Functional Gly297Ser Variant of the Physiological Dysglycemic Peptide Pancreastatin is a Novel Risk Factor for Cardiometabolic Disorders

by

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Running title: Pancreastatin variant and cardiometabolic disorder

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Pancreastatin is a potent physiological anti-insulin peptide that regulates plasma glucose and insulin levels. We report identification of a gain-of-function variant of the pancreastatin peptide that increases the risk for cardiovascular and metabolic diseases in human populations.

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Summary Figure of the article: **Figure 7**
ABSTRACT

Pancreastatin (PST), a chromogranin A (CHGA)-derived potent physiological dysglycemic peptide, regulates glucose/insulin homeostasis. We have identified a non-synonymous functional PST variant (p.Gly297Ser; rs9658664) that occurs in a large section of human populations. Association analysis of this single nucleotide polymorphism with cardiovascular/metabolic diseases states in Indian populations (n≈4300 subjects) displays elevated plasma glucose, glycosylated hemoglobin, diastolic blood pressure and catecholamines in Gly/Ser subjects as compared to wild-type individuals (Gly/Gly). Consistently, the 297Ser allele confers an increased risk (~1.3-1.6-fold) for type-2 diabetes/hypertension/coronary artery disease/metabolic syndrome. In corroboration, the variant peptide (PST-297S) displays gain-of-potency in several cellular events relevant for cardiometabolic disorders (e.g., increased expression of gluconeogenic genes, increased catecholamine secretion, greater inhibition of insulin-stimulated glucose-uptake) than the wild-type peptide (PST-WT). Computational docking analysis and molecular dynamics simulations show higher affinity binding of PST-297S peptide with glucose-regulated protein 78 (GRP78) and insulin receptor (IR) than PST-WT, providing a mechanistic basis for the enhanced activity of the variant peptide. In vitro binding assays validate these in silico predictions of PST peptides binding to GRP78 and IR. In conclusion, the PST 297Ser allele influences cardiovascular/metabolic phenotypes and emerges as a novel risk factor for type-2 diabetes/hypertension/coronary artery disease in human populations.

Keywords: human genetic variation, anti-insulin, type-2 diabetes, hypertension, coronary artery disease
INTRODUCTION

Pancreastatin (PST) is a proteolytically-derived peptide from the acidic glycoprotein chromogranin A (CHGA) that is primarily expressed in endocrine, neuroendocrine and neuronal tissues (1,2). Human PST exists in different molecular forms including 29-mer PST (CHGA273-301), 48-mer PST (CHGA254-301) and 52-mer PST (CHGA250-301), all containing the conserved functional C-terminal part (2); among these forms, the 52-mer peptide has been reported to be the major one in human plasma (3). Proteases like prohormone convertase 2 and carboxypeptidase H have been reported to be involved in the intracellular processing of PST (4,5). The carboxy-terminus of PST is amidated by peptide α-amidating mono-oxygenase (6).

PST exerts diverse biological effects, including inhibition of insulin secretion stimulated by physiological activators (e.g., glucose/glucagon) and pharmacological agents (e.g., sulphonylurea), inhibition of insulin-stimulated glucose uptake and translocation of GLUT4 in adipocytes, enhancement of hepatic gluconeogenesis and suppression of insulin signaling (7). Mice lacking ChgA protein generated by systemic deletion of the Chga gene (8) showed reduced insulin resistance. Furthermore, the exogenous administration of human PST peptide in these Chga knock-out mice caused increased insulin resistance and increased blood glucose levels along with enhanced synthesis of gluconeogenic genes (9,10). Consistently, infusion of PST through brachial artery profoundly reduced human forearm glucose uptake and arterio-venous glucose gradient (11).

Re-sequencing of the CHGA locus by us and others has led to the identification of several variants within PST encoding domain in various populations of the world [(12,13); dbSNP database, https://www.ncbi.nlm.nih.gov/snp/rs9658664]. Among these naturally occurring PST variants, the G297S variant (rs9658664) is located within the functionally active C-terminus of the peptide. Of note, the p.Gly297Ser variant peptide has been shown to exhibit higher potency in inhibiting insulin-stimulated glucose uptake than the wild-type peptide (11).

In our previous study (13), we analyzed the effect of this genetic variation on cardiovascular and metabolic disease states in a small Indian population (n≈400), where we found an association of the 297Ser allele with higher plasma glucose levels. In this study, we have extended our sample to a larger population (n≈4300) comprising of South Indian and North Indian subjects. This study revealed strong association of the p.Gly297Ser polymorphism with
several cardiometabolic parameters [viz., plasma glucose, glycosylated hemoglobin (HbA1c), catecholamines and blood pressure]. We also employed a combination of experimental (biochemical, cellular and molecular assays, including insulin-stimulated glucose uptake, measurement of endogenous gluconeogenic gene transcripts, receptor-ligand binding experiments) and computational (molecular modeling, docking and molecular dynamics simulations) tools to unravel the mechanistic basis for the higher activity of the variant peptide (PST-297S) in comparison to the wild-type peptide (PST-WT) and to account for the higher disease risk in the carriers of the 297Ser allele. We provide evidence for higher affinity binding of the variant peptide with insulin receptor (IR) and glucose-regulated protein 78 (GRP78) that may mediate the higher disease risk in the carriers of the 297Ser allele.

RESEARCH DESIGN and METHODS

Human subjects and methodologies
We recruited 3602 unrelated volunteers at three study centers in Chennai (South India) and 716 unrelated subjects at the Post Graduate Institute of Medical Education and Research in Chandigarh (North India). Details about the demographic and clinical parameters are given in SI Appendix Tables S1 and S2.

All subjects gave written informed consent for the use of their blood samples for genetic and biochemical analyses. This study was approved by the Institute Ethics Committee at Indian Institute of Technology Madras, Chennai in accordance with Declaration of Helsinki (reference number: IITM IEC No. 2010027).

Exon-7 region of CHGA was re-sequenced in the first set of samples to detect the presence of SNPs in the PST domain (13), followed by genotyping for the p.Gly297Ser SNP (rs9658664) using Taqman® allelic discrimination method in the next set of samples.

Circulating levels of common biochemical parameters (e.g., insulin, glucose, HbA1c and cholesterols) in the subjects were measured using standard pathological laboratory methods, which have been described in detail in the Supplementary Information (SI Appendix). Plasma catecholamine and PST levels were quantified using a radioimmunoassay kit (catalog number: BA R-6500) and an enzyme-linked immunosorbent assay kit (catalog number: TM E-9000), respectively, from LDN (Germany).
Cell culture
HepG2 (human liver cell line), L6 (rat skeletal muscle cell line) and SHSY-5Y (human neuroblastoma cell line) cells were obtained from NCCS, Pune, India. L6 cells were differentiated into myotubes by growing them in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum until they became confluent and then replacing the medium with differentiation medium (DMEM with 2% fetal bovine serum) for 8 d (10). 3T3-L1 cells were grown and differentiated as described previously (13). PC12 cells were maintained and grown in DMEM containing 5% fetal bovine serum and 10% horse serum (14). HEK-293 cells used for the binding assays were cultured in Minimal Essential Media supplemented with 10% fetal bovine serum and appropriate antibiotics. β-TC-6 cells were cultured in DMEM with high glucose supplemented with 15% heat-inactivated fetal bovine serum and 1x penicillin/streptomycin.

Chemical synthesis of human PST peptides
PST-WT (wild-type; PEGKGEQEHSSQKEEEEEMAVVPQGLFRG-amide), PST-297S (Ser-297 variant; PEGKGEQEHSSQKEEEEEMAVVPQSLFRG-amide; the variant serine residue is shown in bold and underlined) peptides were synthesized and purified as described previously (13).

Catecholamine secretion and glucose uptake assays
Nor-epinephrine secretion from SH-SY5Y and PC12 cells in the presence/absence of each PST peptide was estimated according to a previously described method (15). Glucose uptake by differentiated L6 and 3T3-L1 cells was assayed using a modified version of our previously reported protocol (13).

RNA extraction and real-time PCR
Total RNA samples from HepG2 cells treated with/without PST peptides (PST-WT or PST-297S) were isolated using our previously described method (16). Endogenous human phosphoenolpyruvate carboxykinase-1 (PCK-1) and glucose-6-phosphatase (G6PC1), and GRP78 transcript levels were estimated using real-time PCR with gene-specific primers (as
detailed in the SI Appendix). The relative gene expression levels for all the genes were determined using the $2^{-\Delta \Delta C_t}$ method.

**Generation of PST peptide structures and docking with GRP78 and IR**

Molecular modeling of PST-WT and PST-297S 52-mer peptides was carried out using MODELLER version 9v13 (17) with the previously derived 29-mer PST peptide structures (13) as templates. The homology-modeled structures were further refined using molecular dynamics simulations for 200 ns. The stability of the simulated structures was assessed by plotting root mean square deviations of Cα atoms with respect to the starting structure over the simulation period.

Next, we performed unbiased docking of the representative PST structures to GRP78 (PDB ID: 3LDO) monomer (18). We also carried out docking of PST peptides with IR (PDB ID: 2DTG) (19). We used a protein-docking program, ZDOCK (20) to predict the binding of PST peptides to GRP78 monomer and IR. Molecular interactions between PST peptides and GRP78 or IR were calculated using PDBsum database (21). ZDOCK scores between GRP78/IR and PST peptides were calculated by using ZDOCK 3.0.2 scoring function considering IFACE statistical potential, shape complementarity and electrostatic parameters (22).

**GRP78 ATPase activity**

The effect of PST and PST-297S peptides on GRP78 ATPase activity was determined using a spectrophotometric Malachite Green Phosphate Assay Kit (MAK307; Sigma-Aldrich, St. Louis, MO, USA) (23).

**GRP78 expression study in HepG2 cells**

The inhibitory effects of PST-WT or PST-297S peptides on tunicamycin-stimulated GRP78 expression in HepG2 cells was determined using western blot analysis (23).

**Overexpression of IR and GRP78, plasma membrane extraction and western immunoblotting**

GRP78- and IR-overexpression constructs were generated by cloning GRP78 cDNA (obtained from catalog no. 32701, Addgene, Watertown, MA, USA) and IR cDNA (a kind gift from Dr.
Frederick M. Stanley (24)) into pcDNA 3.1. Cells were transfected with either GRP78-FLAG or IR-FLAG overexpression constructs (8 μg/100 mm tissue culture dish) using TurboFect™ (Thermo Fisher Scientific, Waltham, MA, USA) transfection reagent. Forty-eight hours post transfection, the cells were serum starved for 6 h before being used for each of the individual experiments. Isolation of plasma membrane fraction from transfected HEK-293 cells or β-TC-6 insulinoma cells and western immunoblotting were performed as previously described (25). The expression levels of the proteins were assessed using chemiluminescence, post-incubation with an anti-FLAG mouse monoclonal antibody (Sigma-Aldrich, used at a dilution of 1:1000), followed by an anti-mouse secondary antibody (used at a dilution of 1:5000).

**Competitive binding assay**

For the competitive binding assay, 10 μg of plasma membrane (from HEK-293 or β-TC-6 cells) in binding buffer (75 mM Tris-HCl, pH 7.5, 2 mM ethylenediaminetetraacetic acid and 12.5 mM MgCl₂) was used to assess the ability of PST-WT or PST-297S peptides to displace labeled [¹²⁵]I-Tyr PST (catalog no. H-5604, Peninsula laboratories, San Carlos, CA, USA) or [¹²⁵]I-Tyr Insulin (catalog no. NEX420, Insulin, human I¹²⁵, PerkinElmer, Waltham, MA, USA). The peptides were added in increasing concentrations (100 pM through 50 μM for IR binding and 10 pM through 1 μM for GRP78 binding) along with 100 nCi of labeled PST or insulin. The binding reactions were incubated at 37°C for 1 h with constant shaking. Increasing concentrations (100 pM through 50 μM) of cold insulin was used as a positive control for displacement in studies pertaining to IR expression. Displacement was calculated as a percentage of receptors in the control samples that did not contain any competing peptides.

