

# Genome-Wide Association Identifies Nine Common Variants Associated With Fasting Proinsulin Levels and Provides New Insights Into the Pathophysiology of Type 2 Diabetes

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**OBJECTIVE**—Proinsulin is a precursor of mature insulin and C-peptide. Higher circulating proinsulin levels are associated with impaired  $\beta$ -cell function, raised glucose levels, insulin resistance, and type 2 diabetes (T2D). Studies of the insulin processing pathway could provide new insights about T2D pathophysiology.

**RESEARCH DESIGN AND METHODS**—We have conducted a meta-analysis of genome-wide association tests of  $\sim 2.5$  million genotyped or imputed single nucleotide polymorphisms (SNPs) and fasting proinsulin levels in 10,701 nondiabetic adults of European ancestry, with follow-up of 23 loci in up to 16,378 individuals, using additive genetic models adjusted for age, sex, fasting insulin, and study-specific covariates.

**RESULTS**—Nine SNPs at eight loci were associated with proinsulin levels ( $P < 5 \times 10^{-8}$ ). Two loci (*LARP6* and *SGSM2*) have not been previously related to metabolic traits, one (*MADD*) has been associated with fasting glucose, one (*PCSK1*) has been implicated in obesity, and four (*TCF7L2*, *SLC30A8*, *VPS13C/C2CD4A/B*, and *ARAP1*, formerly *CENTD2*) increase T2D risk. The proinsulin-raising allele of *ARAP1* was associated with a lower fasting glucose ( $P = 1.7 \times 10^{-4}$ ), improved  $\beta$ -cell function ( $P = 1.1 \times 10^{-5}$ ), and lower risk of T2D (odds ratio 0.88;  $P = 7.8 \times 10^{-6}$ ). Notably, *PCSK1* encodes the protein prohormone convertase 1/3, the first enzyme in the insulin processing pathway. A genotype score composed of the nine proinsulin-raising alleles was not associated with coronary disease in two large case-control datasets.

**CONCLUSIONS**—We have identified nine genetic variants associated with fasting proinsulin. Our findings illuminate the biology underlying glucose homeostasis and T2D development in humans and argue against a direct role of proinsulin in coronary artery disease pathogenesis.

**G**enome-wide association studies (GWAS) have uncovered dozens of common genetic variants associated with risk for type 2 diabetes (T2D; reviewed in [1]). Known associated variants in these loci account for only a small proportion of the heritable component of T2D (1), suggesting that additional loci await discovery. The Meta-Analyses of Glucose and Insulin-related traits Consortium (MAGIC) was created under the premise that genome-wide analysis of continuous diabetes-related traits could not only identify loci regulating variation in these glycemic traits, but also yield additional T2D susceptibility loci and insights into the underlying physiology of these loci (2–5). In addition, the genetic study of T2D endophenotypes may help clarify the pathophysiologic heterogeneity of this disease by elucidating the respective roles of  $\beta$ -cell function, insulin secretion, processing and sensitivity, and glucose metabolism (6).

Discovery of novel genetic determinants of insulin secretion and action has primarily focused on insulin levels (3,4,7,8). Proinsulin is the molecular precursor for insulin and has relatively low insulin-like activity, and its enzymatic conversion into mature insulin and C-peptide is a critical step in insulin production and secretion (Supplementary Fig. 1). Although hyperinsulinemia typically denotes insulin resistance, high proinsulin in relation to circulating levels of mature insulin can indicate  $\beta$ -cell stress as a result of insulin resistance, impaired  $\beta$ -cell function, and/or insulin processing and secretion abnormalities (9) (Supplementary Fig. 2). There is good evidence that higher proinsulin predicts future T2D (10) and coronary artery disease (CAD) (11–13), even after taking fasting glucose levels into account. Interestingly, some loci previously associated with fasting glucose levels (*MADD*) or risk of T2D (*TCF7L2*, *SLC30A8*, *CDKAL1*) are also associated with higher circulating proinsulin (6,14–17). Therefore, genome-wide analysis of proinsulin levels could reveal additional novel loci increasing susceptibility for T2D and perhaps CAD.

Thus, to identify novel loci influencing proinsulin processing and secretion and potentially increasing susceptibility for T2D, we performed a meta-analysis of  $\sim 2.5$  million directly genotyped or imputed autosomal single nucleotide polymorphisms (SNPs) from four GWAS of fasting proinsulin levels (adjusted for concomitant fasting insulin) including 10,701 nondiabetic adult men and women of European descent. Follow-up of 23 lead SNPs from the most significant association signals in up to 16,378 additional individuals of European ancestry detected nine genome-wide significant associations with proinsulin levels, including two novel signals in or near *LARP6* and *SGSM2*, and the known glycemic loci *ARAP1*, *MADD* (two independent signals), *TCF7L2*, *VPS13C/C2CD4A/B*, *SLC30A8*, and *PCSK1*. Here we describe these genetic associations, perform fine-mapping to identify potential causal variants, assess gene expression in human tissues, and define their impact on other glycemic quantitative traits and risk of both T2D and CAD.

## RESEARCH DESIGN AND METHODS

**Cohort/study description.** Four cohorts contributed to the discovery meta-analysis through the contribution of phenotypic and GWAS data. These included the Framingham Heart Study ( $n = 5,759$ ), PROCARDIS ( $n = 3,259$ ), the Fenland study ( $n = 1,372$ ), and the Diabetes Genetics Initiative (DGI;  $n = 311$ ), for a total of 10,701 participants. Eleven cohorts contributed to the follow-up efforts; these included METSIM ( $n = 5,122$ ), Botnia-PPP ( $n = 2,280$ ), HBCS ( $n = 1,649$ ), the Ely study ( $n = 1,568$ ), the Hertfordshire study ( $n = 1,016$ ), ULSAM ( $n = 939$ ), RISC ( $n = 914$ ), PIVUS ( $n = 912$ ), Segovia ( $n = 911$ ), GHRAS ( $n = 668$ ), and SDPP ( $n = 399$ ), for a total of 16,378 participants (with maximal sample for any one SNP of 15,898). We excluded individuals with known

diabetes, on antidiabetic treatment, or with fasting glucose  $\geq 7$  mmol/L (3); all participants were of European descent.

**Proinsulin and insulin measurements.** Proinsulin (in pmol/L) was measured from fasting whole blood, plasma, or serum or a combination of these using enzyme-linked immunosorbent or immunometric assays. Fasting insulin (pmol/L) was measured using either enzyme-linked immunosorbent, immunofluorescent, or radioimmunoassays (Supplementary Table 1).

**Genotyping.** Genome-wide commercial arrays (Affymetrix 500K, MIPS 50K, and Illumina Human1M/610K) were used by the four discovery cohorts as described in Supplementary Table 1. Imputation and quality control methods are described in the Supplementary Data.

