Soluble CD93 is involved in metabolic dysregulation but does not influence carotid intima-media thickness

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Running title: sCD93 in atherosclerosis and type 2 diabetes

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Word count:

Total number of Figures and Tables: 4 Tables, 4 Figures and 5 Supplemental Tables, 3 Supplemental Figures

Abstract

Type 2 diabetes and cardiovascular disease are complex disorders involving metabolic and inflammatory mechanisms. Here we investigated whether sCD93 also plays a role in metabolic dysregulation or early atherosclerosis, as measured by carotid intima-media thickness (IMT). Whilst no association was observed between sCD93 and IMT, sCD93 levels were significantly lower in subjects with type 2 diabetes (n=901, mean±sd: 156.6±40.0ng/mL) compared to those without (n=2470, 164.1±44.8ng/mL, $p<0.0001$). Diabetes risk SNPs (DIAGRAM consortium) did not influence sCD93 levels (individually or combined in a SNP score). In a prospective cohort, lower sCD93 levels preceded diabetes development. Consistent with this, a *cd93*-deficient mouse model (in addition to *apoe* deficiency) no difference in atherosclerotic lesion development. However, *cd93*-deficient mice showed impaired glucose clearance and insulin sensitivity after a high fat diet. Expression of cd93 was observed in pancreatic islets, and leaky vessels were apparent in *cd93*-deficient pancreases. We further demonstrated that stress-induced release of sCD93 is impaired by hyper-glycaemia. Therefore, we propose that CD93 is an important component in glucometabolic regulation.

Introduction

Subjects with type 2 diabetes have 2 to 4-fold greater risk for developing cardiovascular disease (CVD) than those without. Indeed, preventative strategies targeting CVD have shown little progress in subjects with type 2 diabetes, despite their efficacy in subjects without diabetes. Although complete understanding of mechanisms leading to CVD is lacking, it is generally accepted that a combination of metabolic dysregulation and inflammatory pathways are important contributors to CVD pathologies. Therefore, elucidation of pathways linking metabolic dysregulation and inflammation could pinpoint potential therapeutic targets for reducing CVD, especially in subjects with type 2 diabetes.

CD93 is a group XIV c-type lectin, belonging to the endosialin family, originally described as a component of the complement system (1). CD93 is composed of a cytoplasmic tail containing a PDZ binding domain (2), a transmembrane domain containing metalloproteinase sites, an extracellular region containing a mucin-like domain that is highly glycosylated, 5 EGF domains (4 in mice) and a unique C-type lectin domain. CD93 is predominantly expressed on endothelial cells, but also on innate immune cells such as neutrophils and monocytes and megakaryocytes (3). In response to certain inflammatory molecules, the transmembrane CD93 is cleaved and the extracellular segment is released into the circulation as soluble CD93 (sCD93) (4; 5). It is still unknown whether the released sCD93 has a distinct function, or whether release of this fragment is merely to enable the intra-cellular remnant to respond to the cellular stress. Originally described as a factor involved in removal of apoptotic bodies, CD93 has also been proposed to be involved in B cell maturation and Natural Killer T cell (iNKT cell) survival (6). Furthermore, the EGF domains are believed to be involved in angiogenesis (7) and the moesin-binding domain (8) is required for endothelial cell-cell interactions (9).

Regarding its role in metabolism and CVD: *cd93* is a plausible candidate in the mouse non-obese diabetes *Idd13* locus, (10), we have previously shown that reduced levels of circulating sCD93 are associated with increased risk of myocardial infarction (11) and more recently, the *CD93* gene has been identified as a potential regulator of pathways common to both type 2 diabetes and CVD (12). Interestingly, *CD93* expression is up-regulated by conditions relevant to diabetes or its complications, for example flow-related shear stress (13) due to endothelial dysfunction, or during the development of new but leaky blood vessels (14), as observed in retinopathy or during ischemia-related inflammation of cerebral vascular endothelium (15) thus reflecting myocardial infarction.

In the current study we investigated sCD93 for effects on markers of metabolic dysregulation and early cardiovascular disease in human cohorts and a mouse model with a genetic deficiency in scd93. We further examined the mechanisms by which sCD93 might influence metabolic and cardiovascular processes.

Research Design and Methods

Discovery analyses: IMPROVE

The IMPROVE cohort has previously been described (16; 17). Briefly, subjects with at least 3 established CVD risk factors but no symptoms or history of cardiovascular events were enrolled from 7 European centres. Medical history, anthropometric measurements and blood samples were obtained at baseline and standard biochemical phenotyping was performed. Blood samples were stored at -80^{0}C. Extensive carotid intima-media thickness (cIMT) phenotyping, which can be considered a surrogate measure of early atherosclerosis (18; 19), was performed by ultrasound at baseline, 15 and 30 months after enrolment. A composite measure reflecting the entire carotid tree (IMT) was available, as well as segment-specific measures: common carotid artery excluding 1 cm proximal to the bifurcation (CC-IMT) and the bifurcation (BIF-IMT). For each measure, the mean was calculated ($_{\text{mean}}$) and the maximum ($_{\text{max}}$) of the segment identified (20). In addition, the mean of the maximum values of the entire carotid tree was calculated ($_{\text{mean-max}}$) (20). Linear regression of cIMT change over time was used to calculate cIMT progression of each segment. For each individual, the segment with the greatest progression was identified (fastest-progression). Type 2 diabetes was defined as diagnosis, anti-diabetic medication or fasting glucose ≥7mmol/L. Soluble CD93 was measured using the Mesoscale platform, using previously validated ELISA antibodies (11) and SECTOR Imager 2400. Characteristics of the cohort are presented in Table 1.

Genotyping in IMPROVE

Reported type 2 diabetes risk-associated SNPs (21) were genotyped in the IMPROVE cohort using the Illumina Metabochip (22) and Immunochip (23) platforms. Genotyping was conducted at the SNP&SEQ Technology Platform, Uppsala University, Sweden and standard

quality control was applied: Subject exclusions: low call rate (<95%), cryptic relatedness or ambiguous sex. SNP exclusions: failing call rate (<95%) or Hardy–Weinberg equilibrium ($p<5*10^{-6}$) thresholds. After quality control, multi-dimensional scaling (MDS) components were calculated using PLINK (24) with default settings. The first MDS component demonstrates strong correlations with latitude/recruitment centre (Spearmans Rho 0.935 p<0.0001 and Rho 0.946 p<0.0001 for subjects without and with type 2 diabetes respectively).