**Data representation and statistical analyses**

The experimental data for various cellular functions are representative of three or more separate experiments. All results are represented as mean ± S.D. from replicate cells or wells as stated in the respective figure legends. To evaluate the effect of peptides on cellular activity or to compare between different conditions, one-way ANOVA followed by Tukey’s multiple comparison post-hoc test, student’s t-test or linear regression analysis, as appropriate, were performed using Prism (GraphPad, San Diego, CA, USA) or SPSS software (SPSS Inc., IBM, Chicago, IL, USA). Analysis of genotypic frequencies for Hardy-Weinberg equilibrium was carried out as described.
Odds ratios were calculated using binary logistic regression analysis after age, sex and body mass index (BMI) adjustments. Association of genotypes with various phenotypes was analyzed using SPSS software. The blood pressure data was adjusted for anti-hypertensive treatments as described previously (27) and subjects under medications (specifically hypoglycemic drugs) were not included in our analysis. A two-tailed p value of <0.05 was considered as statistically significant between groups. Power of the study was calculated using Quanto version 1.2.4 (SI Appendix Table S3) (28).

DATA AND RESOURCE AVAILABILITY
The data sets and resources generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

RESULTS

Discovery and occurrence of the PST genetic variation p.Gly297Ser in human populations
In this study, we extended our previous analysis carried out in a small population of 410 individuals (13) to a larger population (~4300 subjects) comprising of subjects from two Indian populations (South Indians at Chennai and North Indians at Chandigarh). The samples were analyzed by direct sequencing of the PST genomic region in the primary set of samples, followed by genotyping (SI Appendix Fig. S1) of the p.Gly297Ser variant in a secondary set. The allele frequencies for this SNP in Chennai populations were: Gly297, 93.6% and Ser297, 6.4%; the frequencies in the Chandigarh population were: Gly297, 93.0% and Ser297, 7.0%. The genotype frequencies were in Hardy-Weinberg equilibrium for the entire population ($\chi^2=0.169$, p=0.681) as well as in each ethnic group ($\chi^2=0.096$, p=0.756 for South Indian ancestry and $\chi^2=0.086$, p=0.7699 for North Indian ancestry). The genotype frequencies do not differ significantly ($\chi^2=0.622$, p=0.733) between the South Indian and North Indian populations.

This SNP has also been detected in other ethnic populations, including people of European, African, American, East Asian and South Asian origin; the distribution of genotypes differed by ethnicity ($\chi^2=13688$, p<0.0001; SI Appendix Table S4). Taken together, the PST p.Gly297Ser genetic variation occurs in a large section of people worldwide, with an especially high prevalence in the South Asian population.
The PST 297Ser allele increases the risk for cardiometabolic diseases

The demographic, physiological and biochemical parameters of the cases [comprising of type-2 diabetes (T2D)/hypertension (HTN)/intermediate glucose tolerance (IGT)/coronary artery disease (CAD) patients] and control subjects are shown in SI Appendix Tables S1 and S2. The cases had significantly higher BMI, systolic blood pressure, diastolic blood pressure (DBP) and mean arterial pressure than the controls; several biochemical parameters relevant to cardiometabolic diseases (e.g., plasma glucose, HbA1c and cholesterols) were also elevated in the cases.

Logistic regression analysis to check for the association of the Ser297 allele with these different disease conditions revealed the occurrence of the allele at higher frequencies in the disease groups than in the controls. As shown in Table 1, upon using the additive model (GG vs. GA), we found that the Ser297 allele increased the risk for T2D by ~1.4-fold (p=0.018); the disease risk remained significant after adjusting for age, sex or BMI, individually or together. Upon using the dominant model (GG vs. GA+AA) as well, the risk for T2D was close to ~1.3 fold (p=0.022), which remained significant after adjusting for age, sex or BMI, individually or together. Using both the models, the risk for HTN was elevated in Ser297 carriers by ~1.4-fold [p=0.013 for the additive model (GG vs. GA) and p=0.017 for the dominant model (GG vs. GA+AA)]; the risks remained significant after adjusting for age or sex in both the models. Similarly, this PST allele increased the risk for CAD by ~1.5-fold [p=0.008 for the additive model (GG vs. GA) and p=0.017 for the dominant model (GG vs. GA+AA)]; the risk remained significant after adjusting for age, sex or BMI, individually under the additive model and for BMI under the dominant model. The Ser297 allele also enhanced the risk for metabolic syndrome (cases with one or more of the condition(s): T2D or HTN or IGT or CAD) by ~1.4-fold [p=0.002 for the additive model (GG vs. GA) and p=0.003 for the dominant model (GG vs. GA+AA)]; the risk remained significant after adjusting for age, sex and BMI, individually or together, under both the additive and dominant models. No association was observed upon carrying out logistic regression using the additive model, to compare wild-types with homozygous variants (GG vs. AA); this could be attributed to the low number of homozygous variants (n=20) in the population.
The PST 297Ser allele is associated with elevated levels of plasma glucose and HbA1c

PST p.Gly297Ser SNP was found to be associated with plasma glucose levels in the overall population. The Gly/Ser and Gly/Ser+Ser/Ser subjects displayed markedly higher random blood sugar (RBS) levels than those of the Gly/Gly individuals, by 11.83 mg/dL (p=0.014) and 10.80 mg/dL (p=0.022), respectively (Fig. 1A). Similarly, Gly/Ser and Gly/Ser+Ser/Ser subjects displayed significantly elevated post-glucose blood sugar (PGBS) levels than those of their Gly/Gly counterparts, by 13.68 mg/dL (p=0.015) and 13.54 mg/dL (p=0.014), respectively (Fig. 1B). Consistent with their elevated plasma glucose values (Fig. 1A-B), the Gly/Ser and Gly/Ser+Ser/Ser individuals displayed higher HbA1c levels (6.00% vs. 5.80%; 42.11 vs. 39.94 mmol/mol; p=0.004) and (6.00% vs. 5.80%; 42.13 vs. 39.94 mmol/mol; p=0.003) than the Gly/Gly subjects (Fig. 1C-D). The p-values for HbA1c remained significant after adjustments for age, sex and BMI (Gly/Gly vs. Gly/Ser p=0.005; Gly/Gly vs. Gly/Ser+Ser/Ser p=0.002).

Stratification of the subjects based on their plasma RBS, PGBS and HbA1c levels showed that the minor allelic frequency of the PST 297Ser allele, in general, gradually increased with increasing RBS, PGBS and HbA1c levels (Fig. 1E-H).

Although not statistically significant, the Gly/Ser (111.6 mg/dL) and Gly/Ser+Ser/Ser (112.5 mg/dL) individuals displayed higher fasting blood sugar levels, when compared to the Gly/Gly (107.8 mg/dL) individuals. Similarly, the log-transformed HOMA-IR (Homeostatic Model Assessment of Insulin Resistance) index levels were also higher (but did not attain statistical significance) for the Gly/Ser (0.27) and Gly/Ser+Ser/Ser (0.26) individuals, as compared to the Gly/Gly (0.23) individuals.

Enhanced dysglycemic/gluconeogenesis activities of the PST-297S peptide

In view of the association of the PST 297Ser allele with higher plasma glucose levels (Fig. 1A-B), we examined whether PST-297S exhibits enhanced ability to inhibit insulin-stimulated glucose uptake as compared to PST-WT peptide in relevant cell types (adipocytes and myotubes). Insulin augmented glucose uptake in differentiated 3T3-L1 adipocytes and differentiated L6 myotubes (by ~135%, p<0.01 and ~150%, p<0.001, respectively; Fig. 2A). PST-WT showed only a modest or no inhibitory effect on insulin-stimulated glucose uptake in these cells; on the other hand, PST-297S significantly diminished the insulin-stimulated glucose
uptake in adipocytes (from ~135% to ~110%; p<0.05) as well as in myotubes (from ~150% to ~128%; p<0.001) (Fig. 2A).

Next, we tested whether PST-297S and PST-WT peptides alter the expression of gluconeogenic genes (viz., \textit{PCK1} and \textit{G6PCL}) in HepG2 liver cells. While PST-WT peptide increased the endogenous expression of \textit{PCK1} by ~2.1-fold, the PST-297S peptide showed a ~4.6-fold increment (p<0.05) (Fig. 2B). Likewise, PST-WT increased the endogenous \textit{G6PCL} gene expression by ~2.2-fold whereas PST-297S caused a ~5.6-fold increment (p<0.05) (Fig. 2C).

**Plasma PST levels are elevated in type-2 diabetes**

In view of the strong association of PST with glucose homeostasis (Figs. 1-2) we checked whether the plasma PST levels differ between type-2 diabetic subjects and age-, sex- and BMI-matched healthy (normal glucose tolerant) controls. Diabetic subjects showed ~1.2-fold (p=0.042) increase in their plasma PST levels than the control subjects (SI Appendix Fig. S2).

**Influence of the 297Ser allele on catecholamine levels in the circulation**

As shown in Table 1, the Ser297 allele displayed association with HTN. Carriers of this allele also showed significantly higher levels of DBP than the Gly/Gly (wild-type) individuals [Gly/Gly (80.15 mm Hg) vs Gly/Ser (81.6 mm Hg; p=0.018) and Gly/Ser+Ser/Ser (81.6 mm Hg; p=0.017)]. As plasma catecholamine levels regulate blood pressure, we measured the catecholamine levels in the plasma samples of Gly/Gly and Gly/Ser individuals. Gly/Ser subjects displayed elevated levels of plasma norepinephrine (by ~1.6-fold, p=0.0003) and epinephrine (by ~1.5-fold, p=0.015) than Gly/Gly subjects (Fig. 3A). The p-values for both remained significant after adjustments for age, sex and BMI (p=0.0004 for norepinephrine and p=0.036 for epinephrine). Consistent with these findings, the PST-297S variant peptide evoked enhanced $^3$H-norepinephrine secretion activity both in neuronal SH-SY5Y (~117% over basal, p<0.01) and neuroendocrine PC12 cells (~123% over basal, p<0.05) than the PST-WT peptide (~109% over basal in case of both SH-SY5Y and PC12 cells) (Fig. 3B).

**Differential interactions of PST peptides with insulin receptor (IR) and glucose-related protein 78 (GRP78): computational analysis**
The identity of a functional receptor for PST remains elusive. Since PST peptides exhibit several anti-insulin-like activities in adipocytes and skeletal muscle cells (10), which is supported by our present study as well, we hypothesized that PST peptides might interact with the insulin receptor (IR). In addition, recently, a few reports have also provided evidence for interaction of PST with glucose-related protein (GRP78) (23,29). Therefore, in the present study, we investigated whether PST-WT and PST-297S peptides display differential binding to IR and GRP78.

To begin with, predictions of interactions of PST peptides with IR and GRP78 were made in silico. First, we modeled the PST 52-mer peptides (PST-WT and PST-297S) and simulated them for 200 ns to obtain the best time-averaged, representative structures (SI Appendix Fig. S3A-B); all the residues were in the allowed regions of the Ramachandran plot (SI Appendix Fig. S3C). The PST-WT structure consisted of a stable helix comprising of the residues from Gln279 to Val293 whereas the PST-297S structure consisted of a stable helix comprising of the residues from Glu278 to Val293 (SI Appendix Fig. S3B). The % persistence of conformations carrying α-helix during the simulation period was higher for PST-297S (~80%) than that for PST-WT (~65%).

Next, we docked PST-WT and PST-297S peptides with IR. The peptides displayed remarkably different interactions with IR (Fig. 4A-B). Interestingly, PST binds to IR at the same region where insulin binds; the binding pocket in the IR is formed by two monomers such that PST peptides and insulin seem to share a common binding site (Fig. 4C). Briefly, 6 residues of PST-WT interact with 8 residues of IR monomer-1 through 1 hydrogen bond and 26 hydrophobic interactions. The hydrogen bond was formed between Glu255 (PST-WT) and Lys484 (IR) with a bond distance of 3.1 Å. Seven residues of PST-WT interact with 8 residues of IR monomer-2 via 102 hydrophobic interactions (Fig. 4D). On the other hand, PST-297S interacts with IR monomer-1 via 5 hydrogen bonds and 372 hydrophobic interactions (between 25 residues of PST-297S and 23 residues of IR monomer-1); 4 residues of PST-297S interact with 4 residues of IR monomer-2 via 1 hydrogen bond and 98 hydrophobic interactions. The hydrogen bonds formed between PST-297S and IR were between: Glu286 and Ser526; Glu290 and Asn711; Leu298 and Tyr708; Glu274 and Gln328; and Glu269 and Arg114 (2 hydrogen bonds) (Fig. 4E). The PST-297S and IR complex displayed lower binding free energy of -12.4 Kcal/mole and dissociation constant of 8.06×10^{-10} M whereas these values for the PST-WT and IR complex were: -12.02 Kcal/mole and 15.3×10^{-10} M (SI Appendix Table S5).
Similarly, each PST peptide was docked onto the GRP78 monomer. Analysis of the docked complexes revealed that PST peptides exerted profoundly different interactions with GRP78 (SI Appendix Fig. S4A-B). In brief, 9 residues of PST-WT interact with 13 residues of GRP78 via 1 hydrogen bond and 133 hydrophobic interactions. The hydrogen bond (distance=2.51 Å) was established between Gly297 of PST-WT and Asn389 of GRP78 (SI Appendix Fig. S4C). On the other hand, 14 residues of PST-297S interacts with 18 residues of GRP78 through 174 hydrophobic interactions and 3 hydrogen bonds; these hydrogen bonds were between Glu269 and Tyr396 (bond distance=2.23 Å), Arg300 and Gly48 (bond distance=2.87 Å) and Asp272 and Arg49 (bond distance=2.77 Å) residues (SI Appendix Fig. S4D). Consequently, the PST-297S and GRP78 complex displayed lower binding free energy (-9.18 Kcal/mol) and dissociation constant (0.185×10^{-6} M) than the PST-WT and GRP78 complex (binding free energy=-7.89 Kcal/mol and dissociation constant=1.64×10^{-6} M) (SI Appendix Table S5).