**Statistical analyses.** We aimed to identify genetic variants associated with high proinsulin levels relative to an individual's fasting insulin levels. This can be done by examining proinsulin-to-insulin ratios or by statistically adjusting proinsulin for fasting insulin. We chose the latter because the adjusted trait has comparable predictive value (18) and displayed better statistical performance in pilot studies and adequate heritability in the Framingham Heart Study, one of the larger cohorts examined here ( $h^2 = 0.36$  vs. 0.34 for the proinsulin-to-insulin ratio). In Framingham, correlation between the adjusted trait and the ratio was 0.95, and the quantile-quantile GWAS plots were comparable.

We used a linear regression model with natural log transformed fasting proinsulin as the dependent variable and genotypes as predictors, with adjustment for natural-log transformed fasting insulin values, sex, age, geographical covariates (if applicable), and age squared (Framingham only) to evaluate the association under an additive genetic model. Association analysis was performed by individual studies using SNPTEST (19), STATA (20), PLINK (21), or LMEKIN (R kinship package) software (22). Genome-wide association inflation coefficients were estimated for each discovery cohort using the genomic control (GC) method (23) and applied subsequently to each individual SNP association test statistics to correct for cryptic relatedness. The  $\lambda$  GC value for the final meta-analysis of proinsulin adjusted for fasting insulin was

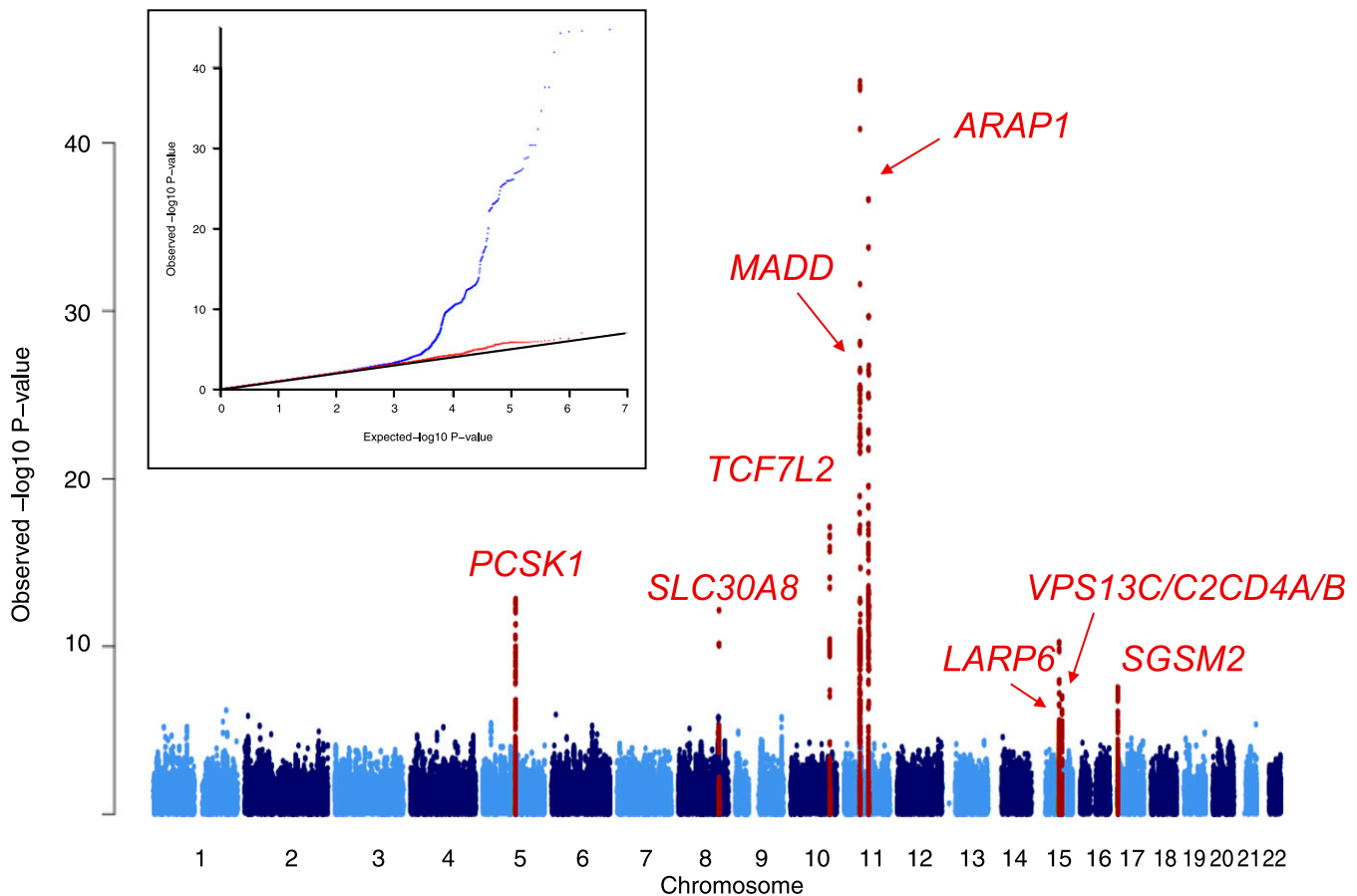
1.01. The inverse-variance fixed effects meta-analysis method was used to evaluate the pooled regression estimates for additively coded SNPs using METAL (24). Sex interaction effects were evaluated with a function in the GWAMA software (25).

**Follow-up SNP selection and analysis.** We carried forward to stage 2 the most significant SNP from each of 21 independent loci that showed association with proinsulin in stage 1 analyses at  $P < 1 \times 10^{-5}$ . Additionally, two SNPs near the  $P < 1 \times 10^{-5}$  threshold (in *ASAP2* and a gene desert region) were carried forward as a result of biological plausibility (*ASAP2* is involved in vesicular transport) and/or consistency of direction of effect in all discovery stage 1 studies (both loci). We genotyped these 23 variants in 11 additional stage 2 studies totaling 16,378 nondiabetic participants of European ancestry (Supplementary Table 1; genotyping assays and conditions are available upon request). We meta-analyzed stage 1 and stage 2 results using inverse-variance weighted fixed effects meta-analysis methods including up to 27,079 participants.

Additional analyses and expression/eQTL studies are described in the Supplementary Material.

## RESULTS

**Genome wide association meta-analysis (stage 1).** We conducted a two-stage association study in individuals of European descent (total  $n = 27,079$ , with  $n = 10,701$  in the discovery stage). Cohort and phenotype information can be found in Supplementary Table 1, and the study design is outlined in Supplementary Fig. 3. A total of 21 independent variants (including two SNPs identified during conditional analyses, see below) met our statistical threshold for follow-up ( $P < 1 \times 10^{-5}$ ; Fig. 1). The clean dataset showed no



**FIG. 1.** Manhattan plot of the association  $P$  values for fasting proinsulin adjusted for fasting insulin. Directly genotyped and imputed SNPs are plotted with their meta-analysis  $P$  values (as  $-\log_{10}$  values) as a function of genomic position (NCBI Build 36). The SNPs that achieved genome-wide significance ( $P < 5 \times 10^{-8}$ ) on follow-up are shown in red. *Insert:* Quantile-quantile (Q-Q) plot for fasting proinsulin adjusted for fasting insulin. The expected null distribution is plotted along the diagonal, the entire distribution of observed  $P$  values is plotted in blue, and a distribution that excludes the nine novel findings is plotted in red.

systematic deviation from the null expectation, with the exception of the tail of the distribution (Fig. 1, insert).

