 165 (2007) 1328—1335.

[17] M. Feinleib, W.B. Kannel, R.J. Garrison, et al., The Framingham offspring study. Design and preliminary data, Prev. Med. 4 (1975) 518—525.

[18] T.R. Dawber, G.F. Meadors, F.E. Moore Jr., Epidemiological approaches to heart disease: the Framingham Study, Am. J. Public Health Nations Health 41 (1951) 279—281.

[19] J.G. Eriksson, Early growth and coronary heart disease and type 2 diabetes: findings from the Helsinki Birth Cohort Study (HBCS), Am. J. Clin. Nutr. 94 (2011) 1799S—1802S.

[20] J. Andersson, J. Sundstrom, T. Gustavsson, et al., Echogenecity of the carotid intima-media complex is related to cardiovascular risk factors, dyslipidemia, oxidative stress and inflammation: the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study, Atherosclerosis 204 (2009) 612—618.

[21] E. Ingelsson, J. Sundstrom, L. Lind, et al., Low-grade albuminuria and the incidence of heart failure in a community-based cohort of elderly men, Eur. Heart J. 28 (2007) 1739—1745.

[22] H. Hedstrand, A study of middle-aged men with particular reference to risk factors for cardiovascular disease, Ups. J. Med. Sci. Suppl. 19 (1975) 1—61.

[23] M. Wohlin, J. Sundstrom, L. Lannfelt, et al., Apolipoprotein E epsilon4 genotype is independently associated with increased intima-media thickness in a recessive pattern, Lipids 42 (2007) 451—456.

[24] M. Farrall, F.R. Green, J.F. Peden, et al., Genome-wide mapping of susceptibility to coronary artery disease identifies a novel replicated locus on chromosome 17, PLoS Genet. 2 (2006) e72.

[25] R. Magi, A.P. Morris, GWAMA: software for genome-wide association meta-analysis, BMC Bioinforma. 11 (2010) 288.

[26] L. Folkersen, F. van't Hooft, E. Chernogubova, et al., Association of genetic risk variants with expression of proximal genes identifies novel susceptibility genes for cardiovascular disease, Circ. Cardiovasc Genet. 3 (2010) 365—373.

[27] G.T. Consortium, The genotype-tissue expression (GTEx) project, Nat. Genet. 45 (2013) 580—585.

[28] J. Persson, R.J. Strawbridge, O. McLeod, et al., Sex-specific effects of adiponectin on carotid intima-media thickness and incident cardiovascular disease, J. Am. Heart Assoc. 4 (2015) e001853.

[29] A.P. Morris, B.F. Voight, T.M. Teslovich, et al., Large-scale association analysis provides insights into the genetic architecture and pathophysiology of type 2 diabetes, Nat. Genet. 44 (2012) 981—990.

[30] F. Fratev, S. Osk Jonsdottir, I. Pajeva, Structural insight into the UNC-45-myosin complex, Proteins 81 (2013) 1212—1221.

[31] J. Bowden, G. Davey Smith, S. Burgess, Mendelian randomization with invalid instruments: effect estimation and bias detection through Egger regression, Int. J. Epidemiol. 44 (2015) 512—525.

[32] W. Ni, O.O. Odunuga, UCS proteins: chaperones for myosin and co-chaperones for Hsp90, Subcell. Biochem. 78 (2015) 133—152.

[33] Y. Jilani, S. Lu, H. Lei, et al., UNC45A localizes to centrosomes and regulates cancer cell proliferation through ChK1 activation, Cancer Lett. 357 (2015) 114—120.

[34] Y. Li, Y. Li, L. Meng, et al., Association between serum C-peptide as a risk factor for cardiovascular disease and high-density lipoprotein cholesterol levels in nondiabetic individuals, PLoS One 10 (2015) e112281.