Differential interactions of PST peptides with IR and GRP78: in vitro validations

To validate the results of in silico docking of PST peptides to IR (Fig. 4), we performed competitive binding assays. An IR expressing construct was generated and transfected into HEK-293 cells and overexpression was confirmed using western blot (Fig. 5A). Saturation binding assays identified that the optimum concentration of the label to be 100 nCi of [125]I-Tyr insulin with reproducible binding upon use of 10 μg IR-expressing plasma membrane (SI Appendix Fig. S5A-B). Since insulin is known to bind to IR with high affinity, cold insulin was used as a positive control to confirm functional IR overexpression by assessing its ability to displace labeled [125]I-Tyr insulin under conditions of endogenous IR expression as well IR overexpression (Fig. 5B). Increasing concentrations of PST-WT and PST-297S were used to evaluate displacement of [125]I-Tyr insulin from the IR-overexpressing plasma membrane. Indeed, both the peptides were capable of displacing [125]I-Tyr insulin (Fig. 5C), though PST-297S resulted in greater displacement leading to lower AUC (area under curve; p=0.0002) of the displacement curve compared to PST-WT (Fig. 5D). Assessment of the potencies of the two peptides with respect to cold insulin in causing displacement of [125]I-Tyr insulin showed that the effect of PST-297S was closer to insulin, as compared to PST-WT. When expressed as a ratio of insulin as 100%, PST-297S (~72%) displayed significantly greater (p<0.0001) binding than PST-WT (~46%) (Fig. 5E). To further validate the binding of endogenous IR with PST peptides, we
used β-TC-6 insulinoma cells. Similar to HEK-293 cells, we also observed both PST peptides displaced $^{[125]}$I-Tyr insulin (Fig. 5F), and PST-297S showed greater displacement compared to PST-WT as evidenced by lower AUC (area under curve; p<0.0001) of the displacement curve (Fig. 5G). The effect of PST-297S was closer to insulin as compared to PST-WT with respect to cold insulin in causing displacement of $^{[125]}$I-Tyr insulin from plasma membrane of β-TC-6 insulinoma cells (p<0.0001) (Fig. 5H). These \textit{in vitro} binding studies support the \textit{in silico} docking studies (Fig. 4) and show that PST peptides interact differentially with IR.

To ascertain if the two PST peptides differ in terms of their binding affinity towards GRP78 as well, we first compared the potencies of PST peptides in causing inhibition of GRP78 ATPase activity \textit{in vitro}. This was achieved using a Malachite green-phosphate assay that measures the liberated free phosphate release that corresponds to an increase in absorbance at 620-640 nm. Inhibition of GRP78 ATPase activity is believed to be central to the dysglycemic role of PST (23). The stronger inhibitory effect of PST-297S (p<0.0001) on GRP78 ATPase activity, as compared to PST-WT (p=0.0051) with respect to control (Fig. 6A), suggests that PST-297S exhibits more potent dysglycemic effects. In addition, PST-297S (p<0.0001) was also capable of inhibiting tunicamycin-stimulated increase in GRP78 expression in HepG2 cells to a greater extent, as compared to PST-WT (p=0.0016) (Fig. 6B-C).

To parse out the interactions of the PST peptides with GRP78, competitive binding assays were performed using the similar strategy that was employed for the IR studies. Overexpression of GRP78 was achieved by generating a GRP78-expressing plasmid and transfecting it into HEK-293 cells (Fig. 6D). Following overexpression, the plasma membrane from the cells was isolated and increasing concentrations of PST-WT and PST-297S were used to evaluate displacement of labeled $^{[125]}$I-Tyr PST peptide. PST-297S peptide was capable of displacing labeled $^{[125]}$I-Tyr PST to a greater extent, as compared to PST-WT (Fig. 6E), as evidenced by the lower AUC (p=0.0021) for the displacement curve of PST-297S than that of PST-WT (Fig. 6F). This finding further suggests that PST-297S displays greater binding affinity toward GRP78 compared to PST-WT peptide, consistent with our \textit{in silico} predictions (\textit{SI Appendix} Fig. S4).

\textbf{DISCUSSION}

\textbf{Pancreastatin: a CHGA-derived physiological dysglycemic peptide}
PST is well-known for its dysglycemic activities such as inhibition of glucose-induced insulin secretion and insulin-stimulated glucose uptake in pancreatic islet beta cells/primary rat adipocytes (2,30). Exogenous PST administration caused insulin resistance in wild-type as well as CHGA-null (i.e., PST-deficient) mice fed with normal chow diet; consistently, the PST deficient mice exhibited protection against high fat diet-induced insulin resistance (31). While there is no report on the association of CHGA with diabetes, elevated levels of circulating CHGA have been observed in several cardiovascular disease states including HTN, myocardial infarction and dilated/hypertrophic cardiomyopathy. There was always a notion, ever since the discovery of PST, that the circulating levels of the peptide might be elevated in diabetic subjects (2,11). To investigate this, we measured the plasma PST levels in type-2 diabetic subjects and age/sex/BMI-matched healthy controls. Indeed, PST levels showed a significant increase in type-2 diabetics as compared to the controls (SI Appendix Fig. S2). Since the plasma immunoassay can detect unprocessed CHGA as well as processed PST, the elevated PST levels in type-2 diabetic subjects could be a reflection of either higher amount of CHGA or a greater degree of CHGA processing to PST. Further analysis of PST levels in larger populations may establish elevated PST levels as an intermediate phenotype for metabolic disorders.

**Association of the PST 297Ser variant with diabetes and metabolic syndrome**

Previous studies discovered several genetic variations within the PST region in various human populations (12,13). Among these variants, p.Gly297Ser occurs within the functionally-important C-terminus of the peptide and is the most frequent one with an estimated prevalence in ~300 million individuals worldwide. The present study revealed association of the PST 297Ser allele with elevated levels of biochemical and physiological parameters that are well-known risk factors for cardiometabolic diseases. Importantly, plasma glucose and HbA1c (Fig. 1A-D) displayed significant associations with the PST 297Ser allele.

The plasma catecholamines levels were also significantly elevated in Gly/Ser subjects (Fig. 3A), an observation in line with more effective catecholamine secretion triggered by PST-297S peptide as compared to the PST-WT peptide in neuroblastoma and adrenal pheochromocytoma cells (Fig. 3B). Catecholamines have an adverse impact on various aspects of cardiac pathology, including atherosclerosis, a key risk factor for CAD (32,33). Indeed, therapeutic strategies to combat myocardial infarction include beta-blockade, further underscoring the contribution of
catecholamine action on myocardial damage. Of note, catecholamines are known to enhance both glycogenolysis and gluconeogenesis leading to higher blood glucose levels and inhibition of insulin-mediated glycogenesis (34). In addition, elevated plasma catecholamine levels are linked to HTN (35). Therefore, it is conceivable that the PST 297Ser allele may enhance the risk for T2D/HTN/CAD/MS (Table 1) concomitant with its adverse effects on biochemical/physiological phenotypes. In addition, upon genotyping chronic kidney disease (CKD) subjects from another cohort consisting of unrelated Indian subjects, we found that the PST 297Ser allele frequency in the CKD population with type-2 diabetes was ~1.7-fold higher than that in the CKD population without type-2 diabetes (data not shown). Thus, the p.Gly297Ser variant also seems to enhance the disease risk in diabetic kidney disease.

Replication of association of PST p.Gly297Ser variant with cardiometabolic conditions using genome-wide association study datasets

To test whether the association of PST p.Gly297Ser variant with cardiometabolic traits in Indian populations is replicable in other populations, we examined several available genome-wide association studies (GWAS) datasets from different world populations (SI Appendix Table S6; data obtained from https://t2d.hugeamp.org/). In general, the variant seemed to increase the disease risk (SI Appendix Table S6). Among the cardiometabolic conditions tested, interestingly, the variant showed modest associations with T2D in two of the studies – the DIAGRAM 1000G GWAS and DIAMANTE T2D GWAS. In the DIAGRAM 1000G GWAS, which primarily focused on samples of European descent, the variant showed an association with T2D upon adjusting for BMI (n=56443, p=0.024, OR=1.234). In the DIAMANTE T2D GWAS carried out in a European population as well, the variant showed an association trend (p=0.099, OR=1.059) with T2D in a large sample size of n=231420. These directionally-concordant findings in other populations of the world support our observation that p.Gly297Ser serves as a risk variant for cardiometabolic disorders. Of note, the findings of the BioMe AMP T2D GWAS suggested that diabetic subjects with the p.Gly297Ser variant are more susceptible to develop several cardiovascular complications, such as peripheral vascular disease (OR=2.588, p=0.038, n=1350).

It is important to note that these GWAS have been carried out in European populations wherein the MAFs for this SNP are much lower than those in South Asian populations (SI Appendix Table S4). Similar GWAS in South Asian populations may yield better associations of
this SNP with cardiovascular phenotypes. In addition, many of the gene chip arrays (for example, Illumina Human610-Quad Bead chip and Illumina Human660-Quad Bead chip) used in GWAS do not probe for this SNP. Therefore, it is not surprising that many GWAS on T2D, particularly even in Indian subjects, have not identified rs9658664 as a risk variant for T2D.

Considering the observed differences in MAF for the p.Gly297Ser variant as well as phenotypic differences of cohorts in publically available databases, we tested the SNP in the context of evolutionary aspect. This variant is under negative selection pressure, as evidenced by \( F_{ST} \) values below 0.05 for all the data sets (SI Appendix Table S4).

**PST-297S peptide: a naturally occurring gain-of-function variant of PST with physiological relevance**

Since we found the PST 297Ser variant to be associated with metabolic disease states (Table 1), we asked whether PST-297S peptide exerts differential activities as compared to PST-WT peptide. While PST-297S peptide significantly blunted insulin-stimulated glucose uptake in differentiated myotubes and adipocytes, the effect of PST-WT peptide was modest/insignificant (Fig. 2A). This observation is consistent with the higher potency and efficacy of the PST-297S peptide in inhibiting 2-deoxyglucose uptake in rat primary adipocytes (11). The enhanced effect of PST-297S might be due to differential interactions with the PST receptor (discussed below). The PST-297S peptide also profoundly augmented the expression of rate-limiting enzymes (viz. \( PCK1 \) and \( G6PC1 \)) involved in the gluconeogenesis pathway in HepG2 cells (Fig. 2B-C), an observation in line with the stronger activation of \( PCK1/G6PC1 \) promoters by the variant peptide in the same cells (13). Since liver is the major site for gluconeogenesis in mammals, such a finding in the liver cell line, HepG2, is of physiological relevance and suggests that the carriers of the PST 297Ser allele might have enhanced gluconeogenesis (36). The observation of elevated RBS and PGBS levels in the carriers of the PST 297Ser allele supports this premise (Fig. 1A-B).

Thus, the PST-297S peptide appears to be a ‘gain of function’ variant showing higher activities for several cellular and molecular processes that we tested. In general, these PST peptides showed effects within the concentration range of 10-100 nM (Figs. 2-3). The effect of PST-WT on forearm glucose-uptake in human subjects was observed at ~200 nM (11). In addition, the glucose-uptake inhibitory effect of PST-WT on adipocytes had an IC\(_{50}\) of ~600 pM (11). Could such concentrations of PST be attained *in vivo*? The plasma PST levels in healthy...
human subjects vary in the range of 25-125 pg/mL \textit{(i.e.,} \textasciitilde5-25 pM) \textit{(11). However, the concentration of the PST precursor protein CHGA in secretory vesicles has been reported to be \textasciitilde4 mM and the concentration of CHGA in the extracellular space in the vicinity of the exocytotic pore just after exocytosis has been estimated as \textasciitilde0.4 mM \textit{(37). Thus, although the steady-state plasma concentration of these peptides may be in the picomolar range, their concentrations in the vicinity of secretory cells after its exocytotic release are likely to be much higher (perhaps, in the nanomolar range). Hence, the effects of the PST peptides on various cellular processes are likely to be physiologically relevant.