**Follow-up studies (stage 2) and global (stage 1 + stage 2) meta-analysis for 23 loci.** We followed up 23 SNPs (the 21 mentioned above plus 2 others that approached our significance threshold and were selected as a result of biological plausibility; see RESEARCH DESIGN AND METHODS) in 11 cohorts totaling up to 16,378 nondiabetic individuals of European descent (Table 1 and Supplementary Table 2). Joint meta-analysis of discovery and follow-up cohorts ( $n = 27,079$ ) revealed nine signals at eight loci reaching genome-wide significance ( $P < 5 \times 10^{-8}$ ), of which two are novel (*SGSM2*, *LARP6*), five have previously been associated with glucose metabolism and/or T2D (*TCF7L2*, *SLC30A8*, *MADD*, *VPS13C/C2CD4A/B*, and *ARAP1*), and one (*PCSK1*) has been previously implicated in obesity and associated with proinsulin levels, although not at genome-wide significance (Table 1 and Fig. 2). Adjusting for BMI, fasting glucose, or both did not attenuate these signals. Of note, when adjusting for fasting glucose or both fasting glucose and BMI (but not BMI alone), one other locus, *SNX7*, reached genome-wide significance ( $P = 5.4 \times 10^{-9}$  and  $1.5 \times 10^{-8}$ , respectively).

Conditional analyses on the two strongest signals revealed that the *MADD* locus harbors two independent signals 19 kb apart (rs10501320 and rs10838687;  $r^2 = 0.068$  in HapMap CEU), whereas a second independent signal near *ARAP1* did not replicate (Fig. 2B, Table 1, and Supplementary Table 2). Among the nine replicated SNPs, individual loci explained between 0.2 and 1.4% of the variance in proinsulin in the discovery samples and up to 2.3% of the variance in the follow-up samples. Together, the nine genome-wide significant SNPs explained between 5.4 and 7.7% of the proinsulin variance in the discovery samples and 8.1% of the variance in the RISC cohort, one of the few follow-up cohorts with genotypes available for all nine SNPs.

**Heterogeneity and sex-stratified analyses.** We noted some degree of heterogeneity in our joint meta-analyses (Table 1). Part of the heterogeneity arose from the METSIM sample, which enrolled only men; exclusion of this cohort from our meta-analysis reduced the heterogeneity. We also stratified our analyses by sex and tested for a SNP  $\times$  sex interaction (26). Our overall findings remained essentially unchanged after sex stratification, and heterogeneity was attenuated (e.g.,  $I^2 = 77.2\%$ , heterogeneity  $P = 1.9 \times 10^{-7}$  for combined men and women, whereas  $I^2 = 64.6\%$ , heterogeneity  $P = 4.5 \times 10^{-4}$  [men] and  $I^2 = 55.6\%$ , heterogeneity  $P = 0.01$  [women] in stratified analyses). Furthermore, tests for interaction with sex among SNPs that reached our follow-up significance threshold revealed a locus (rs306549 in *DDX31*) where a genome-wide significant association was seen in women ( $P = 2.0 \times 10^{-8}$ ; Supplementary Fig. 4A) but not men ( $P = 0.17$ ; Supplementary Fig. 4B; sex interaction  $P = 8.9 \times 10^{-5}$ ). Although removal of the METSIM cohort improved the heterogeneity score and produced nominal significance for the association in men ( $P = 0.02$ ), the effect size remained threefold stronger in women than in men ( $\beta$ -coefficient 0.0427 vs. 0.0165, respectively).

To provide further reassurance regarding any residual heterogeneity, we repeated our meta-analyses based on  $P$  values (rather than  $\beta$ -coefficients) and meta-analyzed the resulting  $z$  scores. Our findings were essentially unchanged, suggesting that heterogeneity in the  $\beta$ -estimates across cohorts has not produced spurious results.

TABLE 1  
Loci associated with fasting proinsulin levels at genome-wide levels of statistical significance

SNP	Nearest gene	CHR	Position	Allele (effect/other)	Freq.	Discovery $P$ value ( $n = 10,538-10,701$ )	Replication $\beta$ (SE) ( $n = 5,894-15,898$ )	Replication $\beta$ (SE), BMI adjusted ( $n = 5,893-15,890$ )	Replication $\beta$ (SE), BMI + FG adjusted ( $n = 5,744-15,741$ )	Combined $P$ value ( $n = 19,168-26,599$ )	Heterogeneity $I^2$ in % (Q-test $P$ value)
rs11603334	<i>ARAP1</i>	11	72110633	A/G	0.15	$3.3 \times 10^{-38}$	0.0928 (0.0053)	0.0943 (0.0056)	0.0938 (0.0052)	$3.2 \times 10^{-102}$	77.2 ( $1.9 \times 10^{-7}$ )
rs10501320	<i>MADD</i>	11	47250375	G/C	0.72	$2.1 \times 10^{-45}$	0.0805 (0.0064)	0.0748 (0.0069)	0.0775 (0.0064)	$1.1 \times 10^{-88}$	68.1 ( $8.7 \times 10^{-4}$ )
rs6235	<i>PCSK1</i>	5	95754654	G/C	0.28	$7.7 \times 10^{-14}$	0.0394 (0.005)	0.0407 (0.0052)	0.0438 (0.0049)	$9.8 \times 10^{-27}$	30.2 (0.14)
rs7903146	<i>TCF7L2</i>	10	114748339	T/C	0.30	$3.5 \times 10^{-18}$	0.0321 (0.007)	0.0330 (0.0076)	0.0303 (0.0063)	$2.3 \times 10^{-20}$	66.2 ( $1.6 \times 10^{-3}$ )
rs4502156	<i>VPS13C/C2CD4A/B</i>	15	60170447	T/C	0.58	$3.8 \times 10^{-11}$	0.0294 (0.0044)	0.0278 (0.0046)	0.0260 (0.0043)	$3.5 \times 10^{-20}$	45.1 (0.03)
rs11558471	<i>SLC30A8</i>	8	118254914	A/G	0.69	$4.2 \times 10^{-13}$	0.0280 (0.0049)	0.0280 (0.0053)	0.0273 (0.0048)	$3.1 \times 10^{-18}$	58.5 (0.01)
rs10838687	<i>MADD</i> (2nd signal)*	11	47269468	T/G	0.80	$8.6 \times 10^{-7}$	0.0253 (0.0050)	0.0240 (0.0049)	0.0246 (0.0049)	$6.9 \times 10^{-12}$	18.4 (0.26)
rs1549318	<i>LARP6</i>	15	68896201	T/C	0.61	$8.0 \times 10^{-8}$	0.0192 (0.0051)	0.0209 (0.0056)	0.0180 (0.005)	$2.4 \times 10^{-10}$	52.7 (0.02)
rs4790333	<i>SGSM2</i>	17	2209453	T/C	0.45	$2.2 \times 10^{-8}$	0.0154 (0.0043)	0.0205 (0.0046)	0.0179 (0.0045)	$3.0 \times 10^{-9}$	56.8 ( $3.5 \times 10^{-3}$ )
rs9727115	<i>SNX7</i> **	1	98949841	G/A	0.64	$5.3 \times 10^{-6}$	0.0133 (0.0051)	0.0130 (0.005)	0.0134 (0.0045)	$2.4 \times 10^{-7}$	0.00 (0.65)