Statistical analyses: Epidemiology in IMPROVE

The trend test for ordered groups was used to assess an effect of latitude (corresponding to recruitment centre). Differences in sCD93 levels between men/women and subjects with/without diabetes were assessed by T-test. Associations between sCD93 levels and established risk factors were assessed by Spearman rank correlation coefficients. Skewed variables, including sCD93, were log-normal transformed for further statistical analyses. Multivariable regression analysis was used to identify established or potential risk markers of metabolism or CVD with significant effects on sCD93 levels. Variables considered for inclusion were: age and sex (forced into the models), height, weight, BMI, waist to hip ratio (WHR), systolic and diastolic blood pressure (SBP and DBP respectively), LDL cholesterol, HDL cholesterol, triglycerides (TGs), fasting glucose, C-reactive protein (CRP), proinsulin, insulin, HOMA indices, adiponectin, leptin, interleukin 5 (IL-5), current smoking, lipid-lowering and anti-hypertensive medication. Multivariable regression, adjusted for established CVD risk markers (age, gender, mds1-3, BMI, SBP, HDL, TGs and current smoking) (25), was used to assess the effect of sCD93 levels on measures of IMT. Analyses were conducted using STATA 11.2 (STATCorp,Texas, USA).

Statistical analyses: Genetics in IMPROVE

Linear regression analyses assuming an additive genetic model were conducted to assess the influence of type 2 diabetes risk-associated SNPs on sCD93 levels, adjusting for age, sex and population structure (MDS1-3). Analysis was conducted in PLINK (24). Genotypes of 52 (of 62 known (21)) type 2 diabetes-risk associated SNPs were combined in an unwieghted SNP score by summing the reported (21) type 2 diabetes risk-increasing alleles for each subject (representing the total burden of genetically determined type 2 diabetes risk). Only subjects without type 2 diabetes and with complete genotyping were analysed. The score was tested for influence on levels of sCD93, using a linear regression model as above, in STATA 11.2 (STATCorp,Texas, USA).

Replication analyses: Stockholm Diabetes Prevention Program (SDPP)

The SDPP is a prospective cohort of subjects from the Stockholm area, aged 35-55 years (26). Briefly, blood samples, oral glucose tolerance tests (OGTT), basic clinical phenotyping and questionnaires were conducted on participants at baseline and after 8-10 years of follow-up. Levels of sCD93 was measured by Mesoscale (as above), in baseline samples and in a subset of follow-up samples (Online Supplemental Figure 1). Baseline samples were from subjects newly diagnosed as normal glucose tolerant (NGT, n=843), pre-diabetes (defined as impaired glucose tolerance and/or impaired fasting glucose, n=326) and type 2 diabetes (n=113). Follow-up samples from the subjects NGT at baseline were also analysed. Some subjects remained NGT (n=370) whilst others had progressed to pre-diabetes (n=314) or type 2 diabetes (n=158). sCD93 levels were measured by Mesoscale as above. ANOVA (adjusted for age and sex) was used to compare levels of sCD93 between glucose tolerance groups.

Cd93-deficient mice

The *cd93*-deficient mouse was generated by the trans-NIH Knock-Out Mouse Project (KOMP) and obtained from the KOMP Repository (www.komp.org). Embryonic stem cells were generated from C57BL/6N mice and kept on the C57BL/6N background. Breeding of the *cd93*-

deficient mice did not show a Mendelian ratio, with a very low ratio of homozygous knockout mice observed. However, cd93 heterozygous (*cd93*$^{+/-}$) mice had half the circulating concentration of sCD93 compared to their wild-type (*cd93*^{wt}) littermates (Supplemental Table 1), rendering this comparable with humans, as humans have varying levels, rather than complete absence, of sCD93 (11). Therefore, this study focuses on *cd93*^{wt} and *cd93*$^{+/-}$ animals. All mice were bred and kept at the Karolinska Institutet animal facility and with 12 hour day/night cycle with food and water *ad libutim*. All procedures were approved by the regional animal ethics authority (Norra Stockholm).

Characterization cd93-deficient mice

Mouse scd93 was measured using Mesoscale technology with antibodies directed against murine cd93 (capture antibody clone 223437, detection antibody BAF1696, R&D systems) using EDTA plasma from male mice (N=8 from each genotype) fed on western diet for 16 weeks. Expression of cd93 on the B cell population was determined by flow cytometry. Single cell suspensions of spleen cells from male mice (N=8 from each genotype) fed on western diet for 16 weeks were used. Firstly, Fc receptors were blocked with anti-FcRII and III (clone 24.G2 in-house preparation). B cells were stained with anti-mouse CD45R eFlour450® (clone RA3-6B2 ebioscience) and anti-mouse-CD19 conjugated with APC-Cy7 (clone 6D5 biolegend). The percent of IgG and IgM positive B cells was determined by using anti-mouse IgG conjugated with FITC (Biolegend Poly4060) and anti-mouse-IgM conjugated with APC (Biolegend RMM-1). Expression of cd93 on B cells was determined by anti-mouse cd93 conjugated with PE (Biolegend clone AA4.1) on Beckman Coulter Gallios™ flow cytometer equipped with blue (405nm) green (488nm) and red (633nm) lasers. The percentage of iNKT cells in the liver was determined using the previously published method (10) with the addition of a violet viability dye (Live/Dead Life technologies) using the Beckman Coulter Gallios™ flow cytometer.

Tissue collection and assessment of atherosclerotic lesions in mice ($apoe^{-/-}cd93^{wt}$ and *$apoe^{-/-}cd93^{+/-}$).*

For atherosclerosis studies, mice were crossed into the *apoe*-deficient (*$apoe^{-/-}$*) mice (originally from Jackson Laboratory) and backcrossed 6 generations to C57BL/6N. Homozygous *apoe*-deficient mice, with 2 or 1 copies of *cd93* (*$apoe^{-/-}cd93^{wt}$* and *$apoe^{-/-}cd93^{+/-}$* respectively) were fed a normal rodent diet for 32 weeks, at which point they were sacrificed with CO_2 and blood sampled via cardiac puncture. Plasma samples (EDTA) were stored at -80^{0}C. Organs were perfused with sterile PBS and the descending thoracic aorta was collected into 4% paraformaldehyde. The thoracic aorta was pinned onto a paraffin bed and *En face* lipid content was determined by staining with Sudan IV (Sigma-Aldrich). Images were captured using a DC480 camera connected to a MZ6 stereomicroscope (both from Leica). Quantification of the total plaque area for each aortic arch were summed and expressed as the percentage of the total surface area of the aorta using ImageJ software (NIH).