\textbf{Plausible molecular basis for the increased dysglycemic activities of the PST-297S peptide}

Most of the effects of PST peptides are likely to be receptor-mediated since these effects are very prompt \textit{(within seconds to minutes). Although previous studies have suggested that PST activates a receptor signaling pathway that involves seven-transmembrane spanning receptor(s) coupled to Gq-PLC\textbeta-Ca\textsuperscript{2+}-PKC signaling and NO-dependent pathways, the identity of the PST receptor/cellular interacting partner(s) has remained elusive \textit{(38–42).}

In addition, the mechanism of antagonistic action of PST on insulin signaling also remains unclear. It may be hypothesized that the circulating plasma PST may encounter many receptors expressed on the membrane. Since the exact receptor for PST is still unknown and PST peptides consistently show insulin counter-regulatory effects, we asked whether PST peptides might interact with IR. Our \textit{in silico} docking analysis revealed that both PST peptides and insulin bind to the IR at the same binding pocket (Fig. 4C) and PST-297S shows less binding free energy and dissociation constant \textit{(i.e., more affinity) values towards IR than the PST-WT peptide \textit{(SI Appendix Table S5). In corroboration, IR-peptide binding assays \textit{in vitro} \textit{(in both HEK-293 cells and \textbeta-TC-6 insulinoma cells) provided evidence for stronger binding affinity for PST-297S (Fig. 5). Thus, enhanced anti-insulin effects of the PST-297S peptide might be due its higher affinity and stronger interactions with IR.}

Recent studies suggested that PST binds with GRP78 \textit{(23,29). GRP78 is an endoplasmic reticulum chaperone which serves as an endoplasmic reticulum stress indicator \textit{(43) and is involved in various cellular processes \textit{(44) such as, facilitating the folding and assembly of proteins \textit{(45), and regulating the Unfolded Protein Response \textit{(46). GRP78 plays key roles in cardiac development, cardiomyocyte survival and function \textit{(47). Moreover, GRP78 exerts\textit{}}}}
cardioprotective effects in the face of I/R injury (48). GRP78 overproduction in pancreatic beta cells protects against high-fat-diet-induced glucose intolerance and insulin resistance (49). Given such an influence of GRP78, we asked whether the PST peptides could be differentially interacting with GRP78, resulting in the differential glucose-related phenotypes observed. Indeed, the docking of the PST peptides onto GRP78 revealed that PST-297S exerted higher affinity interactions as compared to PST-WT (SI Appendix Fig. S4A-D). In corroboration, binding assays demonstrated that PST-297S peptide displaced labeled $^{125}$I-Tyr PST from GRP78-expressing plasma membranes to a greater extent as compared to PST-WT (Fig. 6).

Taken together, PST-297S peptide showed higher binding affinity to both IR and GRP78 than PST-WT peptide. These differential interactions provide a molecular basis for the enhanced activities of PST-297S over PST-WT for various cellular processes and may mediate the underlying deleterious phenotype for increased disease risk.

Limitation of the study

Very few PST homozygous variant (i.e., Ser/Ser) individuals (n=20) were identified in our study population (n=4318). Moreover, these subjects did not have phenotypic data for all the parameters. Therefore, a direct comparison of their parameters with the wild-type (i.e., Gly/Gly) subjects was not meaningful. In view of this limitation, we combined the Ser/Ser homozygotes with the Gly/Ser heterozygotes for most of the analyses. It would be interesting to test genotype-phenotype associations between PST Gly/Gly and PST Ser/Ser subjects in larger study populations in future.

Conclusions and perspectives

We discovered a common genetic variation (p.Gly297Ser) within the physiological dysglycemic peptide pancreastatin, a proteolytic fragment of the prohormone CHGA that is expressed in secretory vesicles across endocrine/neuroendocrine/neuronal cell types. The 297Ser allele was associated with elevated levels of several cardiometabolic traits including plasma glucose, HbA1c, diastolic blood pressure and catecholamines in human subjects. In corroboration, human carriers of the 297Ser allele were associated with increased risk for cardiometabolic diseases in human populations. Our multidisciplinary studies provided molecular basis for enhanced effects of the variant peptide (as compared to the wild-type peptide) on several relevant
cellular/molecular functions that might lead to an increased disease risk in the carriers of 297Ser allele. A schematic of our findings is presented in Fig. 7. These results have implications for inter-individual variations in glucose/catecholamine homeostasis and ultimately for pathogenesis of cardiometabolic disease states.

**AUTHOR CONTRIBUTIONS**

NRM conceived and designed the study. PKRA, MKi, SDM, VRC, RG, DV, SR, LS, BSS, DRI, SM and SSh carried out the experimental and computational analyses. MSR, MKh, AKM, JRG, SSe, ASM, VM, VR, SVNP and NRM provided equipment/reagents/clinical samples and reviewed/edited the manuscript. PKRA, MKi, VRC and NRM wrote the manuscript.

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NRM is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the integrity of the data and accuracy of the data analysis.

**DISCLOSURES/ CONFLICT OF INTEREST**

None

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### Table 1 Association of PST 297Ser allele with the risk for cardiometabolic diseases *

| Model   | Disease Condition | OR (95% CI)                  | p-value | OR (95% CI)                  | p-value | OR (95% CI)                  | p-value | OR (95% CI)                  | p-value |
|---------|-------------------|-------------------------------|---------|-------------------------------|---------|-------------------------------|---------|-------------------------------|---------|
|         |                   | Unadjusted | Age-adjusted | Sex-adjusted | BMI-adjusted | Age-, Sex- and BMI-adjusted |         | Age-, Sex- and BMI-adjusted |         |
| T2D     |                  | 1.361      | 1.372         | 1.356         | 1.425         | 1.475                           |         | 1.055-1.755                  | 1.058-1.779 | 1.051-1.749 | 1.064-1.909 | 1.072-2.028 | p=0.018 | p=0.017 | p=0.019 | p=0.017 |
|         |                   | 1.429      | 1.455         | 1.431         | 1.303         | 1.244                           |         | 1.077-1.897                  | 1.093-1.938 | 1.075-1.905 | 0.889-1.909 | 0.835-1.853 | p=0.013 | p=0.010 | p=0.014 | p=0.175 |
| GG vs.  |                  | 1.520      | 1.425         | 1.468         | 1.672         | 1.253                           |         | 1.113-2.076                  | 1.037-1.960 | 1.050-2.053 | 1.141-2.451 | 0.786-1.997 | p=0.008 | p=0.029 | p=0.025 | p=0.343 |
| GA      |                  | 1.416      | 1.404         | 1.402         | 1.474         | 1.454                           |         | 1.138-1.763                  | 1.127-1.750 | 1.125-1.748 | 1.128-1.925 | 1.094-1.932 | p=0.008 | p=0.025 | p=0.008 | p=0.343 |
|                | GG vs. GA+AA | p=0.002 | p=0.003 | p=0.003 | p=0.004 | p=0.010 |
|----------------|--------------|---------|---------|---------|---------|---------|
| **T2D**        |              |         |         |         |         |         |
|                | p=0.022      | 1.337   | 1.336   | 1.331   | 1.440   | 1.476   |
|                | 1.042-1.715  | (1.036-1.724) | (1.038-1.708) | (1.080-1.919) | (1.077-2.022) |         |
| **HTN**        |              |         |         |         |         |         |
|                | p=0.017      | 1.401   | 1.413   | 1.386   | 1.335   | 1.285   |
|                | 1.062-1.848  | (1.067-1.872) | (1.047-1.835) | (0.919-1.939) | (0.870-1.898) | p=0.207 |
| **CAD**        |              |         |         |         |         |         |
|                | p=0.017      | 1.457   | 1.367   | 1.388   | 1.612   | 1.237   |
|                | 1.071-1.982  | (1.098-1.873) | (1.098-1.932) | (1.102-2.357) | (0.778-1.969) | p=0.368 |
| **MS**         |              |         |         |         |         |         |
|                | p=0.003      | 1.378   | 1.360   | 1.360   | 1.473   | 1.455   |
|                | 1.113-1.707  | (1.097-1.687) | (1.097-1.686) | (1.133-1.915) | (1.099-1.926) | p=0.009 |

* Logistic regression analysis was carried out in the overall Indian population (consisting of both South Indian and North Indian subjects). Odds ratio for T2D, HTN, CAD and MS (subjects with T2D, HTN, T2D+HTN, IGT and CAD) in subjects having GA (i.e., Gly/Ser) or GA+AA (i.e., Gly/Ser+Ser/Ser) genotypes with respect to subjects having the wild-type GG (i.e., Gly/Gly) genotype were analyzed. Statistically significant values have been shown in bold. The association data for GG vs. AA has not been included in this
table as none of the disease conditions reached significant association; this could be attributed to the low number of homozygous variants (n=20) in the population.

T2D, type-2 diabetes; HTN, hypertension; CAD: coronary artery disease; MS, metabolic syndrome; IGT, intermediate glucose tolerance; BMI, body mass index.
FIGURE LEGENDS

Fig. 1 Association of p.Gly297Ser variation with phenotypic traits for type-2 diabetes. The relevant phenotypes of subjects having Gly/Gly genotype were compared to those of subjects having Gly/Ser and Gly/Ser+Ser/Ser genotypes. (A) RBS (mg/dL), (B) PGBS (mg/dL), (C) HbA1c (%) and (D) HbA1c (mmol/mol) levels of the genotype groups in the overall population. Data shown in panels A-D have been represented as mean ± S.D. To evaluate the significance of allele-specific associations, adjusted linear regression analyses were carried out. RBS: Gly/Gly vs. Gly/Ser, unadjusted p=0.014 and age-, BMI- and sex-adjusted p=0.071; Gly/Gly vs. Gly/Ser+Ser/Ser, unadjusted p=0.022 and age-, BMI- and sex-adjusted p=0.102; PGBS: Gly/Gly vs. Gly/Ser, unadjusted p=0.015 and age-, BMI- and sex-adjusted p=0.092; Gly/Gly vs. Gly/Ser+Ser/Ser, unadjusted p=0.014 and age-, BMI- and sex-adjusted p=0.071; HbA1c: Gly/Gly vs. Gly/Ser, unadjusted p=0.004, age-, BMI- and sex-adjusted p=0.005; Gly/Gly vs. Gly/Ser+Ser/Ser, unadjusted p=0.003 and age-, BMI- and sex-adjusted p=0.002. Frequency of occurrence of Gly and Ser alleles in the three different groups divided on the basis of (E) random blood sugar levels - normal (<100 mg/dL), pre-diabetic (100-125 mg/dL) and diabetic (>125 mg/dL), (F) post-glucose blood sugar levels - normal (<140 mg/dL), pre-diabetic (140-200 mg/dL) and diabetic (>200 mg/dL), (G) HbA1c levels - normal (<5.7%), pre-diabetic (5.7%-6.4%) and diabetic (>6.4%) and (H) HbA1c levels - normal (<39 mmol/mol), pre-diabetic (39-46 mmol/mol) and diabetic (>46 mmol/mol).

PST, pancreastatin; RBS, random blood sugar; PGBS, post-glucose blood sugar; HbA1c, glycosylated hemoglobin; BMI, body mass index.

Fig. 2 Effect of PST peptides on insulin-stimulated glucose uptake in adipocytes/skeletal muscle cells and gluconeogenic gene expression in liver cells. (A) Insulin-stimulated glucose uptake in differentiated 3T3-L1 (adipocytes) and L6 (myotubes) post treatment with human PST peptides (100 nM). One-way ANOVA followed by Tukey’s multiple comparison post-hoc test analysis was carried out to compare the values across different conditions. PST-297S peptide showed a stronger inhibitory effect on insulin-stimulated glucose uptake in both the cell types.
For 3T3-L1 adipocytes, one-way ANOVA F=10.02, p=0.0044; for L6 myotubes: one-way ANOVA F=110.3, p<0.0001. Each data point represents mean ± S.D. value from three wells of a representative experiment. (B) Abundance of PCK1 and (C) G6PC1 transcripts, expressed as AU in HepG2 cells upon treatment with PST-WT and PST-297S peptides were determined using quantitative real-time PCR. Augmented expressions of endogenous levels of PCK1 (one-way ANOVA F=6.125, p=0.029; panel B) and G6PC1 (one-way ANOVA F=5.277, p=0.04; panel C) were observed. β-Actin was used as the housekeeping control.