$\beta$ -Coefficients for the effect allele are shown after adjustments for sex, age, geographic covariates (if applicable), and age squared (Framingham only), besides the additional adjustments denoted in the column headings. The combined  $P$  values are shown for the joint analysis with the basic adjustments listed above. CHR, chromosome; Freq, frequency. \*This second SNP in *MADD* was selected for further testing based on a suggestive  $P$  value ( $8.6 \times 10^{-7}$ ) when analyses for chromosome 11 were conditioned for the top two signals (*MADD* and *ARAP1*). The conditional analysis results are shown. \*\**SNX7* only reached genome-wide significance after adjusting for fasting glucose ( $P = 5.4 \times 10^{-9}$ ). Another signal (rs306549 in *DDX31*) reached genome-wide significance in women ( $P = 2.0 \times 10^{-8}$ ) but not in men ( $P = 0.17$ ); see text for details.

### Exploration of proinsulin processing mechanisms.

Proinsulin is initially cleaved to split 32,33-split proinsulin and further to insulin and C-peptide before secretion (Supplementary Fig. 1); we were therefore interested in the effects of the nine top SNPs on these traits. The proinsulin-raising alleles of each SNP were consistently associated with higher 32,33-split proinsulin levels, with effect sizes following the rank order of proinsulin effect sizes. Nearly all associations reached nominal conventional levels of statistical significance in this smaller dataset of 4,103–6,343 individuals with measures of 32,33-split proinsulin levels (all  $P < 1.5 \times 10^{-3}$ , with the exception of the conditional signal at *MADD*). The insulinogenic index (27), which measures dynamic insulin secretion during the first 30 min after an oral glucose load and was available in 14,956 subjects, showed nominal associations for four loci. Of these, the proinsulin-raising alleles were associated with a lower insulinogenic index at *VPS13C/C2CD4A/B*, *TCF7L2*, and *SLC30A8* and higher at *ARAP1* (Table 2).

We detected no nominal associations with fasting C-peptide ( $P > 0.05$ ). Given the differences in hepatic clearance of insulin and C-peptide, we also performed sensitivity analyses to account for any possible impact this may have had on our results. We adjusted proinsulin levels for fasting C-peptide rather than fasting insulin in two cohorts (Ely and Botnia-PPP); comparison of  $\beta$ -estimates showed that the majority of loci had very similar effect sizes and the same rank order was preserved, arguing against noticeable discrepancies between the two adjustment schemes.

**Association with other glycemic traits.** To clarify potential mechanisms, the top nine signals (*ARAP1*, two at *MADD*, *PCSK1*, *TCF7L2*, *VPS13C/C2CD4A/B*, *SLC30A8*, *LARP6*, and *SGSM2*) were also examined in relation to other glucometabolic traits (fasting and 2-h postload glucose and insulin, homeostasis model assessment estimates of  $\beta$ -cell function [HOMA-B] and insulin resistance [HOMA-IR] [28], glycated hemoglobin [A1C], T2D, and BMI [Table 3]). We investigated results available from MAGIC meta-analyses of GWAS of glycemic traits (3–5) and obtained T2D and BMI results in collaboration with the DIAGRAM (29) and GIANT (30) consortia, respectively. Nominal associations ( $P < 0.05$ ) were found for fasting glucose (with the proinsulin-raising allele increasing fasting glucose levels at *MADD*, *SLC30A8*, *TCF7L2*, and *VPS13C/C2CD4A/B* and decreasing fasting glucose levels at *ARAP1* and *PCSK1*), fasting insulin (increased levels at *ARAP1*, *LARP6*, and *SGSM2* and decreased levels at *TCF7L2*), HOMA-B (decreased at *MADD*, *SLC30A8*, *VPS13C/C2CD4A/B*, and *TCF7L2* and increased at *PCSK1*, *ARAP1*, and *LARP6*), insulin resistance as measured by HOMA-IR (increased at *LARP6* and *SGSM2* and decreased at *TCF7L2*), and 2-h postload glucose (decreased at *SLC30A8* and *VPS13C/C2CD4A/B* and increased at *ARAP1* and *TCF7L2*). We detected no significant associations for 2-h postload insulin or insulin sensitivity as estimated by the Matsuda index (31) (Table 3).

Associations with T2D were confirmed for four known T2D loci (*SLC30A8*, *ARAP1*, *VPS13C/C2CD4A/B*, and *TCF7L2*; Table 3). Counterintuitively, the proinsulin-raising allele of *ARAP1* (formerly known as *CENTD2* and reported as such in DIAGRAM+) (29) was associated with a lower fasting glucose (0.019 mg/dL per A allele;  $P = 1.7 \times 10^{-4}$ ), lower A1C (0.023%;  $P = 0.02$ ), and a lower risk of T2D (odds ratio [OR] 0.88;  $P = 7.8 \times 10^{-6}$ ; Table 3).

The two novel loci (*LARP6* and *SGSM2*) did not show significant associations with T2D (OR [95% CI]: 1.01 [0.95–1.07] and 1.01 [0.96–1.08], respectively), indicating that if they increase T2D risk they do so to an extent confined within the bounds of narrow 95% CI.

**Fine-mapping, copy number variants, and tissue expression.** We used MACH (32) or IMPUTE (19) applied to the 1000 Genomes CEU reference panel (www.1000genomes.org) to carry out imputation of ~8 million autosomal SNPs with minor allele frequency >1%. Analysis of 1000 Genomes-imputed data in the four discovery cohorts indicates that although there are low-frequency (1–5%) genetic variants that influence levels of circulating proinsulin, these are found in the same loci that contain common proinsulin-influencing variants, and none of them yield substantially stronger signals than the index SNP at each locus (Supplementary Fig. 5).