Mouse metabolic studies ($cd93^{wt}$ and *$cd93^{+/-}$)*

For metabolic studies, *cd93*-deficient mice were fed a western diet (SDS custom diet: 21% fat 0.2% cholesterol mixed in standard CRM (p) maintenance diet) for 16 weeks. Glucose and insulin tolerance tests were conducted. After 4 hours of fasting, a bolus of glucose (1g/kg for glucose tolerance test) or insulin (0.75 U/kg for insulin tolerance test) was given by intra-peritoneal injection. Blood was sampled from the tail vein at 30 min intervals.

Pancreatic morphology in the cd93-deficient mouse model ($cd93^{wt}$ vs $cd93^{+/-}$)

Differences in pancreas morphology between genotypes were assessed by immuno-histochemistry (IHC). Mice were fed a western diet for 16 weeks prior to removal of pancreas. Embedding and sectioning of the pancreas as well as rehydration and dehydration of sections were conducted as per standard protocols. Four pancreases were analysed per genotype. To assess presence and location of insulin, cd93 and von Willebrand Factor (vWF), sections were

boiled for 20 minutes in Diva Decloaker (Biocare Medical) and sections were treated with 3% hydrogen peroxidase before blocking in 5% goat serum in 1% bovine serum albumin. Serial sections were stained using antibodies against insulin (guinea pig anti-insulin, Abcam), vWF (rabbit anti-vWF, Abcam) and cd93 (rat anti-cd93, R&D). Of note, the anti-cd93 antibody targets an extracellular epitope, thus is able to detect cell surface-attached, as well as soluble, cd93. After overnight incubation at 4^0C, sections were incubated with biotinylated secondary antibodies (Goat anti-guinea pig, Abcam; Goat anti-rabbit, Vector; and Rabbit anti-rat, Vector Laboratories respectively) for 1hr at room temperature. Peroxidase-Avidin/biotin complex was achieved using Vectorstain ABC Elite kit (Vector Laboratories) and detected using Novo Red (Vector Laboratories) as per manufacturer's directions and counterstained with haematoxylin. The numbers of islets were counted in parallel by 2 researchers, using 3-5 haematoxylin and eosin-stained sections. The size of insulin-stained islets were measured using ImageJ software (NIH). The number of vWF-positive and total islets were counted and a percent of positive islet staining calculated (number vWF positive islets / total number islets and multiplied by 100). The pancreas from one *cd93*^{ko} mouse also fed on western diet for 16 weeks was included for confirmation of anti-cd93 staining.

Blood vessel integrity (*cd93*^{wt} *vs cd93*$^{+/-}$)

An *in vivo* blood vessel permeability assay was used by i.v. injection of 0.5% Evans blue into anesthetised 4 week old male mice. After 30mins, mice were euthanized and perfused with PBS. After collection, the pancreases were treated with 50% trichloroacetic acid at a 1:4 ratio (ug/mL) and homogenised using Bio-Gen Pro200 (Pro Scientific) for 30 seconds. The amount of Evans blue was determined as previously published (27) and detected using GlowMax Multi with fluorescence 625/660-720 (Promega).

Peripheral markers of endothelial damage (*cd93*^{wt} *vs cd93*$^{+/-}$)

Soluble E-selectin and vWF A2 were measured in the plasma of mice fed either a western diet of chow diet for 16 weeks. E-selectin was measured using DuoKit for E-selectin (R&D) on a Mesoscale platform as per manufacturer's directions, with addition of SULFO-TAG labelled Streptavidin and reading on SECTOR Imager 2400 (Mesoscale). vWF A2 was measured using SimpleStep Elisa kit from Abcam as per manufacturer's directions.

Analysis of sCD93 release from endothelial cells

To assess the impact of diabetes-relevant conditions on sCD93 release, the human carotid endothelial cell line (HctAEC, Cell Applications, in complete endothelial cell growth media (Cell Applications)) and human endothelial hybrid cell (EA.Hy 926, ATCC, in RPMI, 10% foetal calf serum and 1% Penicillin and Streptomycin (all from Sigma-Aldrich)) were cultured in gelatin-coated (Sigma-Aldrich) flasks. During passage 5, cells were seeded onto gelatin-coated 48 well plates. After overnight incubation with glucose-free DMEM (Sigma-Aldrich), HctAEC was supplemented with 1% Heparin (Sigma-Aldrich), 0.5% endothelial cell growth supplement (Sigma-Aldrich) and both HctAEC and EA.Hy were supplemented with 10% foetal bovine serum and 1% Penicillin and Streptomycin. Cells were then stimulated with or without 50nM Phorbol 12-myristate 13-acetate (PMA) or 50ug/mL lipopolysaccharide (LPS) in 5 or 30mM Glucose (Braun). sCD93 was measured by Mesoscale technology as above.

Results

Plasma levels of sCD93 in IMPROVE

It has been previously reported that in IMPROVE latitude demonstrates the strongest independent effect on cIMT (17), however there was no significant association was observed between sCD93 and latitude (p=0.942). Furthermore, in accordance with previous reports of sCD93 (11), there was no significant difference between men and women (mean±sd: 162±42ng/mL vs 163±45ng/mL, p=0.3833). However, levels of sCD93 were significantly

lower in subjects with type 2 diabetes (157±40ng/mL) compared to those without (164±45ng/mL, $p<0.0001$). Thus, the cohort was stratified for diabetes status as this is likely to impact upon further analysis of cIMT or other CVD risk factors.

Associations between sCD93 levels and putative metabolic and cardiovascular risk markers

In the subjects without diabetes, levels of sCD93 were correlated with age and height, as well as metabolic markers including BMI, insulin, HOMA indices, vitamin D and adiponectin (Table 2). Consistent with lower levels being associated with poor metabolic control, sCD93 levels were positively correlated with adiponectin and vitamin D, but inversely correlated with BMI, insulin and HOMA indices. The association between sCD93 and lipid levels was confounded by lipid-lowering medication (Table 2). In subjects without lipid-lowering medication, sCD93 levels were associated with an advantageous metabolic profile, namely positively associated with HDL levels and negatively associated with TGs.

Consistent with above, type 2 diabetic subjects without lipid-lowering medication demonstrated a negative association (albeit non-significant) with TG levels. Whilst a negative correlation was observed between sCD93 levels and SBP, when analysing subjects without anti-hypertensive medication, this association was lost. It should be noted that this analysis was limited to 316 subjects, thus limited power is an issue.