PST, pancreastatin; PST-WT, wild-type pancreastatin peptide; PST-297S, variant pancreastatin peptide; ANOVA, analysis of variance; PCK1, human phosphoenolpyruvate carboxykinase-1; G6PC1, human glucose-6-phosphatase; AU, arbitrary units; PCR, polymerase chain reaction.

**Fig. 3** Influence of PST 297Ser allele on plasma catecholamine levels and catecholamine secretion from neuronal/neuroendocrine cells. (A) Plasma catecholamine levels in Gly/Gly and Gly/Ser subjects recruited at Madras Medical Mission, Chennai. Data are expressed as mean ± S.D. Statistical significance between genotype groups was analyzed using linear regression analysis; unadjusted p=0.0003 for norepinephrine and p=0.015 for epinephrine; age-, sex- and BMI-adjusted p=0.0004 for norepinephrine and p=0.036 for epinephrine. (B) Effect of PST peptides on 3H-norepinephrine release from SH-SY5Y (neuroblastoma) and PC12 (adrenal pheochromocytoma) cells. Each data point represents mean ± S.D. from three wells of a representative experiment. Statistically significant differences between groups were calculated using one-way ANOVA followed by Tukey’s multiple comparison post-hoc test. One-way ANOVA F=11.51, p=0.0088 for SH-SY5Y cells and one-way ANOVA F=8.481, p=0.0364 for PC12 cells.

PST, pancreastatin; ANOVA, analysis of variance.

**Fig. 4** Differential interactions of human PST peptides with insulin receptor. (A) A snapshot of the docked complex between PST-WT and IR. Hydrogen bond interactions (between Glu255 and Lys484) and atoms forming hydrogen bonds have been highlighted; PST-WT has been shown in orange color. (B) A snapshot of the docked complex between PST-297S and IR. Hydrogen bonds between Glu269 and Arg114 (2 hydrogen bonds); Glu274 and Gln328; Glu286 and Ser526; Glu290 and Asn711; and Leu298 and Tyr708 have been shown. PST-297S has been
shown in blue color. IR monomers have been distinguished using different colors (cyan and grey). (C) Insulin, PST-WT and PST-297S peptides were docked individually to IR using ZDOCK and all docked complexes were subsequently superimposed. Docking complex for PST-WT, PST-297S and insulin showed a common binding site in the IR structure. Insulin, PST-WT and PST-297S have been shown in brown, orange and blue, respectively. IR has been represented in grey and cyan colors. (D) IR showed binding to PST-WT through both its monomers (monomer 1 and monomer 2) differently. PST-WT binds to IR through one hydrogen bond (to the monomer 1) and 128 non-bonded contacts (26 with monomer 1 and 102 with monomer 2); the interacting residues have been shown. (E) PST-297S peptide and IR were docked using ZDOCK. PST-287S binds to IR through six hydrogen bonds (five with monomer 1 and one with monomer 2) and 470 hydrophobic interactions (372 with monomer 1 and 98 with monomer 2). In (D) and (E), hydrogen bonds have been shown as sky blue colored lines and hydrophobic interactions have been represented as dotted lines.

PST, pancreastatin; PST-WT, wild-type pancreastatin peptide; PST-297S, variant pancreastatin peptide; IR, insulin receptor.

**Fig. 5 In vitro interaction studies between human PST peptides and IR.** (A) Western blot for IR expression post-transfection of HEK-293 cells with increasing amounts of IR-overexpressing construct – lane 1: 0 μg, lane 2: 0.5 μg, lane 3: 1 μg, lane 4: 2 μg and lane 5: 3 μg of IR-overexpressing construct. (B) The ability of cold insulin to displace labeled $^{[125]}$I-Tyr insulin was assessed under endogenous and IR-overexpression conditions. HEK-293 cells were transfected with IR-overexpressing construct (8 μg/100 mm tissue culture dish) using TurboFect™ transfection reagent. The different conditions were compared using one-way ANOVA followed by Tukey’s multiple comparisons test; n=6, one-way ANOVA F=151.3, p<0.0001. (C) Competitive binding assay was performed to assess the ability of PST-WT or PST-297S peptides to displace labeled $^{[125]}$I-Tyr Insulin from the plasma membrane of IR-overexpressing HEK-293 cells by adding increasing concentrations (0, 100 pM through 50 μM) of the PST peptides along with 100 nCi of labeled insulin to 10 μg of isolated plasma membrane. Displacement was calculated as a percentage of receptors in the control samples that did not contain any competing peptides. (D) Quantitative analysis of the graph in (C) was performed by comparing the AUC of the displacement curves of PST-WT and PST-297S peptides using an unpaired two-tailed
Student’s t-test (n=3). (E) Competitive binding assay was performed to compare the ability of PST-WT or PST-297S peptides with that of cold insulin in displacing labeled $^{125}$I-Tyr insulin from the plasma membrane of IR-overexpressing HEK-293 cells. Increasing concentrations (0, 100 pM through 50 μM) of the PST peptides and cold insulin along with 100 nCi of labeled $^{125}$I-Tyr insulin were added to 10 μg of isolated plasma membrane. Quantitative representation of the binding ability of PST peptides to IR, with respect to binding of insulin to IR (taken as 100%). The PST-WT and PST-Ser groups were compared using unpaired Student’s t-test (n=3).

(F) Competitive binding assay was performed to assess the ability of PST-WT or PST-297S peptides to displace labeled $^{125}$I-Tyr Insulin from the plasma membrane of β-TC-6 cells by adding increasing concentrations (0, 100 pM through 50 μM) of the PST peptides along with 100 nCi of labeled insulin to 10 μg of isolated plasma membrane. Displacement was calculated as a percentage of receptors in the control samples that did not contain any competing peptides. (G) Quantitative analysis of the graph in (F) was performed by comparing the AUC of the displacement curves of PST-WT and PST-297S peptides using an unpaired two-tailed Student’s t-test (n=3).

(H) Competitive binding assay was performed to compare the ability of PST-WT or PST-297S peptides with that of cold insulin in displacing labeled $^{125}$I-Tyr insulin from the plasma membrane of β-TC-6 cells. Increasing concentrations (0, 100 pM through 50 μM) of the PST peptides and cold insulin along with 100 nCi of labeled $^{125}$I-Tyr insulin were added to 10 μg of isolated plasma membrane. Quantitative representation of the binding ability of PST peptides to IR, with respect to binding of insulin to IR (taken as 100%). The PST-WT and PST-Ser groups were compared using unpaired two-tailed Student’s t-test (n=3).

PST, pancreastatin; PST-WT, wild-type pancreastatin peptide; PST-297S, variant pancreastatin peptide; IR, insulin receptor; ANOVA, analysis of variance; AUC, area under curve; Ins, insulin.

**Fig. 6 In vitro interaction studies between human PST peptides and GRP78.** (A) Spectrophotometric Malachite green-phosphate assay was performed to compare the effect of 2 μM PST-WT and PST-297S on inhibition of GRP78 ATPase activity. The different groups were compared using one-way ANOVA followed by Tukey’s multiple comparisons test; n=4, one-way ANOVA F=30.93, p<0.0001. (B) Human HepG2 hepatocytes were treated with 100 nM PST-WT or PST-297S along with 5 μg/mL tunicamycin or with 5 μg/mL tunicamycin alone for 24 h. Changes in GRP78 expression following this treatment were visualized using an
immunoblot with anti-GRP78 antibody (control: anti-β-actin). (C) Quantitative analysis of the immunoblot in (B) was performed using one-way ANOVA followed by Tukey’s multiple comparisons test; n=5, one-way ANOVA F=35.29, p<0.0001. (D) Western blot for GRP78 expression post-transfection of HEK-293 cells with increasing amounts of GRP78-overexpressing construct – lane 1: 0 μg, lane 2: 0.5 μg, lane 3: 1 μg, lane 4: 2 μg, lane 5: 3 μg of GRP78-overexpressing construct. (E) Competitive binding assay was performed to assess the ability of PST-WT or PST-297S peptides to displace labeled [125I]-Tyr PST by adding increasing concentrations (0, 10 pM through 1 μM) of the PST peptides along with 100 nCi of labeled PST to 10 μg of isolated plasma membrane. Displacement was calculated as a percentage of receptors in the control samples that did not contain any competing peptides. (F) Quantitative analysis of the graph in (E) was performed by comparing the AUC of the displacement curves of PST-WT and PST-297S using an unpaired two-tailed Student’s t-test (n=4).

PST, pancreastatin; PST-WT, wild-type pancreastatin peptide; PST-297S, variant pancreastatin peptide; GRP78, glucose-regulated protein 78; ANOVA, analysis of variance; AUC, area under curve.

Fig. 7 A schematic representation of the plausible mechanistic basis for increased cardiometabolic disease risk associated with the PST 297Ser allele. Occurrence of a non-synonymous genetic variation within the PST region results in the PST p.Gly297Ser variant. PST-297S peptide differs from PST-WT in its secondary structure (especially, α-helical content). Structural differences between PST-WT and PST-297S peptides and their consequent differential interactions with GRP78 and IR may cause enhanced potencies of the PST-297S peptide for inhibition of insulin-stimulated glucose uptake and activation of the gluconeogenesis pathway. These and associated cellular/molecular processes may elevate the levels of plasma glucose, HbA1c and catecholamines in the carriers of the PST 297Ser allele, thereby increasing their risk for type-2 diabetes, hypertension, coronary artery disease and metabolic syndrome.

PST, pancreastatin; PST-WT, wild-type pancreastatin peptide; PST-297S, variant pancreastatin peptide; GRP78, glucose-regulated protein 78; IR, insulin receptor; HbA1c, glycosylated hemoglobin.
Figure 1

A. RBS (mg/dL)
- Gly/Gly: 111.07 (n=450), 122.90 (n=67), 121.87 (n=70)
- Gly/Ser: 143.59 (n=1231), 157.27 (n=159)
- Ser/Ser: 5.80 (n=1104), 6.00 (n=136), 6.00 (n=142)

B. PGBS (mg/dL)
- Gly/Gly: 143.59 (n=1231), 157.27 (n=159)
- Gly/Ser: 5.80 (n=1104), 6.00 (n=136)
- Ser/Ser: 39.94 (n=1104), 42.11 (n=136), 42.13 (n=142)

C. HbA1c (%)
- Gly/Gly: 5.80, 6.00, 6.00
- Gly/Ser: 39.94, 42.11, 42.13
- Ser/Ser: 8.72, 8.72

D. HbA1c (mmol/mol)
- Gly/Gly: 39.94, 42.11, 42.13
- Gly/Ser: 8.72, 8.72
- Ser/Ser: 5.80, 6.00, 6.00

E. MAF (%) for RBS
- <100: 93.45 (n=374), 92.34 (n=124), 88.64 (n=22)
- <140: 94.56 (n=900), 93.32 (n=202)
- <200: 95.19 (n=603), 93.88 (n=425)
- >46: 95.19 (n=603), 93.88 (n=425)

F. MAF (%) for PGBS
- <100: 93.45 (n=374), 92.34 (n=124), 88.64 (n=22)
- <140: 94.56 (n=900), 93.32 (n=202)
- <200: 95.19 (n=603), 93.88 (n=425)
- >46: 95.19 (n=603), 93.88 (n=425)

G. MAF (%) for HbA1c
- <100: 93.45 (n=374), 92.34 (n=124), 88.64 (n=22)
- <140: 94.56 (n=900), 93.32 (n=202)
- <200: 95.19 (n=603), 93.88 (n=425)
- >46: 95.19 (n=603), 93.88 (n=425)
Figure 2
Figure 3

A

Norepinephrine

Epinephrine

Plasma catecholamines (pg/ml)

PST Gly/Gly Gly/Ser
(n=122) (n=17)
PST Gly/Gly Gly/Ser
(n=119) (n=14)

p=0.0003

p=0.015

B

Effect on \(^3\)H-Norepinephrine release

(% over basal)

SHSY-5Y
PC12

Basal PST PST 297S
Basal PST PST 297S

p<0.01

p<0.05

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Figure 5
Figure 6
| Structural/Functional aspects | PST G297S | PST WT |
|-----------------------------|-----------|--------|
| α-helical content           | Higher    | Lower  |
| Insulin-stimulated glucose uptake | Less    | More   |
| Gluconeogenesis pathway     | More activated | Less activated |
| Binding affinity to GRP78 and IR | Higher | Lower |
| **Cellular and Molecular level** |           |        |
| Plasma blood sugar, HbA1c and catecholamine levels | Elevated in the carriers of 297Ser allele | Normal in the carriers of wild-type allele |
| **Biochemical level**       |           |        |
| Risk for type-2 diabetes, hypertension, coronary artery disease and metabolic syndrome | ↑ | ↓ |
| **Physiological level**     |           |        |
SUPPLEMENTAL MATERIAL

Functional Gly297Ser Variant of the Physiological Dysglycemic Peptide Pancreastatin is a Novel Risk Factor for Cardiometabolic Disorders

by

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Running title: Pancreastatin variant and cardiometabolic disorder

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SUPPLEMENTARY INFORMATION

Human subjects and methodologies

We recruited 3602 unrelated volunteers from urban Chennai cosmopolitan population at three study centers: (i) Madras Diabetes Research Foundation [n=2464 subjects consisting of 1573 with hypertension (HTN) and/or type-2 diabetes (T2D) and/or intermediate glucose tolerance (IGT) and 891 healthy controls]; (ii) Madras Medical Mission (n=625 subjects with HTN/T2D); (iii) Madras Medical College [n=513 subjects with coronary artery disease (CAD)]. These three collection centers from Chennai mainly consisted of people from South Indian ancestry. We also recruited another group of unrelated subjects (n=716; consisting of 355 hypertensives and 361 normotensive controls) from North India at the Post Graduate Institute of Medical Education and Research, Chandigarh. The detailed demographic and clinical parameters are given in SI Appendix Tables S1 and S2.