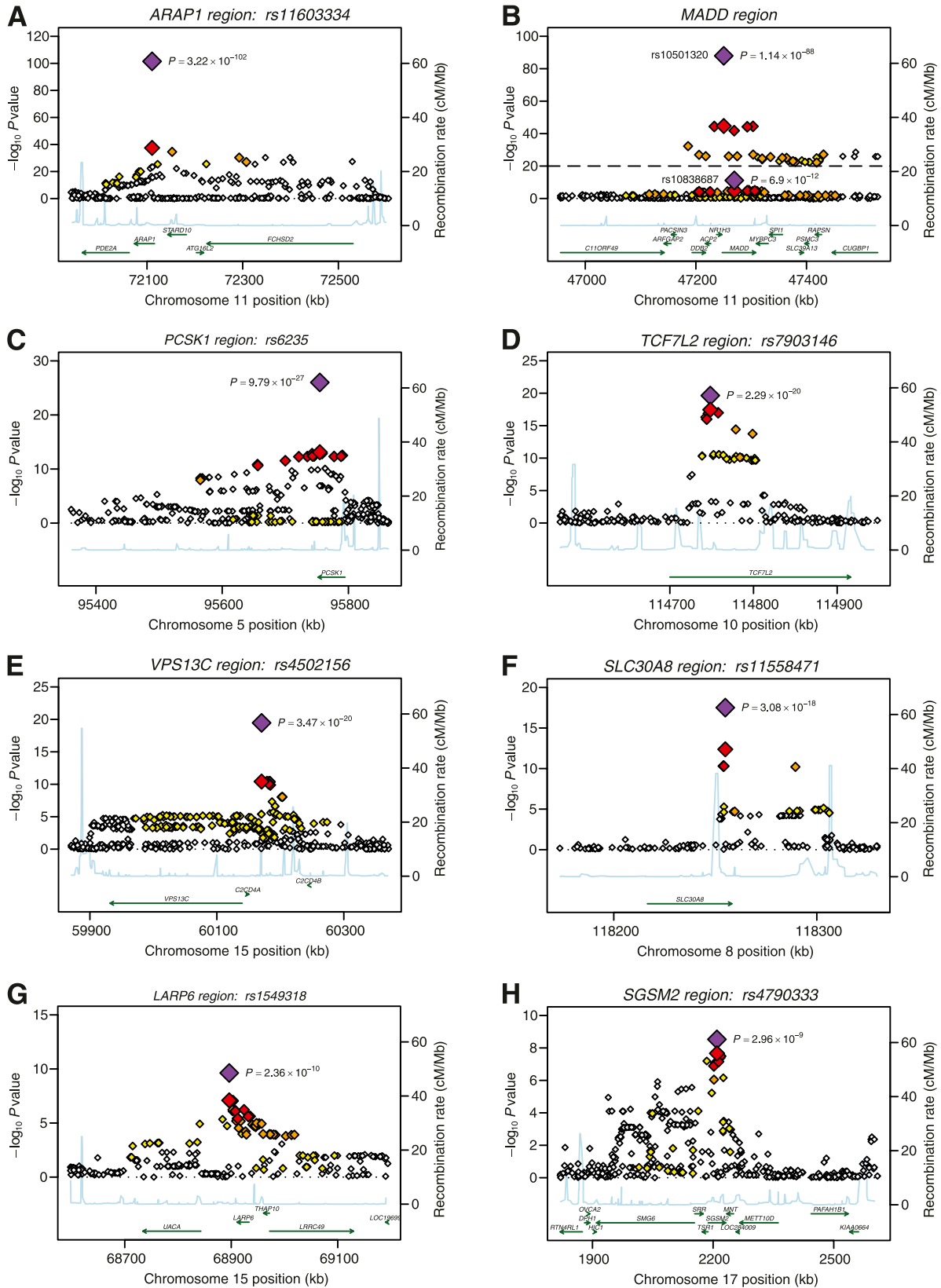
Using current databases of copy number variants (33) and the SNAP software (<http://www.broadinstitute.org/mpg/snap/index.php>; CEU, HapMap release 22), we checked whether any of the proinsulin-associated SNPs were within 500 kb and in linkage disequilibrium (LD) with any of the SNPs known to tag copy number variants in the human genome. No copy number variant tag SNPs with  $r^2 > 0.3$  were found within 500 kb of our lead SNPs.

To guide identification of the gene responsible for each association signal, we also examined the gene expression profile of selected genes in each associated region across a range of human tissues, including islets and fluorescence-activated cell (FAC)-sorted  $\beta$ -cells (Fig. 3A–F and Supplementary Fig. 6). We defined 1-Mb intervals around the lead SNP at each locus and prioritized biologically plausible genes as gleaned from the literature (see Box in Supplementary Data). We were able to demonstrate  $\beta$ -cell expression of most genes examined (Fig. 3F). However, at the *LARP6* locus, *CT62* is expressed exclusively in testis, likely excluding it as a relevant gene in this context. At the *ARAP1* locus, *STARD10* is expressed more strongly in pancreatic and islet tissue than any other tissue type; similarly, at the *VPS13C* locus both *C2CD4A* and *C2CD4B* demonstrate higher expression in pancreas and islets than all other tissue types.

We also studied the expression of the transcript for the gene closest to the index SNP at each of the nine replicated loci in human islets isolated from 55 nondiabetic and 9 diabetic individuals. Of the nine loci, *PCSK1* ( $P = 0.02$ ) and *MADD* ( $P = 0.07$ ) demonstrated 35–45% lower expression in subjects with T2D compared with control subjects.

**Functional exploration.** We evaluated whether any of the associated SNPs was in strong LD with a potentially causal variant. We used SNPper (34) to classify all SNPs in strong LD with the lead SNP ( $r^2 \geq 0.8$ ) within a 1-MB region. We found that *PCSK1* rs6235 codes for a non-synonymous variant (S690T), which is in perfect LD with rs6234, another missense variant (Q665E); both were predicted to be nondamaging by PolyPhen (35) and SIFT (36). At *SLC30A8*, the proinsulin-associated SNP rs11558471 is a perfect proxy for the known T2D-associated SNP rs13266634, encoding R325W. The T allele (encoding tryptophan) is predicted to be benign by PolyPhen, but damaging by SIFT. We found no other strong ( $r^2 > 0.8$ ) correlations in HapMap CEU with potentially functional SNPs within 1 Mb of the lead signals.

We also tested whether any of the proinsulin-associated SNPs might influence proximal (*cis*) expression of human



**FIG. 2.** Regional plots of eight genomic regions containing novel genome-wide significant associations. For each region, directly genotyped and imputed SNPs are plotted with their meta-analysis  $P$  values (as  $-\log_{10}$  values) as a function of genomic position (NCBI Build 36). In each panel, the stage 1 discovery SNP taken forward to stage 2 follow-up is represented by a purple diamond (with global meta-analysis  $P$  value), with its stage 1 discovery  $P$  value denoted by a red diamond with bolded borders. Estimated recombination rates (taken from HapMap) are plotted to reflect the local LD structure around the associated SNPs and their correlated proxies (according to a white to red scale from  $r^2 = 0$  to 1, based on pairwise  $r^2$  values from HapMap CEU). Gene annotations were taken from the University of California Santa Cruz genome browser. **A:** *ARAP1* region; **B:** *MADD* region; **C:** *PCSK1* region; **D:** *TCF7L2* region; **E:** *VPS13C/C2CD4A/B* region; **F:** *SLC30A8* region; **G:** *LARP6* region; **H:** *SGSM2* region.