Associations between sCD93 levels and cIMT in subjects

As cardiovascular risk factors have a large impact on cIMT measures (16; 17), these parameters were considered for inclusion in multiple regression models. Proinsulin and insulin measurements are not informative in subjects with diabetes (due to influence of medication and disease pathology) thus these variables were omitted. Diabetes-stratified multiple regression analysis gave rise to 3 models: A) age and sex. B) with variables significant in both subjects with and without type 2 diabetes, where DBP, TGs, creatinine and current smoking were added

to model A. C) further inclusion of variables significant in one stratum (LDL, IL5, adiponectin and SBP). It was clear that levels of sCD93 were not associated with any baseline or progression measures of cIMT in subjects with or without type 2 diabetes, when adjusting for age and sex (Supplemental Table 2), nor in the regression models adjusting for established CVD risk markers (data not shown). We could exclude lack of power as a reason for failing to detect an association (power =0.99 for subjects without type 2 diabetes and 0.81 for the subjects with type 2 diabetes). Thus we conclude that sCD93 levels do not influence on cIMT.

Mendelian randomisation of type 2 diabetes risk-associated SNPs on sCD93 levels

To assess whether reduced sCD93 levels are a result of type 2 diabetes, rather than a possible cause, a Mendelian randomisation experiment was conducted. If reduced sCD93 levels are a result of diabetes-related processes and/or susceptibility, then genetic variants which influence risk of type 2 diabetes would be expected to influence sCD93 levels. Genotypes of 53 (of 62 known (21)) type 2 diabetes risk-associated SNPs were available for the IMPROVE cohort and were analysed for association with sCD93 levels (adjusting for age, sex and population structure in subjects without diabetes). Individually, no SNP met the Bonferroni-corrected p value for significance ($p<9.43E-4$, Supplemental Table 4), nor was there any correlation with sCD93 levels for SNPs combined in an un-weighted SNP score (Spearman Rank rho=0.0045, p=0.8248). These findings indicate that genetic susceptibility to type 2 diabetes is not a cause of reduced CD93 levels; hence, it is possible that reduced sCD93 levels precede development of type 2 diabetes.

Soluble CD93 levels in the prospective SDPP cohort

IMPROVE subjects demonstrate multiple metabolic aberrations, thus is not the optimal setting for investigating potential diabetes biomarkers. To address this the prospective SDPP cohort, specifically designed to assess potential biomarkers of T2D, was investigated. Characteristics of the baseline and follow-up samples are presented in Supplemental Table 3 and Table 3,

respectively. Baseline levels of sCD93 demonstrated a non-significant trend (ANOVA p=0.2335, adjustment for age and sex) whereby lower levels were observed in subjects with poor glucose regulation: NGT 163±44ng/mL for NGT, 158±44ng/mL for pre-diabetes and 158±41ng/mL for type 2 diabetes. Similarly, there was no significant difference between follow-up levels of NGT, pre-diabetes or type 2 diabetes (153±42ng/mL, 154±51ng/mL and 154±48ng/mL, respectively). To assess whether sCD93 influenced progression to prediabetes or type 2 diabetes over the follow-up time, baseline levels were compared between the subjects (all NGT at baseline) who were diagnosed as NGT, prediabetes or type 2 diabetes at follow-up. Subjects who remained NGT had significantly higher levels of sCD93 than those who progressed from NGT to type 2 diabetes during follow-up (166±44ng/mL vs 158±45ng/mL respectively, T-test p=0.016). A similar non-significant trend of higher sCD93 levels was observed in subjects who remained NGT compared to those who progressed to pre-diabetes during follow-up (166±44ng/mL vs 161±44ng/mL, respectively, p=0.058). These results support the hypothesis that reduced sCD93 levels occur before onset of type 2 diabetes.

Cd93-deficient mouse model

A *cd93*-deficient mouse model has previously been described (6), where there was no gross phenotypic abnormality. However, mice demonstrated reduced phagocytic activity (6) and defective maturation of B cells and iNKT cells (26; 28) and associated with altered vascular permeability in glioma (9). These mice lack only exon 1 of the *cd93* gene and had a mixed genetic background, with embryonic stem cells originating from 129/sv mice and crossed into C57BL/6J mice. In contrast, our strategy maintained a genetically pure strain, namely C57BL6/N, with the entire *cd93* gene being deleted. This *cd93*-deficient mouse model again showed no gross phenotypic defect, however there was partial lethality. Importantly, the mice carrying one *cd93* gene ($cd93^{+/-}$) had approximately half the concentration of scd93 in the periphery compared to wild type mice ($cd93^{wt}$, 104±18 ng/mL vs 254±63 ng/mL respectively,

P =0.008, Supplemental Table 1). Compared to *cd93*^{wt}, the *cd93*$^{+/-}$ mice showed no difference in mature B cell populations (as determined by percentage IgG or IgM positive B cells) or iNKT cells (Supplemental Table 1). Therefore, these mice were appropriate for our studies aimed at investigating whether reduced levels of scd93 influence development of atherosclerosis and type 2 diabetes.

Atherosclerosis in the cd93-deficient mouse model (apoe$^{-/-}$*cd93*^{wt} *vs apoe*$^{-/-}$*cd93*$^{+/-}$*)*

In order to confirm the impact of CD93 on atherosclerosis in a tightly controlled setting, specifically reduced levels of scd93, the *cd93*-deficient mouse model was crossed with the *apoe*-deficient (*apoe*$^{-/-}$) model, which is commonly used to study atherosclerosis. *Apoe*$^{-/-}$ mice have dramatically higher VLDL cholesterol levels and mimic the early phases of the human atherosclerosis by developing atherosclerotic lesions in the aorta. *Apoe*$^{-/-}$*cd93*^{wt} and *apoe*$^{-/-}$*cd93*$^{+/-}$ mice were fed a chow diet until being sacrificed at 32 weeks. Whilst atherosclerotic lesions were visible in the descending aorta, there was no difference between *apoe*$^{-/-}$*cd93*^{wt} and *apoe*$^{-/-}$*cd93*$^{+/-}$ mice regarding the lesion area observed (Figure 1). Thus, these data are consistent with the human findings that scd93 levels do not influence early atherosclerosis.

Metabolic characteristics of the cd93-deficient mouse model (cd93^{wt} *vs cd93*$^{+/-}$*)*

To mirror the human metabolic findings, we investigated whether mice with reduced sCD93 levels also had impaired glucose metabolism. When fed a chow diet, both genotypes demonstrated a similar rate of glucose clearance, however *cd93*$^{+/-}$ mice had higher basal level of glucose compared with *cd93*^{wt} (Figure 1 and Online Supplement Figure 2). However, when fed a western diet (21% fat 0.2% cholesterol), male *cd93*$^{+/-}$ mice demonstrated impaired clearance of glucose and reduced sensitivity to insulin compared to *cd93*^{wt} mice (Figure 2). This was not seen in female mice (data not shown). Levels of fasting insulin as well as biomarkers of metabolic dysregulation (leptin, glucagon, resistin and GLP-1) were measured and compared between *cd93*$^{+/-}$ and *cd93*^{wt} mice (Table 4). Whilst not statistically different, a trend was

observed whereby *cd93*$^{+/-}$ mice had increased levels of insulin and leptin levels compared to *cd93*^{wt} mice and were more insulin resistant (as measured by HOMA-IR).