In the overall population (Chennai and Chandigarh populations) (n=4318), the mean age of the subjects was ~48 years; ~27.2% of the subjects were type-2 diabetics; ~18.0% subjects were hypertensives; ~8.7% subjects had both T2D and HTN; ~5.1% had IGT; ~11.9% subjects had CAD; and ~29% subjects were healthy controls. The following criteria were adopted for disease classifications - diabetics: fasting blood sugar (FBS) ≥7.0 mmol/L (126 mg/dL), treatment with anti-diabetics drugs, or history of disease; hypertensives: systolic blood pressure (SBP) ≥140 mm Hg and/or diastolic blood pressure (DBP) ≥90 mm Hg or a history of hypertension and anti-hypertensive treatments; CAD: subjects with at least one block in their coronary artery or initial stage of myocardial infarction based on findings from angiogram and electro/echo-cardiogram. Normotensives served as the control subjects for hypertensives, while individuals with normal glucose tolerance served as controls for type-2 diabetics. All the
controls had no history of disease and medication. None of the subjects had kidney disease or any type of cancer.

Demographic (age, gender), physical [height, weight, body mass index (BMI)] and physiological parameters [SBP, DBP, mean arterial pressure (MAP)], medication status and blood samples [in ethylenediaminetetraacetic acid (EDTA)-containing tubes] were collected from the study subjects. Plasma samples were also collected, aliquoted and stored at -80°C. Genomic DNA from the subjects was isolated as described previously (1). Exon-7 region of CHGA was PCR-amplified, purified and sequenced to detect the presence of single nucleotide polymorphisms (SNPs) in the pancreastatin (PST) domains in the first set of samples, as described previously (2). The next set of DNA samples was genotyped for the p.Gly297Ser SNP (rs9658664) using PCR-mediated allelic discrimination method (SI Appendix Fig. S1); 5.0 μL reaction mixture contained 10 ng of genomic DNA, 2.5 μL of 2× TaqMan® Universal PCR master mix (Applied Biosystems, Waltham, MA, USA), 0.125 μL of 40× TaqMan® SNP genotyping assay mix (assay ID: C__25598362_10; containing 8 μM fluorescently-tagged probes specific for the wild-type and the variant alleles, 36 μM each of forward and reverse primers). PCR amplification conditions were according to the manufacturer’s protocol: 2 min at 50°C, 10 min at 95°C, followed by 15 s at 92°C and 60 s at 60°C for 40 cycles. Allelic discrimination with end-point detection of fluorescence was carried out using ABI 7900HT sequence detection system followed by analysis with SDS software (Applied Biosystems). Non-template and positive controls were routinely included to ensure specificity and accuracy in genotyping.

**Estimation of biochemical parameters**

BMI was calculated by dividing the weight in kilograms with the square of the height in meters. Biochemical analyses were carried out on a Roche Cobas 8000 and Roche Cobas c702
Autoanalyzer. Blood glucose and cholesterol were measured using GOD-POD and CHOD-POD methods, respectively. Serum creatinine and urea were measured using creatinine amido hydrolase and urease methods, respectively. The blood pressure readings were made in the sitting position using a brachial oscillometric cuff by experienced nursing staff. Plasma PST levels in age-/sex-/BMI-matched diabetic and control subjects were measured using an enzyme-linked immunosorbent assay kit (that utilized an antibody raised against the PST domain of CHGA; catalog number: TM E-9000, LDN), according to the manufacturer’s protocol.

**Catecholamine secretion and glucose uptake assays**

Briefly, SH-SY5Y and PC12 cells grown in poly-L-lysine-coated 12-well plates were incubated with 0.5 μCi of L-[7-3H] norepinephrine (PerkinElmer, Waltham, MA, USA) in DMEM with 10% fetal bovine serum (FBS) (for SHSY-5Y), DMEM with 10% horse serum and 5% FBS (for PC12 cells) for 2 h at 37°C. Cells were washed twice with release buffer (150 mM NaCl, 5 mM KCl, 2 mM CaCl₂ and 10 mM HEPES pH 7.0) and then incubated with serum-free media for 30 min at 37°C. The cells were then treated with release buffer along with 100 nM of PST peptides (PST-WT or PST-297S). After 20 min of treatment with the peptides, the secretion media was collected and the cells were lysed with lysis buffer [i.e., release buffer plus 0.1% (v/v) Triton™ X-100]. Secretion media and cell lysates were assayed for [3H]-norepinephrine counts using a Tricarb™ liquid scintillation counter (PerkinElmer) and the results have been expressed as % secretion: (amount released/[(amount released+amount in cell lysates)] × 100.

Estimation of glucose uptake by differentiated L6 and 3T3-L1 cells was carried out using a modified version of our previously reported protocol (2). In brief, cells were serum starved for 2 h and incubated in Krebs-Ringer HEPES buffer (120 mM NaCl, 5 mM KCl, 1.2 mM
MgSO₄, 1.3 mM CaCl₂, 1.3 mM KH₂PO₄ and 20 mM HEPES pH 7.4) without glucose in the presence or absence of 100 nM of PST peptides (PST-WT or PST-297S) for 10 min before stimulation with 20 nM insulin for 20 min. The cells were then incubated with 0.5 µCi [³H]-2-deoxy-D-glucose (PerkinElmer) for an additional 20 min. Cells were washed with ice-cold phosphate-buffered saline (PBS), lysed with 0.5 M NaOH and 0.1% SDS, following which the radioactivity in the cell lysates was measured. Total protein was estimated using the Bradford method.

**RNA extraction and real-time PCR**

To estimate endogenous phosphoenol pyruvate carboxykinase-1 (*PCK1*) transcript, real-time PCR was performed using the DyNAmo™ HS SYBR® Green qPCR Kit (Thermo Fisher Scientific) and human *PCK1* specific primers: forward, 5'-GAGAAAGCGTTCAATGCCA-3' and reverse, 5'-ACGTAGGGTGAATCCGTCAG-3'. Similarly, to estimate endogenous glucose-6-phosphatase (*G6PC1*), the following primers were used: forward, 5'-TGCCCCTGATAAAGCAGTTC-3' and reverse, 5'-GGTCGGCTTTATCTTTCCCT-3'. For normalization of the expression levels of human *PCK1* and *G6PC1* genes, *β-actin* abundance was measured using the following primers: forward, 5'-CTGGTGCCTGGGGCG-3' and reverse, 5'-AGCCTCGCCTTTGCGCA-3'. The relative gene expression levels of both the genes were determined using the 2⁻ΔΔCt method.

In this study, we have carried out cellular assays in different cell lines – HepG2, L6, SHSY-5Y, 3T3-L1 and PC12. The expression of *IR* and *GRP78* in all these cell lines has been reported previously – HepG2 (https://www.proteinatlas.org/ENSG00000044574-HSPA5/cell), L6 [IR: (3) and GRP78: (4)], SHSY-5Y (www.proteinatlas.org), 3T3-L1 [IR: (5) and GRP78: (6)] and PC12 [IR: (7) and GRP78: (8,9)]. In addition, using quantitative PCR, we found *GRP78* to be expressed in HepG2, L6 and SHSY-5Y as well (*SI Appendix* Fig. S6). Total RNA
samples from HepG2, SHSY-5Y and L6 cells were isolated as described previously (10). To estimate the endogenous GRP78 transcript, real-time PCR was performed using the DyNAmo™ HS SYBR® Green qPCR Kit and human GRP78 specific primers: forward, 5'-GGAAAGAAGGTTACCCATGC-3’ and reverse, 5'-AGAAGAGACACATCGAAGGT-3’ for the human cell lines, HepG2 and SHSY-5Y and rat GRP78 specific primers: forward, 5'-GAAACTGCGAGGCGTAT-3’ and reverse, 5'-ATGTTCTTCTCTCCCTCTCTTCA-3’ for the rat cell line L6. For normalization of human GRP78 gene expression, β-actin abundance was measured using the following primers: forward, 5'-CTGGTGCTGCTGAGGCG-3’ and reverse 5'-AGCCTCGCCTTTGGCGA-3’. For normalization of rat GRP78 gene expression, β-actin abundance was measured using the following primers: forward, 5'-GCTGTGCTATGTTGCCCTAG-3’ and reverse, 5'-CGCTCATTGCCCAGATAGTG-3’. The relative gene expression levels were determined using the 2^-ΔΔCt method.

**GRP78 ATPase activity**

To compare the effect of PST-WT and PST-297S on GRP78 ATPase activity, we performed spectrophotometric assay using recombinant human GRP78 protein (catalog no. ab78432, Abcam, Cambridge, MA, USA). PST-WT or PST-297S (2 µM) was co-incubated with GRP78 (1 µM) in 50 µL of assay buffer (20 mM Tris pH 7.5, 50 mM KCl and 1.5 mM MgCl₂) along with GRP78 alone in control wells. The reaction was started by adding 400 µM ATP and incubated at 37°C for 1 h. Liberated free phosphate (Pi) was measured using Malachite green-phosphate assay (catalog no. MAK307, Sigma-Aldrich, St. Louis, MO, USA) (n=4).

**GRP78 expression study in HepG2 cells**

To determine the inhibitory effect of PST-WT and PST-297S peptides on tunicamycin-stimulated GRP78 expression in HepG2 cells, we performed western blot. HepG2 cells were
cultured in 60-mm culture dishes. Upon reaching 80% confluence, the cells were treated with PST-WT or PST-297S (100 nM) along with tunicamycin (5 µg/mL) or with tunicamycin (5 µg/mL) alone for 24 h. No treatment was given in the control wells. After treatment, the cells were lysed in lysis buffer containing phosphatase and protease inhibitors. After separating equal amounts of protein using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), gels were transferred to a polyvinylidene difluoride (PVDF) membrane. The non-specific binding proteins on the membrane were blocked using 5% skim milk powder in PBS with Tween (PBST; 2 h, room temperature). Membranes were then probed with the primary antibody, anti-GRP78 Bip (clone C50B12, Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:1000 dilution in PBST, overnight at 4°C. After five washes with PBST, the membranes were incubated with HRP-conjugated anti-rabbit secondary IgG at a dilution of 1:3000 for 2 h at room temperature. After five washes, the blots were visualized using ECL detection kit (cat no. WBKLS0500, Immobilon Western Chemiluminescent HRP Substrate, Merck Millipore, Darmstadt, Germany). The signals (n=5) were normalized with β-actin and the blots were analyzed using ImageJ software (NIH, Bethesda, MD, USA).