TABLE 2  
Association of proinsulin loci with insulin-processing traits

SNP	Nearest gene	Alleles proinsulin-raising/ other	32,33-split proinsulin ( <i>n</i> = 4103–6343)		Fasting insulin ( <i>n</i> = 36,775–38,335)		HOMA-B ( <i>n</i> = 35,046–36,606)		Insulinogenic index ( <i>n</i> = 10,902–14,956)		C-peptide ( <i>n</i> = 1,537–2,956)	
			$\beta$	<i>P</i> value	$\beta$	<i>P</i> value	$\beta$	<i>P</i> value	$\beta$	<i>P</i> value	$\beta$	<i>P</i> value
rs11603334	<i>ARAP1</i>	A/G	0.146	$1.2 \times 10^{-25}$	0.012	0.02	0.016	$1.4 \times 10^{-4}$	0.049	$1.1 \times 10^{-5}$	0.017	0.22
rs10501320	<i>MADD</i>	G/C	0.124	$1.4 \times 10^{-20}$	-0.003	0.45	-0.015	$8.1 \times 10^{-5}$	-0.005	0.67	$-3.0 \times 10^{-4}$	0.98
rs6235	<i>PCSK1</i>	G/C	0.057	$4.5 \times 10^{-6}$	0.001	0.78	0.008	0.02	-0.004	0.70	-0.008	0.46
rs7903146	<i>TCF7L2</i>	T/C	0.042	$1.5 \times 10^{-3}$	-0.012	0.005	-0.020	$1.4 \times 10^{-7}$	-0.063	$9.6 \times 10^{-7}$	0.000	0.98
rs4502156	<i>VPS13C/C2CD4A/B</i>	T/C	0.039	$4.5 \times 10^{-4}$	-0.002	0.67	-0.010	0.004	-0.053	$3.5 \times 10^{-9}$	-0.017	0.11
rs11558471	<i>SLC30A8</i>	A/G	0.059	$1.1 \times 10^{-5}$	-0.005	0.21	-0.017	$1.4 \times 10^{-6}$	-0.032	0.001	0.003	0.81
rs10838687	<i>MADD</i> (2nd signal)	T/G	0.006	0.71	0.006	0.22	0.007	0.06	0.029	0.79	-0.0084	0.47
rs1549318	<i>LARP6</i>	T/C	0.043	$1.2 \times 10^{-4}$	0.009	0.02	0.009	0.007	-0.001	0.88	-0.014	0.18
rs4790333	<i>SGSM2</i>	T/C	0.040	$3.2 \times 10^{-4}$	0.008	0.04	0.002	0.55	0.009	0.33	-0.015	0.15

$\beta$ -Coefficients are adjusted for age, sex, and study-specific covariates (if applicable).

transcripts, in tissues available to us that had been paired to genetic data. We found a significant association ( $P = 0.01$  permutation threshold) of rs1549318 with expression levels of *LARP6* in adipose tissue. SNP rs1549318 is located  $\sim 37$  kb from *LARP6*, and the proinsulin-raising T allele is associated with lower levels of expression. Analysis of an eQTL database from human liver indicated that the proinsulin-raising A allele of the lead SNP at the *SGSM2* locus (rs179456) was associated with increased liver expression of *TRPS1* ( $P = 0.004$ ).

**CAD.** We constructed unweighted and weighted genotype scores composed of the nine genome-wide significant proinsulin-raising alleles, with weights defined by the  $\beta$ -coefficients from our replication meta-analysis, and tested the association of these scores with CAD in the CARDIoGRAM (37) ( $n = \sim 22,000$  CAD case subjects and 60,000 control subjects) and C4D (38) ( $n = 15,420$  CAD case subjects and 15,062 control subjects) datasets. Neither weighted nor unweighted genotype scores reached nominal significance in either dataset ( $P = 0.47$  and  $0.81$  for unweighted and weighted scores in CARDIoGRAM, respectively;  $P = 0.60$  and  $0.43$  for unweighted and weighted scores in C4D, respectively).

## DISCUSSION

We report the first meta-analysis of genome-wide association datasets for circulating fasting proinsulin. We adjusted proinsulin for fasting insulin levels, aiming to capture an increase in proinsulin relative to the nonspecific activation of the insulin processing pathway induced by generalized insulin resistance (Supplementary Fig. 2). Loci that simply influence insulin resistance are typically sought by a GWAS for fasting insulin or more sophisticated measures of insulin sensitivity (3,4,6). Thus, we hoped to identify loci that indicate the inability of the  $\beta$ -cell to process proinsulin adequately in response to metabolic demands.