Assessment of the pancreas morphology (cd93^{wt} *vs cd93*$^{+/-}$ *and cd93*^{ko}*)*

The number of islets and the average size of islets did not differ between *cd93*^{wt} and *cd93*$^{+/-}$ mice (21.7 vs 24.1, T-test p =0.3398 and 595 pixels vs 622 pixels, T-test p=0.4180 respectively). Insulin staining was visible in islets in all genotypes, however some interstitial insulin staining was apparent in the sections from the *cd93*$^{+/-}$ mice (Figure 3, top panel). As expected, vWF staining was restricted to endothelium in all genotypes (Figure 3, bottom panel). In *cd93*^{wt} mice, cd93 demonstrated endothelial staining (as expected with cell surface-attached cd93, Figure 3, middle panel) similar to that of vWF. Diffuse cd93 staining was also observed in the islets. Whilst this could reflect a previously unappreciated expression of cd93 by beta cells, we believe that it is more likely that the diffuse staining reflects the scd93 released from the endothelial cells. In *cd93*$^{+/-}$ mice, the endothelial scd93 staining was less obvious, but the diffuse scd93 staining was clearly visible. However, in the pancreas obtained from a *cd93*$^{-/-}$mouse, there was no cd93 staining observed.

Interestingly, *cd93*$^{+/-}$ mice had a trend (P= 0.08) of decreased percentage of vWF positive islets compared to *cd93*^{wt} mice, indicating that there is endothelial disturbances in the western diet fed *cd93*$^{+/-}$ mice (Figure 4A).

Pancreatic blood vessel leakage

Given the previously indicated role of cd93 in vessel leakage (9), and the interstitial insulin staining in the *cd93*$^{+/-}$ mice, we performed an *in vivo* blood vessel permeability assay using Evans blue. Under physiologic conditions the endothelium is impermeable to albumin, so Evans blue-bound albumin remains restricted within blood vessels. Presence of Evans blue within a tissue after perfusion with PBS indicates leakage out of blood vessels into the

interstitial space. Interestingly, young *cd93*$^{+/-}$ mice had an increase in Evans blue in compared to *cd93*^{wt} littermates (Figure 4B). The finding that higher levels of Evans blue were detected in *cd93*$^{+/-}$ than *cd93*^{wt} mice provided confirmation that the (albeit weak) interstitial insulin staining in the pancreas of *cd93*$^{+/-}$ mice was not merely an artefact. Thus lacking cd93 even at a young age results in leaky vessels, however, neither young nor mice fed on rodent chow displayed a diabetes phenotype. Therefore, we questioned whether after metabolic stress of western diet there was signs of endothelial damage in plasma, indicated by soluble e-selectin and vWF A2. Indeed, *cd93*$^{+/-}$ mice fed on a western diet demonstrated an increase in both endothelial damage markers compared with *cd93*^{wt} (Figure 4C and D), indicating endothelial damage in the western diet fed *cd93*$^{+/-}$ mice.

Influence of high glucose levels on release of sCD93 from endothelial cells

As diffuse cd93 staining was observed in islets and metabolic regulation in mice was impaired after dietary stress, we investigated whether the release of scd93 (by known stimuli) might be influenced by hyper-glycaemia, mimicking the prediabetes state. Glucose levels didn't not influence the release of sCD93 from primary HctAEC under basal (media) or LPS-stimulated conditions (Online Supplemental Figure 2A), however hyper-glycaemia (30mM glucose) reduced PMA-stimulated release of sCD93 compared to normo-glycaemia (5mM glucose). This was experiment was repeated with the EA.Hy 926 cell line, with comparable results (Online Supplemental Figure 2B).

Discussion

The main aim of this study was to elucidate whether sCD93 might play a role in metabolic or cardiovascular disease. Our results refute sCD93 as an important factor in the early vascular changes indicative of atherosclerosis, however they do provide solid evidence for a role of sCD93 in glucometabolic regulation and a starting point for understanding the role of CD93 in these diseases.

The most striking result from this study is the finding that reduced levels of sCD93 were associated with metabolic dysregulation. Here we show that: i) lower sCD93 levels were observed in high CVD risk subjects with type 2 diabetes than those without diabetes; ii) insulin-related processes were associated with sCD93 levels in subjects without diabetes; iii) lower levels of sCD93 were not due to genetic susceptibility to type 2 diabetes; iv) lower sCD93 levels preceded development of type 2 diabetes; v) dietary stress in a *cd93*-deficient mouse model causes impaired metabolic regulation and increased endothelial damage; vi) cd93 (cell surface-bound and soluble) is detected in islets; vii) hyper-glycaemia impaired the release of sCD93 by specific stimuli. The lack of association between sCD93 levels and early atherosclerosis measures was consistent between human and mouse data.

We propose that CD93 expression and sCD93 release in pancreatic islets is important for endothelial integrity and thereby metabolic control. CD93-deficiency leads to leaky blood vessels. Under normal metabolic conditions this is tolerated or compensated for. However, when stressed (inflammatory or metabolic) release of scd93 is further impaired, possibly leading to endothelial damage. Leaky vessels and endothelial damage which permits insulin diffusion into the interstitial space leads to sub-optimal insulin delivery to distal tissues. These results are the first direct evidence for the recently proposed role for CD93 in type 2 diabetes (12).

In view of previous publications on CD93, it should be noted that there was no evidence to suggest that changes in iNKT cells were responsible for the effects reported here, in contrast to previous reports (10). In addition, 2 SNPs associated with sCD93 levels in control subjects have been described (11). No associations were observed between these SNPs and sCD93 levels or insulin sensitivity (Supplementary Table 5) in IMPROVE, nor do they demonstrate any association with type 2 diabetes (DIAGRAM consortium, n=100,589, rs2749812 p=0.940, rs3746731 p=0.870 (29)). Whilst IMPROVE is the largest cohort to date with data on sCD93 levels, this cohort is metabolically disturbed, in contrast to the MI cases and healthy controls (11), where few subjects were on lipid-lowering or anti-hypertensive medication and very few subjects had type 2 diabetes. Therefore comparisons between the Mälarstig and IMPROVE data should be approached with caution. We admit that the size of the SDPP replication study is limited, however, the use of OGTT to define glucose control categories and the length of follow-up compensate for the restricted sample size. A further caveat is that the murine model demonstrated a sex difference which was not seen in the clinical data. The murine studies were conducted in, mice of reproductive age, thus it is plausible that age-related differences in hormones might contribute to this discrepancy. With an average age of 64 years, the women of IMPROVE are most likely to be post-menopausal, thus this effect was not seen. The SDPP cohort was younger so it cannot be assumed that female participants in SDPP are post-menopausal, however the size of the cohort precluded assessment of sex-specific effects.