**Generation of constructs for insulin receptor (IR) and glucose-regulated protein 78 (GRP78) overexpression**

IR-FLAG and GRP78-FLAG overexpressing constructs were generated by cloning IR cDNA (a kind gift from Dr. Frederick M. Stanley) and GRP78 cDNA (catalog no. 32701, Addgene, Watertown, MA, USA) flanked by the restriction sites HindIII and XbaI at the 5' and 3' ends, respectively into the MCS of pcDNA 3.1. The primers were designed such that post-cloning the recombinant plasmids expressed IR and GRP78 with a FLAG tag at the C-terminal and N-terminal, respectively. The following primers were used for cloning: IR-FP: 5'
CCCAAGCTTATGCACCTGTACCCCGAGAG-3', IR-RP: 5'-GCTCTAGATTA\textbf{CTTGTCGTCATCGTCTTTGTAGTC}GGAAGGATTGGACCGAGG-3', GRP78-FP: 5'-CCCAAGCTTATG\textbf{GACTACAAAGACGATGACGACAAG}ATGAAGCTCTCCCTGGT G-3' and GRP78-RP: 5'-GCTCTAGACTACA\textbf{ACCTCCCTTTTTCTGCTG}-3'. The clones generated were confirmed using Sanger sequencing. The bold and underlined nucleotides indicate the FLAG coding sequences.

The IR-FLAG and GRP-FLAG overexpressing plasmids were transformed into DH5α cells using heat shock protocol and the transformed cells were plated on ampicillin-containing plates. Couple of colonies were selected and inoculated for mini-preparation of the plasmid. The plasmid sequence was authenticated using sequencing. Following authentication, large-scale preparation of the plasmids was performed and used for transfection into HEK-293 cells. HEK-293 cells used for the binding assays were cultured in Minimal Essential Media supplemented with 10% FBS and appropriate antibiotics. To assess the expression, increasing concentrations of the plasmids were transfected to determine the optimum concentration required for robust expression of the GRP78 and IR proteins. Cells were transfected with either GRP78- or IR-overexpressing constructs (8 μg/100 mm tissue culture dish) using TurboFect™ (Thermo Fisher Scientific) transfection reagent. 48 h following transfection, the cells were serum starved for 6 h before being used for each of the individual experiments.

**Plasma membrane extraction and western immunoblotting**

β-TC-6 cells were cultured in DMEM with high glucose supplemented with 15% heat-inactivated FBS and 1× penicillin/streptomycin. β-TC-6 cells (2.5×10^6) were plated on 150 mm tissue culture dishes and cultured by changing the medium every 24 h for 3 d (~60% confluency). Isolation of plasma membrane fraction (from HEK-293 and β-TC-6 cells) and
western immunoblotting was performed as previously described (11). Briefly, the cells were scraped using ice-cold non-detergent lysis buffer (5 mM Tris-HCl pH 7.4, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 2 μg/mL of protease inhibitors leupeptin and aprotinin). The cells were dounced, toggled for 30 min at 4°C and centrifuged at 2500 rpm for 5 min at 4°C to remove cell debris and nuclei. The supernatant was centrifuged at 37,000 ×g for 30 min at 4°C. The pellet containing the plasma membrane was re-suspended in binding buffer (75 mM Tris-HCl pH 7.5, 2 mM EDTA and 12.5 mM MgCl₂) and protein estimation was performed using standard DC protein estimation (Bio-Rad, Hercules, CA, USA). 80 μg of the plasma membranes were resolved on an SDS-PAGE gel and transferred to a PVDF membrane for western immunoblotting.

**Generation of PST peptide structures and docking with IR and GRP78**

Molecular modeling of 52-mer PST-WT and PST-297S peptides was carried out using MODELLER program version 9v13 (12) with the previously derived 29-mer PST peptide structures (2) as templates because crystal or nuclear magnetic resonance structures of these peptides are not available. Initially, the PST-WT 52-mer and PST-WT 29-mer peptide sequences were aligned. On the basis of this alignment output and using the 29-mer PST peptide structure as a template, the PST-WT 52-mer structure was generated using MODELLER. Subsequently, Gly-297 was mutated to Ser-297 to generate the PST-297S peptide structure. The detailed methodologies for selecting the best models for wild-type and variant peptides from all the models generated by MODELLER have been presented (2). To mimic the physiological conditions, the modeled 52-mer PST-WT and PST-297S peptides were amide capped at the C-terminal end. The homology-modeled structures were further refined using molecular dynamics (MD) simulations for 200 ns. The stability of the simulated structures was assessed by plotting root mean square deviations (RMSD) of Cα atoms with
respect to the starting structure over the simulation period. The best models have all residues in allowed region of the Ramachandran plot. While RMSD for the PST-WT showed major fluctuations during the initial 80-90 ns, the PST-297S peptide did not display such fluctuations throughout the simulation time; the mean RMSD values for PST-WT and PST-297S were more or less similar after 90 ns. We also analyzed the secondary structural elements during the MD simulation. Consistent with the RMSD pattern the PST-297S peptide showed more stable secondary structural content throughout the trajectory (data not shown). The % of conformations carrying α-helix differed between the two peptides: 65% for PST-WT and 80% for PST-297S.

We used the protein-docking program, ZDOCK, to predict the binding of PST peptides to GRP78 monomer or IR. ZDOCK performs efficient global docking search on a 3D grid by using Fast Fourier Transform algorithm and scores docked complexes based on a combination of shape complementarity, electrostatics and statistical potential terms (13). ZDOCK predicted 100 binding modes of PST peptides with GRP78 and IR and these docked complexes were ranked according to ZDOCK score. Molecular interactions between PST peptides and GRP78 or IR were calculated using PDBsum database (14). ZDOCK scores between GRP78 and PST peptides were calculated by using ZDOCK 3.0.2. The binding energies (ΔG) and dissociation constants (K_d) between GRP78/IR and PST peptides were calculated using Protein-Protein Affinity Predictor (PPA-Pred) program (15). All the figures were rendered using PyMOL (The PyMOL Molecular Graphics System, version 1.5.0.4 Schrödinger, LLC).
SUPPLEMENTAL FIGURES

Fig. S1 Representative amplification plot for genotyping of the p.Gly297Ser polymorphism using TaqMan allelic discrimination method. Occurrence of G9358A SNP (rs9658664) that causes substitution of Gly by Ser at the 297th residue of the protein (i.e., the PST p.Gly297Ser variation) was analyzed using the allele discrimination method. Representative amplification plot demonstrates the segregation of the GG, GA, AA genotypes and no template control. A substantial increase in only VIC-dye fluorescence indicated homozygosity for allele A (shown in red), a considerable increase in only FAM-dye fluorescence indicated homozygosity for allele G (shown in blue), while the presence of both VIC- and FAM-dye fluorescence indicated allele A-allele G heterozygosity (shown in green). SNP, single nucleotide polymorphism; PST, pancreastatin.
**Fig. S2 Plasma PST levels are elevated in type-2 diabetic subjects.** Plasma PST levels in freshly thawed plasma samples from age-/sex-/BMI-matched subjects were estimated using an ELISA kit. The relative plasma PST values have been expressed as mean ± S.D. Type-2 diabetic subjects (n=45) displayed ~20% higher plasma PST levels (p=0.042) as compared to control subjects (n=43) upon being compared using linear regression analysis. The BMI-adjusted p=0.043 and age-/BMI-adjusted p=0.041.

ELISA, enzyme-linked immunosorbent assay; PST, pancreastatin.
Fig. S3 Root mean square deviation analysis, modeled structures and Ramachandran plots of human PST peptides.  
(A) RMSD profiles of backbone atoms of human PST-WT (black) and variant PST-297S (red) peptides with respect to the corresponding starting structures were generated using g_rms module in Gromacs-4.5.5. RMSD values have been shown as a function of time (ns). The PST-WT peptide got stabilized and reached a plateau state within the first 80 ns; on the other hand, the PST-297S peptide stabilized and reached a plateau within the first 40 ns. Homology-modeled structures of PST-WT and its variant PST-297S were optimized, equilibrated and simulated for 200 ns.  
(B) Representative final snapshots of PST-WT and PST-297S peptides are shown as cartoons, the mutation sites are shown in ball and stick and the C-terminal amide capping is shown in licorice representation.  
(C) The quality of the modeled structures was analyzed using Procheck (16). Ramachandran plots for the PST-WT and PST-297S structures have about 80.5% and 85.7% of residues, respectively, in the most-allowed regions and no residues in the disallowed regions suggesting that the modeled structures are of good quality. Amino acid residues falling in the allowed region are represented using blue colored dots. The yellow, brown and red colored areas represent generously allowed, additionally allowed and most favored regions in the plot, respectively. Regions A, B and L correspond to the residues involved in the formation of right-handed α-helix, β-sheet and left-handed helix, respectively.

RMSD, root mean square deviation; PST: pancreastatin; PST-WT, wild-type pancreastatin peptide; PST-297S, variant pancreastatin peptide.
Fig. S4 Differential interactions of human PST peptides with GRP78. (A) A snapshot of the docked complex between PST-WT and GRP78. The hydrogen bond interaction (between Gly297 and Asn389) and atoms forming hydrogen bond are highlighted; PST-WT is shown in light orange color. (B) A snapshot of the docked complex between PST-297S and GRP78. Hydrogen bond interactions (between Glu269 and Tyr396; Arg300 and Gly48; Asp272 and Arg49) are highlighted and PST-297S is shown in blue color. GRP78 is shown in grey color. (C) PST-WT was found to interact with GRP78 via 1 hydrogen bond and 133 non-bonded contacts, the interacting residues are shown. (D) PST-297S was found to interact with GRP78 through 3 hydrogen bonds and 174 non-bonded contacts, the interacting residues are shown. The hydrophobic interactions have been represented using dotted lines, while hydrogen bonds have been represented using blue-colored lines.

PST, pancreastatin; PST-WT, wild-type pancreastatin peptide; PST-297S, variant pancreastatin peptide; GRP78, glucose-regulated protein 78.
Fig. S5 Optimization of *in vitro* competitive binding experiments and comparison of the IR-binding abilities of PST peptides and insulin. (A) A binding assay with increasing concentrations of labeled PST (0, 5, 25, 50, 100 and 200 nCi) and 10 μg IR-expressing plasma membrane was performed in order to identify the optimal concentration of labeled $^{125}$I-Tyr PST to be used in the competitive binding experiments. (B) A binding assay with increasing amounts of IR-expressing plasma membrane (2.5, 5, 7.5, 10, 20 and 30 μg) and 100 nCi labeled $^{125}$I-Tyr PST was performed, to identify the optimal amount of IR-expressing plasma membrane to be used in the competitive binding experiments.

PST, pancreastatin; IR, insulin receptor.
**Fig. S6** Assessment of expression of GRP78 in the cell lines used in the study.  
(A) Relative abundance (expressed as arbitrary units) of human \textit{GRP78} (in HepG2 and SHSY-5Y) and rat \textit{GRP78} (in L6) was determined using quantitative RT-PCR. The \textit{GRP78} mRNA levels were normalised to \textit{β-actin} mRNA levels.  
(B) Gel picture showing the semi-quantitative PCR amplification of \textit{GRP78} (top) and \textit{β-actin} (bottom) using mRNA isolated from HepG2, SHSY-5Y and L6 cells as templates.  