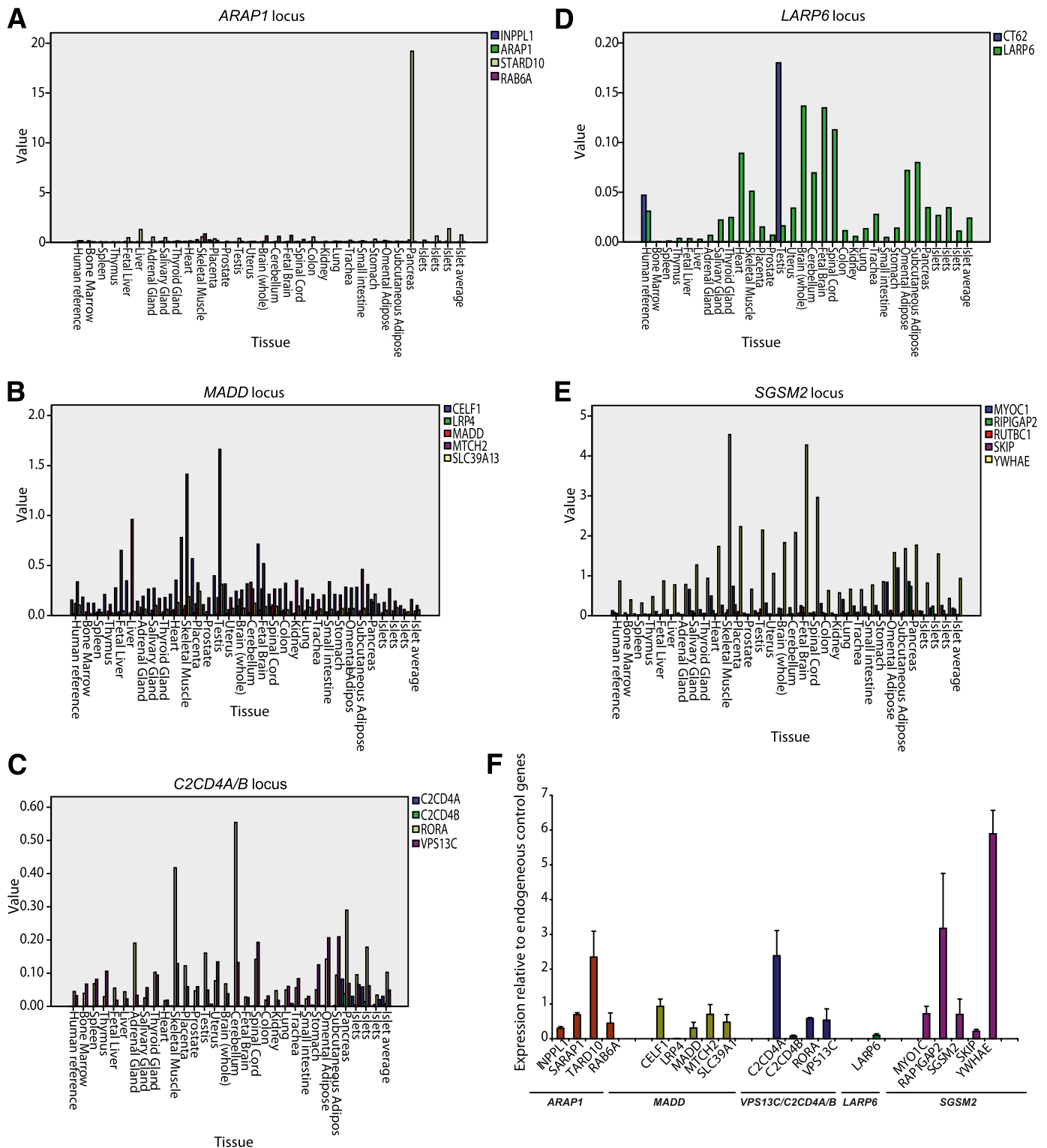
We have identified nine signals at eight loci associated with higher proinsulin levels (see Box in Supplementary Data). Two of these loci (*LARP6* and *SGSM2*) have not been previously related to metabolic traits. A 10th signal emerged after sex-stratified analyses; an explanation for the female-specific genome-wide significant association at *DDX31* requires fine-mapping to identify the causal gene. Although the function of the *DDX31* gene product is unknown, other members of the DEAD-box protein family have been implicated in sex-specific processes such as spermatogenesis (39). We have also replicated at the genome-wide level previously reported nominal associations of *MADD*, *TCF7L2*, *VPS13C/C2CD4A/B*, *SLC30A8*, and *PCSK1* with proinsulin (6,14–17,40). The knowledge that *TCF7L2*, *SLC30A8*, *VPS13C/C2CD4A/B*, and *ARAP1* are established T2D loci provides reassurance that a quest for genetic determinants of proinsulin can serve to identify disease-associated signals. Interestingly, the proinsulin-raising alleles at *TCF7L2*, *SLC30A8*, and *VPS13C/C2CD4A/B* cause impairment of  $\beta$ -cell function, as estimated by HOMA-B and the insulinogenic index. By raising proinsulin but lowering insulin secretion, these loci point to defects in the insulin processing and secretion pathway, distal to the first enzymatic step. Such a hypothesis is consistent with postulated modes of action for *TCF7L2* (41) and *SLC30A8* (42); *VPS13C*, by influencing protein trafficking across membrane compartments, could also affect the same process. Further fine-mapping and functional experiments will be required to establish the precise mechanism at this locus.

TABLE 3  
Association of proinsulin loci with other glycemic traits

SNP	Nearest gene	Alleles Proinsulin- raising/other	Fasting glucose ( <i>n</i> = 44,601– 46,186)	HOMA-IR ( <i>n</i> = 35,512– 37,072)	Matsuda index ( <i>n</i> = 7,055– 9,561)	2-h glucose ( <i>n</i> = 15,088– 15,252)	2-h insulin ( <i>n</i> = 6,923– 7,083)	HbA <sub>1c</sub> ( <i>n</i> = 33,736– 44,731)	T2D ( <i>n</i> = 8,130 case subjects/ 38,987 control subjects)	BMI ( <i>n</i> = 123,260– 123,865)
rs11603334	<i>ARAP1</i>	A/G <i>P</i> value	−0.019 $1.7 \times 10^{-4}$	0.016 0.105	−0.014 0.226	0.009 0.043	−0.050 0.294	−0.023 0.016	0.88 $7.8 \times 10^{-6}$	0.012 0.065
rs10501320	<i>MADD</i>	G/C <i>P</i> value	0.025 $3.1 \times 10^{-9}$	−0.015 0.775	0.009 0.413	−0.001 0.605	−0.012 0.459	0.004 0.595	1.01 0.629	0.014 0.012
rs6235	<i>PCSK1</i>	G/C <i>P</i> value	−0.014 $4.8 \times 10^{-4}$	0.001 0.837	−0.008 0.435	0.034 0.109	0.015 0.273	0.013 0.053	1.02 0.401	0.016 0.002
rs7903146	<i>TCF7L2</i>	T/C <i>P</i> value	0.023 $2.8 \times 10^{-8}$	−0.010 0.034	0.014 0.244	0.108 $4.3 \times 10^{-7}$	0.004 0.772	0.015 0.029	1.40 $2.2 \times 10^{-51}$	−0.019 $2.4 \times 10^{-4}$
rs4502156	<i>VPS13G/C2CD4AB</i>	T/C <i>P</i> value	0.020 $8.4 \times 10^{-8}$	0.002 0.648	−0.004 0.658	−0.071 $2.1 \times 10^{-4}$	0.012 0.321	0.002 0.779	1.07 0.001	−0.003 0.528
rs11558471	<i>SLC30A8</i>	A/G <i>P</i> value	0.027 $2.6 \times 10^{-11}$	−0.017 0.714	0.014 0.197	−0.002 $1.4 \times 10^{-4}$	0.080 0.275	0.013 0.080	1.14 $3.3 \times 10^{-8}$	−0.012 0.027
rs10838687	<i>MADD</i> (2nd signal)	T/G <i>P</i> value	−0.011 0.014	0.004 0.383	−0.006 0.578	0.014 0.512	0.003 0.830	−0.001 0.876	0.98 0.383	−0.013 0.020
rs1549318	<i>LARP6</i>	T/C <i>P</i> value	−0.002 0.698	0.009 0.044	0.001 0.906	0.008 0.621	−0.010 0.556	−0.005 0.444	1.01 0.667	0.003 0.583
rs4790333	<i>SGSM2</i>	T/C <i>P</i> value	0.004 0.328	0.009 0.023	0.009 0.338	−0.0002 0.992	0.008 0.519	−0.005 0.425	1.01 0.626	−0.005 0.261

β-Coefficients are adjusted for age, sex, and study-specific covariates (if applicable). OR is for T2D only as a dichotomous trait.