Previously, release of sCD93, has been implicated as a response to stressors such as inflammatory, immune or angiogenic mediators. Our demonstration of clear cd93 staining in pancreatic islets is novel and might reflect a protective function, whereby a deficiency in cd93 is permissive of beta-cell destruction. Furthermore, the *in vitro* studies showed that sCD93 was not released from endothelial cells as efficiently under hyper-glycaemia fits with the documented downward spiral of glycaemic control characteristic of type 2 diabetes progression.

Differences in sCD93 levels between subjects with and without diabetes is subtle, therefore it is unlikely that measurement of sCD93 levels would have clinical utility as biomarker. However, given that this molecule might mediate both inflammatory and metabolic pathways, further investigation and understanding of CD93s functions is warranted and might provide opportunities for future preventative strategies. Having established the *cd93*-deficient mouse model and confirmed the human relevance, we are able to continue to conduct a deeper functional evaluation of cd93.

Acknowledgements: We would like to thank all participants of the IMPROVE and SDPP studies and acknowledge the advice of Dr Neil Portwood and technical assistance of Nancy Simon.

Author Contributions: AB and RJS designed and conducted study and drafted the manuscript. Measurement and analysis of sCD93 were conducted by RJS, AS, PT, FF and AB. The IMPROVE cohort collection and phenotyping was conducted by ET, DB, RR, AJS, PG, SK, EM, EG, SH, UdF and AHa. Genotyping was overseen by A-CS. Management and quality control of phenotypic and genetic data for IMPROVE was conducted by RJS and BS. AHi and C-GÖ collected and phenotyped the SDPP cohort. FF, PT, LM, AHa and AB were responsible for the animal studies. CÖC, AB and RS conducted the immunohistochemistry. All authors edited and approved the manuscript. RJS and AB are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Duality of Interest: The authors declare that no conflict of interest exists.

Funding: IMPROVE was supported by the European Commission (Contract number: QLG1-CT-2002-00896), the Swedish Heart-Lung Foundation, the Swedish Research Council (projects 8691 and 0593), the Knut and Alice Wallenberg Foundation, the Foundation for Strategic

Research, the Stockholm County Council (project 592229), the Strategic Cardiovascular and Diabetes Programmes of Karolinska Institutet and Stockholm County Council, the European Union Framework Programme 7 (FP7/2007-2013) for the Innovative Medicine Initiative under grant agreement n° IMI/115006 (the SUMMIT consortium), the Academy of Finland (Grant #110413), the British Heart Foundation (RG2008/08, RG2008/014) and the Italian Ministry of Health (Ricerca Corrente). SDPP (Stockholm Diabetes Prevention Programme) was supported by Stockholm County Council, the Swedish Research Council, the Swedish Diabetes Association, the Swedish Council of Working Life and Social Research, and Novo Nordisk Scandinavia. KOMP obtained NIH grants to Velocigene at Regeneron Inc (U01HG004085) and the CSD Consortium (U01HG004080) funded the generation of gene-targeted embryonic stem cells for 8500 genes in the KOMP Program and archived and distributed by the KOMP Repository at UC Davis and CHORI (U42RR024244). For more information or to obtain KOMP products go to www.komp.org or email service@komp.org. . The SNP&SEQ Technology Platform in Uppsala is part of the National Genomics Infrastructure funded by the Swedish Council for Research Infrastructures hosted by Science for Life Laboratory. RJS is supported by SRP Diabetes Program at Karolinska Institutet and the KI Geriatric Foundation. BS acknowledge funding from the Magnus Bergvall Foundation and the Foundation for Old Servants. LM is a Ragnar Söderberg fellow in Medicine (M-14/55), and received funding from the Karolinska Institute Cardiovascular Program Career Development Grant and the Swedish Heart-Lung-Foundation (20120615, 20130664, 20140186).

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Table 1: IMPROVE cohort characteristics

		without diabetes	type 2 diabetes
N		2470	901
Male		1138 (44.7)	533 (57.4)
Age (years)		64.2 (5.4)	64.2 (5.6)
Height (m)		1.67 (0.09)	1.69 (0.09)
BMI (kg/m2)		26.6 (3.9)	29.2 (4.6)
WHR		0.91 (0.08)	0.95 (0.09)
SBP		141 (19)	145 (18)
DBP		82 (10)	82 (10)
LDL (mmol/L)		3.71 (0.97)	3.07 (0.95)
HDL (mmol/L)		1.31 (0.36)	1.14 (0.33)
Triglycerides (mmol/L)		1.47 (0.90)	1.91 (1.82)
Fasting glucose (mmol/L)		5.29 (0.67)	7.71 (2.18)
C reactive protein (mmol/L)		2.89 (6.16)	3.20 (4.22)
CD93 (ng/mL)		164 (45)	157 (40)
Fasting proinsulin (pmol/L)		6.03 (6.26)	10.5 (8.88)
Fasting insulin (pmol/L)		44.4 (61.5)	66.5 (88.4)
HOMA B		68.9 (54.3)	50.8 (50.8)
HOMA IR		0.83 (1.09)	1.33 (1.66)
Uric acid (mmol/L)		309 (70)	333 (76)
Creatinine (mmol/L)		80.3 (17.7)	82.8 (17.7)
Vitamin D (nmol/L)		50.7 (21.5)	48.2 (20.2)
Adiponectin (ug/mL)		14.2 (9.9)	9.43 (7.19)
Leptin (ng/mL)		20.0 (17.0)	21.6 (17.4)
IL-5 (pg/mL)		0.67 (1.82)	0.86 (3.50)
Pack years		9.84 (16.3)	14.1 (18.6)
Current smoking (%)		381 (15.0)	143 (15.4)
Lipid-lowering medication (%)		1268 (49.8)	449 (48.6)
Anti-hypertensive medication (%)		1397 (54.8)	604 (65.0)
Baseline	CC-IMT_{mean}	0.738 (0.141)	0.758 (0.145)
	BIF-IMT_{mean}	1.131 (0.396)	1.190 (0.429)
	IMT_{mean}	0.880 (0.196)	0.918 (0.206)
	CC-IMT_{max}	1.185 (0.196)	1.225 (0.412)
	BIF-IMT_{max}	1.840 (0.750)	1.954 (0.829)
	IMT_{max}	1.998 (0.792)	2.140 (0.862)
	$IMT_{mean-max}$	1.239 (0.292)	1.290 (0.312)
Progression	CC-IMT_{mean}	0.008 (0.025)	0.011 (0.034)
	BIF-IMT_{mean}	0.032 (0.070)	0.040 (0.087)
	IMT_{mean}	0.018 (0.030)	0.022 (0.035)
	CC-IMT_{max}	0.013 (0.087)	0.019 (0.113)
	BIF-IMT_{max}	0.047 (0.153)	0.058 (0.178)