RT-PCR: real-time PCR, mRNA: messenger RNA.
SUPPLEMENTAL TABLES

Table S1 Demographic profile of the study subjects *

| Study center/location | Parameter                      | Cases     | Controls  |
|-----------------------|--------------------------------|-----------|-----------|
|                       | N                              | 1573      | 891       |
| MDRF (South India)    | Age                            | 52.0 ± 0.30 | 40.7 ± 0.42 |
|                       | Male (%)/Female (%)            | 49.0%/51.0% | 42.4%/57.6% |
|                       | **Disease state**              |           |           |
|                       | Type-2 diabetes                | 1110 (70.57%) | -         |
|                       | Hypertension                   | 34 (2.16%) | -         |
|                       | Diabetes and hypertension      | 207 (13.16%) | -         |
|                       | Intermediate glucose tolerance | 222 (14.11%) | -         |
|                       | N                              | 625       | -         |
| MMM (South India)     | Age                            | 41.3 ± 0.29 | -         |
|                       | Male (%)/Female (%)            | 73.3%/26.7% | -         |
|                       | **Disease state**              |           |           |
|                       | Type-2 diabetes                | 65 (10.40%) | -         |
|                       | Hypertension                   | 390 (62.4%) | -         |
|                       | Diabetes and hypertension      | 170 (27.2%) | -         |
|                       | N                              | 513       | -         |
| MMC (South India)     | Age                            | 51±0.49   | -         |
|                       | Male (%)/Female (%)            | 87.10%/12.90% | -       |
|                       | **Disease state**              |           |           |
|                       | Coronary artery disease        | 513 (100%) | -         |
|                       | N                              | 355       | 361       |
| PGIMER (North India)  | Age                            | 45.8 ± 0.64 | 60.0 ± 0.40 |
|                       | Male (%)/Female (%)            | 49.1%/50.9% | 71.4%/28.6% |
|                       | **Disease state**              |           |           |
|                       | Hypertension                   | 355 (100%) | -         |

* Subjects were recruited at four study centers: MDRF (Madras Diabetes Research Foundation, Chennai), MMM (Madras Medical Mission, Chennai), MMC (Madras Medical College, Chennai) and PGIMER (Post Graduate Institute of Medical Education and Research, Chandigarh). Majority of the subjects at the Chennai centers had South Indian ancestry while majority of the subjects at the Chandigarh center had North Indian ancestry. The values have been represented as mean ± S.E.M. Disease states of the cases are shown. Criteria for disease classifications were as follows. Type-2 diabetes - fasting blood sugar ≥7.0 mmol/L (126 mg/dL), treatment with anti-diabetic drugs or history of disease; hypertension: systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg or history of hypertension and anti-hypertensive treatments; coronary artery disease: subjects with at least one block in
their coronary artery or initial stage of myocardial infarction based on findings from angiogram and electro/echo-cardiogram. Normotensives served as the control subjects for hypertensives, while individuals with normal glucose tolerance served as controls for diabetics. All the controls had no history of disease and medication. None of the subjects had kidney disease or any type of cancer.
Table S2 Clinical characteristics of case and control subjects *

| Parameters       | Cases                          | Controls                      | p-value   |
|------------------|-------------------------------|-------------------------------|-----------|
| **Physical**     |                               |                               |           |
| Age (years)      | 48.95 ± 11.78 (n=3041)        | 46.30 ± 14.30 (n=1250)        | <0.0001   |
| BMI (kg/m²)      | 25.45 ± 4.20 (n=2514)         | 24.07 ± 4.71 (n=887)          | <0.0001   |
| **Physiological**|                               |                               |           |
| SBP (mm Hg)      | 132.19 ± 20.58 (n=2958)       | 118.35 ± 16.22 (n=1247)       | <0.0001   |
| DBP (mm Hg)      | 82.33 ± 12.27 (n=2958)        | 74.64 ± 10.18 (n=1247)        | <0.0001   |
| MAP (mm Hg)      | 98.95 ± 13.88 (n=2958)        | 89.20 ± 11.10 (n=1247)        | <0.0001   |
| **Biochemical**  |                               |                               |           |
| Urea (mg/dL)     | 23.32 ± 8.47 (n=2515)         | 21.03 ± 6.94 (n=886)          | <0.0001   |
| Creatinine (mg/dL)| 0.85 ± 0.285 (n=2531)        | 0.85 ± 0.260 (n=886)          | 0.411     |
| FBS (mg/dL)      | 143.01 ± 64.49 (n=1859)       | 87.37 ± 16.43 (n=867)         | <0.0001   |
| PGBS (mg/dL)     | 230.31 ± 100.87 (n=1800)      | 109.71 ± 33.74 (n=886)        | <0.0001   |
| FBS Insulin      | 11.46 ± 7.31 (n=572)          | 8.81 ± 6.06 (n=646)           | <0.0001   |
| PGBS Insulin     | 73.29 ± 60.06 (n=532)         | 61.92 ± 51.3 (n=499)          | 0.001     |
| HOMA-IR index    | 3.81 ± 2.96 (n=571)           | 1.88 ± 1.36 (n=646)           | <0.0001   |
| HbA1c (%)        | 7.92 ± 2.31 (n=1485)          | 5.56 ± 0.65 (n=887)           | <0.0001   |
| TC (mg/dL)       | 185.82 ± 41.92 (n=2520)       | 177.38 ± 36.37 (n=887)        | <0.0001   |
| TG (mg/dL)       | 165.82 ± 121.90 (n=2521)      | 119.14 ± 82.13 (n=887)        | <0.0001   |
| HDL-C (mg/dL)    | 40.69 ± 9.41 (n=2461)         | 42.58 ± 9.70 (n=887)          | <0.0001   |
| LDL-C (mg/dL)    | 116.23 ± 35.76 (n=2391)       | 111.25 ± 31.11 (n=886)        | <0.0001   |
| VLDL-C (mg/dL)   | 32.26 ± 20.61 (n=1169)        | 23.02 ± 13.22 (n=867)         | <0.0001   |

* Clinical parameters of the overall study population (Chennai and Chandigarh populations) (n=4318) stratified as cases and controls were analyzed using independent samples t-test. Values have been expressed as mean ± S.D. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; FBS, fasting blood sugar; PGBS, post-glucose blood sugar; HOMA-IR index, insulin resistance index [fasting insulin (mIU/L) × fasting glucose (mg/dL)/405]; HbA1c, glycosylated hemoglobin; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; VLDL-C, very low-density lipoprotein cholesterol.
Table S3 Statistical power of the study *

| Disease state              | Power of the study |
|----------------------------|--------------------|
| Type-2 Diabetes            | 0.6839             |
| Hypertension               | 0.7127             |
| Coronary Artery Disease    | 0.6980             |
| Metabolic Syndrome         | 0.9071             |

* Power of the study was analyzed using Quanto version 1.2.4 (17). By using case-control design/outcome and population risk as disease risk, the power of the sample size was calculated with 0.05 as the type 1 error rate. Statistical power of the study was calculated for all disease states (type-2 diabetes, hypertension, coronary artery disease and metabolic syndrome) involved in our study and the values are shown.
Table S4 Distribution PST p.Gly297Ser variant genotypes in different ethnic populations*

| Ethnicity/Study       | European | African | American | East Asian | South Asian | FST Score |
|-----------------------|----------|---------|----------|------------|-------------|-----------|
| **1000 Genomes**      | N        | 1006    | 1322     | 694        | 1008        | 978       | 0.0377    |
|                       | MAF (%)  | 0.89    | 0.08     | 0.00       | 0.00        | 5.80      |           |
| **ExAC**              | N        | 9500    | 2882     | 464        | 858         | 7970      | 0.0355    |
|                       | MAF (%)  | 1.45    | 0.21     | 1.51       | 0.12        | 8.57      |           |
| **GnomAD-Genomes**    | N        | 18844   | 8690     | 848        | 1560        | NA        | 0.0023    |
|                       | MAF (%)  | 0.94    | 0.15     | 0.47       | 0.00        | NA        |           |
| **GnomAD-Exomes**     | N        | 78196   | 9174     | 24928      | 11780       | 22960     | 0.0420    |
|                       | MAF (%)  | 0.94    | 0.16     | 0.43       | 0.02        | 8.33      |           |
| **HapMap**            | N        | 176     | 298      | 592        | 168         | NA        | 0.0115    |
|                       | MAF (%)  | 1.10    | 0.30     | 3.20       | 0.00        | NA        |           |
| **ALFA**              | N        | 117750  | 3708     | 1788       | 324         | 62        | 0.0006    |
|                       | MAF (%)  | 0.99    | 0.32     | 0.61       | 0.00        | 10.00     |           |
| **PAGE Study**        | N        | NA      | NA       | 68744      | 8218        | 838       | 0.0128    |
|                       | MAF (%)  | NA      | NA       | 0.32       | 0.06        | 6.80      |           |
| **GenomeAsia 100K**   | N        | 228     | 208      | 52         | 1394        | 1448      | 0.0278    |
|                       | MAF (%)  | 0.89    | 0.00     | 0.00       | 0.50        | 6.15      |           |
| **Mean**              | MAF (%)  | 0.99    | 0.18     | 0.37       | 0.06        | 8.19      | 0.0334    |

* MAFs of the PST p.Gly297Ser variation (rs9658664) in various populations of the world, as calculated in different studies. The MAFs differed by ethnicity (χ²=13688, p<0.0001). Of note, South Asian populations showed much higher MAFs than other populations. Data from the ExAC, GnomAD-Genomes and GnomAD-Exomes studies have been obtained from https://gnomad.broadinstitute.org/. Data for the 1000 genomes, HapMap, ALFA and PAGE studies have been obtained from dbSNP. Data for the GenomeAsia 100k study was taken from https://browser.genomeasia100k.org/. In the PAGE study, African American, Mexican, Puerto Rican, Native Hawaiian, Cuban, Dominican, Central American, South American and Native American populations have been considered as American. In the ALFA study, Latin American 1 and 2 populations have been considered as American. In the GenomeAsia 100k study,
Southeast Asian and Northeast Asian populations have been considered as East Asian. The global $F_{ST}$ scores of the PST-G297 variant were calculated for the five major world populations from several studies, using the previously described equation (with all figures rounded off to fourth place after decimal):

$$F_{ST} = \frac{H_T - H_S}{H_T} \text{ with } H_T = 2 \times p \times q, H_S = \frac{H_{S_1} \times n_{s_1} + H_{S_2} \times n_{s_2}}{n_{s_1} + n_{s_2}}, \text{ and } H_{S_i} = 2 \times p_{S_i} \times q_{S_i}$$  \hspace{1cm} (18),

where $p_{S_i}$ and $q_{S_i}$ are the allele frequencies in subpopulation $i$, $n_{s_1}$ and $n_{s_2}$ are the number of individuals of the subpopulations $S_1$ and $S_2$, respectively, $H_S$ is the average heterozygosity of the subpopulations and $H_T$ is the heterozygosity based on the total population. $F_{ST}$ values below 0.05 indicate low differentiation (i.e., negative selection pressure) and values above 0.65 indicate extreme differentiation (i.e., positive selection pressure) (19). All the studies had $F_{ST}$ values <0.05, suggesting that the altered function arising as a consequence of this variant results in it being selected against, hence leading to little genetic differentiation.

MAF, minor allelic frequency; GnomAd, genome aggregation database; ExAC, exome aggregation consortium; ALFA, allele frequency aggregator; PAGE, population architecture using genomics and epidemiology; NA, data not available.
| Ligand-receptor complex                  | Binding free energy (ΔG) (Kcal/mole) | Dissociation constant (K_d) (M) |
|----------------------------------------|--------------------------------------|---------------------------------|
| Insulin and insulin receptor           | -12.32                               | 9.22 × 10^{-10}                 |
| PST-WT and insulin receptor            | -12.02                               | 15.3 × 10^{-10}                 |
| PST-297S and insulin receptor          | -12.40                               | 8.06 × 10^{-10}                 |
| PST-WT and GRP78                       | -7.89                                | 1.64 × 10^{-6}                  |
| PST-297S and GRP78                     | -9.18                                | 0.185 × 10^{-6}                 |

* Binding free energy and dissociation constant values were calculated using PPA-Pred program (15).

PST, pancreastatin; PST-WT, wild-type pancreastatin peptide; PST-297S, variant pancreastatin peptide; GRP78, glucose-regulated protein 78; IR, insulin receptor
Table S6 GWAS-identified associations of rs9658664 with disease conditions*

| Disease                  | Odds ratio | p-value | Direction of effect | Study                      | Sample Size (n) |
|--------------------------|------------|---------|---------------------|----------------------------|-----------------|
| T2D                      | 1.127      | 0.1     | ↑                   | DIAGRAM 1000G GWAS         | 82594           |
| T2D adj BMI              | 1.234      | 0.024   | ↑                   | DIAGRAM 1000G GWAS         | 56443           |
| Claudication in T2D      | 2.140      | 0.134   | ↑                   | DIAGRAM 1000G GWAS         | 1095            |
| PVD in T2D               | 2.588      | 0.038   | ↑                   | BioMe AMP T2D GWAS         | 1350            |
| Stroke in T2D            | 2.074      | 0.162   | ↑                   | BioMe AMP T2D GWAS         | 790             |
| Hypertension in T2D      | 0.478      | 0.106   | ↓                   | DIAMANTE (European) T2D GWAS | 1110            |
| T2D                      | 1.059      | 0.099   | ↑                   | DIAMANTE (European) T2D GWAS | 231420          |

*The association of PST 297Ser allele with various cardiometabolic conditions has been tested in different GWAS studies carried out till date including DIAGRAM 1000G GWAS (20), BioMe AMP T2D GWAS (21–24) and DIAMANTE (European) T2D GWAS (25). Data for generating this figure have been obtained from https://t2d.hugeamp.org/ as accessed on 15th March 2021.
T2D, type-2 diabetes; BMI, body mass index; PVD, peripheral vascular disease; CAD, coronary artery disease; MI, myocardial infarction
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