**FIG. 3. Expression profiles of biologically plausible genes within each associated locus across a range of human tissue types, including islet preparations from three donors. Expression levels determined with respect to the geometric mean of three endogenous control assays. A: *ARAP1* region; B: *MADD* region; C: *VPS13C/C2CD4A/B* region; D: *LARP6* region; E: *SGSM2* region. F: Expression levels of genes near the proinsulin associated variants in human FAC-sorted  $\beta$ -cells. Data are expression means  $\pm$  SD of the relative expression measured by quantitative PCR obtained from three human nondiabetic donors.**

*ARAP1*, which harbors the strongest proinsulin association, provides an intriguing counterpoint. Under its previous designation of *CENTD2* it was recently associated with T2D (29); however, the T2D-associated allele is associated with lower proinsulin levels, as well as lower  $\beta$ -cell function

(HOMA-B and insulinogenic index). This suggests that the genetic defect that gives rise to T2D at this locus causes a generalized downregulation of insulin secretion (e.g., through a reduction in  $\beta$ -cell mass/function or very early defects in insulin processing) and stands in contrast with

*TCF7L2*, *SLC30A8*, and *VPS13C/C2CD4A/B*. A corollary of the divergent effect of these loci on T2D is that both disproportionate elevations and reductions in proinsulin can indicate  $\beta$ -cell dysfunction. Of the genes that lie within 1 Mb of the *ARAP1* association signal, we have demonstrated islet expression in the four strong biological candidates we examined (*ARAP1*, *INPPL1*, *STARD10*, and *RAB6A*); however, expression of *STARD10* was much higher in pancreas than in any other human tissue, and of all genes tested at the *ARAP1* locus *STARD10* was expressed most strongly in islets, indicating that the role of its protein product in the transfer of phospholipids to membranes may be particularly relevant to this cell type.

*LARP6* is a ribonucleoprotein identified in the current study as a novel locus associated with increased fasting proinsulin levels. It is involved in the regulation of translation and subcellular localization of collagen I, in a manner dependent upon both the RNA-binding and La domains (43). The associated SNP rs1549318 is located within a region of high LD, which spans the gene and includes a number of SNPs within the RNA-binding domain. Although the link between *LARP6* and proinsulin levels is not clear, it is nominally associated with fasting insulin and HOMA-IR, but not T2D. It may therefore represent a marker of insulin resistance and perhaps other related common dysmetabolic conditions.

In previous publications we have reported the association of *C2CD4B* with fasting glucose (3) and that of the nearby locus *VPS13C* with 2-h glucose (4); *C2CD4B* is also associated with T2D in Japanese (44), with supportive evidence found in Europeans (3,44). Here we show that the same genomic region is associated with fasting proinsulin. The strongest association with proinsulin reported here (rs4502156) and those associated with fasting glucose and 2-h glucose may represent independent signals, since they are all in relatively weak LD in HapMap CEU Europeans: rs4502156 versus rs11071657 (best fasting glucose signal),  $r^2 = 0.306$ ; rs4502156 vs. rs17271305 (best 2-h glucose signal),  $r^2 = 0.450$ ; and rs11071657 versus rs17271305,  $r^2 = 0.287$ . On the other hand, in Europeans our proinsulin-associated SNP is in strong LD ( $r^2 = 0.967$ ) with the T2D-associated SNP reported by Yamauchi et al. (44). Although four strong biological candidates (*C2CD4A*, *C2CD4B*, *VPS13C*, and *RORA*, a gene that encodes a member of the NR1 subfamily of nuclear hormone receptors) are expressed in FAC-sorted  $\beta$ -cells, the relative expression of the first two is much higher in islets than in other human tissues, again suggesting that these two genes, encoding nuclear factors that are upregulated in response to inflammation, may be particularly relevant to endocrine pancreatic function.

The genome-wide association of a missense variant in *PCSK1* with fasting proinsulin also serves as a positive control. *PCSK1* encodes the protein prohormone convertase 1/3 (PC1), which is the first enzyme in the proinsulin processing pathway, where it cleaves proinsulin to 32,33-split proinsulin (Supplementary Fig. 1). A related enzyme, PC2, acts on 32,33-split proinsulin in the second processing step. People deficient in PC1 become obese at an early age and exhibit pituitary hypofunction because of the lack of several mature peptide hormones (45), whereas PC2-null mice demonstrate increased levels of 32,33-split proinsulin (46). The rs6235 SNP reported here results in the substitution of a serine residue for threonine at position 690 of the molecule; the minor allele (Thr) is associated with higher proinsulin levels. A nominal association

of the same allele with higher proinsulin levels has recently been reported (40); its association with higher BMI is only nominal here, but confirms a previous report (47). This specific amino acid change has been shown not to affect enzyme catalysis or maturation of the protein in vitro (47), but the C-terminus of the protein (where S690T is located, adjacent to a conserved proline residue) is known to direct the correct subcellular targeting of the protein as well as stabilizing and partially inhibiting PC1. Although one might expect lower levels of the reaction product (32,33-split proinsulin) in carriers of the risk allele, the potential diversion of the substrate down its alternate path (giving rise to 65,66-split proinsulin, whose assay typically has 60% cross-reactivity with 32,33-split proinsulin) requires further study. Alternatively, if changes in the activity of PC1 also affect that of PC2 (for instance, by competing for inhibitory peptides) one might see reductions in the catalytic function of both enzymes and accumulation of both proinsulin and 32,33-split proinsulin.

Because of the reported relationship between proinsulin levels and coronary events (11–13), the identification of genetic determinants of proinsulin levels might help shed light on whether hyperproinsulinemia is a mediator of CAD or a byproduct of a shared etiological mechanism. If hyperproinsulinemia is causally associated with an increased risk of CAD, one might expect that SNPs that specifically and selectively raise proinsulin levels should increase the risk of CAD given an adequately powered study. We have not observed such an effect for a genotype score constructed with the genome-wide significant proinsulin association signals. Assuming conservative approximations of the reported effect sizes of proinsulin on CAD (OR  $\sim 1.5$  per 1-SD increase in proinsulin) (12,13), and of the nine SNPs reported here on circulating proinsulin (5%), a CAD cohort like CARDIoGRAM has 99% power to detect an effect of proinsulin SNPs on CAD. The absence of statistical significance argues against a direct etiological role of proinsulin on CAD.

In summary, we have identified nine loci that associate with fasting proinsulin levels. Several of these loci increase risk of T2D; interestingly, both proinsulin-raising and lowering alleles can lead to T2D through decreases in insulin secretion, indicating defects distal or proximal to the first enzymatic step in proinsulin conversion, respectively. Other genetic determinants of proinsulin levels do not necessarily lead to higher T2D risk, suggesting that it is not a mere elevation in proinsulin, but rather the specific impairment in proinsulin processing and the reaction of the  $\beta$ -cell to this defect that determine whether ultimately  $\beta$ -cell insufficiency will cause pathological hyperglycemia. The direct elevation of fasting proinsulin out of proportion to fasting insulin does not seem to increase risk of CAD.

#### ACKNOWLEDGMENTS

Please see the Supplementary Data.

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