IMT_{max}	0.040 (0.157)	0.056 (0.178)
$IMT_{mean-max}$	0.162 (0.140)	0.188 (0.155)
fastest_progression	0.024 (0.051)	0.028 (0.054)

Where: values are presented as mean (standard deviation) for continuous measures and n (%) for categorical measures. T2D was defined as diagnosis, anti-diabetic medication or fasting glucose >=7mmol/L; Vitamin D, adjusted for season of blood sampling; all IMT measured in mm.

Table 2: Spearmans rank correlation coefficients between sCD93 and cardiovascular risk markers

	without diabetes		type 2 diabetes	
	Rho	P	Rho	P
Sex	-0.001	0.9671	-0.045	0.1882
Age (years)	0.080	**0.0001**	0.166	**<0.0001**
Height (m)	-0.063	**0.0022**	-0.054	0.1163
BMI (kg/m2)	-0.073	**0.0003**	-0.042	0.2199
WHR	0.023	0.2672	0.007	0.8511
SBP (mmHg)	-0.033	0.1067	-0.099	**0.0042**
SBP (mmHg)*	-0.023	0.4501	0.038	0.5048
DBP (mmHg)	-0.020	0.3319	-0.016	0.6428
DBP (mmHg)*	-0.050	0.0940	-0.003	0.9619
LDL cholesterol (mmol/L)	0.042	**0.0422**	-0.028	0.4210
LDL cholesterol (mmol/L)#	0.006	0.8265	0.048	0.3165
HDL cholesterol (mmol/L)	-0.073	**0.0003**	-0.081	**0.0195**
HDL cholesterol (mmol/L)#	0.056	**0.0343**	-0.019	0.6872
Triglycerides (mmol/L)	-0.051	**0.0131**	-0.010	0.7663
Triglycerides (mmol/L)#	-0.098	**0.0002**	-0.074	0.1106
Fasting glucose (mmol/L)	-0.014	0.5101	NA	NA
C reactive protein (mmol/L)	-0.014	0.5101	0.020	0.5704
Current smoking	0.022	0.2833	-0.004	0.9027
Lipid lowering medication	-0.024	0.2455	0.000	0.9915
Anti-hypertensive medication	-0.025	0.2191	0.033	0.3467
fasting proinsulin (pmol/L)	-0.025	0.2223	NA	NA
fasting insulin (pmol/L)	-0.078	**0.0001**	NA	NA
HOMA B	-0.054	**0.0076**	NA	NA
HOMA IR	-0.080	**0.0001**	NA	NA
Uric Acid (micromol/L)	0.019	0.3567	0.023	0.5073
Creatinnine (micromol/L)	0.173	**<0.0001**	0.237	**<0.0001**
Vitamin D (nmol/L)	0.067	**0.0009**	0.032	0.3310
Adiponectin (ug/mL)	0.063	**0.0022**	0.028	0.4159
Leptin (ng/mL)	-0.025	0.2197	0.000	0.9932
IL-5 (pg/mL)	0.091	**<0.0001**	0.034	0.3060
FRS	0.022	0.2719	0.103	**0.0019**

Where: T2D was defined as diagnosis, anti-diabetic medication or fasting glucose >=7mmol/L;* subjects not on Anti-hypertensive medication (n= 1120 and 316 for subjects without diabetes and with type 2 diabetes respectively); # subjects not on lipid lowering medication (n= 1426 and 462 for subjects without diabetes and with type 2 diabetes respectively); FRS, Framingham risk score; Vitamin D, adjusted for season of blood sampling; NA, not applicable.

Table 3: SDPP followup characteristics (of baseline NGT subjects) by followup glucose regulation status

		without diabetes	prediabetes	type 2 diabetes
n*		370	314	158
male (%)		200 (54)	179 (57)	110 (69)
baseline	Age (years)	47.3 (4.7)	48.2 (4.4)	48.2 (4.6)
	Height (m)	1.73 (0.09)	1.72 (0.09)	1.74 (0.09)
	Weight (kg)	74.9 (12.4)	81.8 (14.1)	85.6 (15.2)
	BMI (kg/m2)	24.9 (3.2)	27.6 (4.1)	28.4 (4.7)
	WHR	0.84 (0.07)	0.87 (0.07)	0.90 (0.06)
	SBP	121 (14)	128 (15)	130 (15)
	DBP	76 (9)	80 (9)	81 (9)
	Fasting glucose (mmol/L)	4.6 0 (0.49)	4.94 (0.50)	5.06 (0.56)
	Fasting insulin (mU/L)	14.2 (6.3)	17.5 (9.0)	21.2 (10.2)
	sCD93 (ng/mL)	166 (44)	161 (44)	158 (45)
	scurrent smokers (%)	89 (22.2)	105 (29.2)	63 (36.8)
	BP treatment (%)	19 (4.8)	38 (10.6)	18 (10.6)
followup	followup time	9.1 (1.3)	9.2 (1.2)	9.5 (1.2)
	Age (years)	56.5 (4.8)	57.4 (4.5)	57.7 (4.7)
	Height (m)	1.72 (0.09)	1.71 (0.09)	1.73 (0.09)
	Weight (kg)	77.1 (13.3)	86.5 (15.9)	91.0 (18.2)
	BMI (kg/m2)	25.9 (3.4)	29.4 (4.8)	30.3 (5.8)
	WHR	0.88 (0.06)	0.91 (0.06)	0.94 (0.07)
	SBP	133 (17)	143 (17)	144 (18)
	DBP	82 (10)	87 (10)	87 (11)
	Fasting glucose (mmol/L)	4.86 (0.46)	5.72 (0.68)	7.35 (2.18)
	Fasting insulin (mU/L)	14.6 (6.0)	21.2 (11.9)	26.6 (13.2)
	sCD93 (ng/mL)	153 (42)	154 (51)	154 (48)
	delta sCD93	13 (46)	7 (52)	4 (55)
	scurrent smokers (%)	61 (15.3)	73 (20.3)	34 (20.0)
	T2D treatment (%)	0	0	39 (22.8)
	BP treatment (%)	64 (16.0)	133 (36.9)	72 (42.1)

Where: values are presented as mean (standard deviation) for continuous measures and n (%) for categorical measures; Prediabetes defined as impaired glucose tolerance and/or impaired fasting glucose;* smallest n for any variable; delta sCD93, baseline sCD93 – follow-up sCD93.

Table 4: Peripherial fasting levels of diabetes relavant analytes

	cd93+/-	*cd93wt*	p-value
Glucose (nmol/L)	12.6	11.1	0.03
Insulin (ng/ml)	17.4	12.6	0.24
Leptin (ng/ml)	62.2	51.5	0.24
Resistin (ng/ml)	164	183	0.30
Glucagon (ng/ml)	0.09	0.07	0.50
GLP-1 (ng/ml)	0.03	0.01	0.26
Homa-IR*	0.25	0.16	0.15
Total cholesterol (mg/dL)	442	433	0.86
Triglycerides (mg/dL)	138	139	0.89

where: Homa-IR* was calculated by G0 x I0 /22.5 where I0 is fasting blood insulin (µU/ml) and G0 fasting blood glucose (mmol/l)

Figure legends

Figure 1: Representative image of A) *apoe*$^{-/-}$*cd93*$^{+/-}$ and B) *apoe*$^{-/-}$*cd93*^{wt} descending aorta stained with Sudden IV. C) Quantification of lesions in the descending aorta of female and male *apoe*-deficient mice with 1 or 2 copies of *cd93* (n=9-12).

Figure 2: Glucose metabolism of *cd93*$^{+/-}$ male mice compared to *cd93*^{wt} male mice A) Glucose tolerance test of *cd93*$^{+/-}$ and *cd93*^{wt} male mice (n=10-13), aged 4 months, before given a western diet. B) Weight of *cd93*$^{+/-}$ and *cd93*^{wt} male mice (n=9-12 respectively), after 16 weeks of western diet. C) Glucose tolerance test of *cd93*$^{+/-}$ and *cd93*^{wt} male mice (n=9-12 respectively), after 16 weeks of western diet. D) Insulin tolerance test of *cd93*$^{+/-}$ and *cd93*^{wt} male mice (n=9-11 respectively), after 16 weeks of western diet. ** $p \leq 0.01$ * $p \leq 0.05$ 2 way ANOVA between genotypes, error bar SEM.

Figure 3: Immunohistochemistry of pancreas sections demonstrating the location of insulin, sCD93 and vWF in mice with 2, 1 or 0 copies of the *cd93* gene (*cd93*^{wt}*, cd93*$^{+/-}$ *and cd93*^{ko} respectively).

Figure 4: A. Percent of vWF positive islets in pancreas from mice fed 16 weeks on western diet B. Quantification of Evans blue in pancreas in mice with 1 or 0 copies of *cd93* gene, * $p \leq 0.05$ sudent's T-test error bar SEM, Amount of soluble vWF A2 (C) and E-selectin (D) in plasma of mice with 1 or 0 copies of *cd93* gene and fed 16 weeks on western diet. B, D, * $p \leq 0.05$ sudent's T-test, D * $p \leq 0.05$ one way ANOVA between genotypes and diets, error bar SEM.

List of Online Supplemental Materials

Online Supplemental Affiliations: Additional members of the IMPROVE study group members

Online Supplementary Table 1: Cell populations in mice with one or 2 copies of the cd93 gene

Online Supplementary Table 2: No association between cd93 and measures of baseline or progression of IMT in IMPROVE

Online Supplemental Table 3: SDPP baseline characteristics by baseline glucose regulation status

Online Supplemental Table 4: Genotypes of 53 (of 62 known (21)) type 2 diabetes-risk associated SNPs were available for the IMPROVE cohort and were analysed for association with sCD93 levels

Online Supplemental Figure 1: Schematic demonstrating the study design used for analysis of the Stockholm Diabetes Prevention Program (NGT, normal glucose tolerance; prediabetes, impaired glucose tolerance or impaired fasting glucose; T2D, type 2 diabetes).

Online Supplemental Figure 2: Effect of normo-glycaemia or hyper-glycaemia on release of sCD93 from endothelial cells. A. Human carotid artery endothelial cells (HctAEC) were cultured in the presence of 5nM or 30nM glucose, with and without inflammatory stimuli (50nM PMA or 50ug/ml LPS) for 24 hours. B. Human endothelial hybrid cells EA.Hy 926 were cultured in the presence of 5nM or 30nM glucose, with and without inflammatory stimuli (50nM PMA) for 24 hours.

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Online Supplemental Table 1: Cell populations in mice with one or 2 copies of the *cd93* gene

	cd93$^{+/-}$	*cd93wt*	P value
scd93 (ng/mL)	104 (18)	254 (63)	**0.008**
% cd93+ B cells*	2.79 (1.08)	6.43 (1.61)	**0.018**
% iNKT cells#	2.49 (1.29)	3.43 (2.45)	0.410
% IgG+ B cells*	79 (8.02)	81 (8.64)	0.590
% IgM+ B cells*	0.81 (0.29)	1.08 (0.75)	0.270

Where: *, Defined as CD45R+CD19+ from spleen; #, Defined as CD4+α-galCer tetramer + from liver. values are presented as mean (standard deviation).

Online Supplemental Table 2: Association of sCD93 levels with measures of IMT

		without diabetes			type 2 diabetes		
		Beta	Se	P	Beta	Se	P
baseline	CC-IMT_{mean}	0.007	0.005	0.168	-0.016	0.010	0.102
	BIF-IMT_{mean}	-0.006	0.010	0.575	0.012	0.019	0.527
	IMT_{mean}	0.002	0.006	0.706	0.006	0.012	0.606
	CC-IMT_{max}	0.002	0.008	0.762	-0.012	0.016	0.438
	BIF-IMT_{max}	-0.002	0.012	0.873	0.031	0.023	0.185
	IMT_{max}	0.003	0.012	0.808	0.035	0.022	0.116
	$IMT_{mean-max}$	0.002	0.007	0.716	0.008	0.013	0.536
Progression	CC-IMT_{mean}	-0.002	0.002	0.296	-0.002	0.005	0.658
	BIF-IMT_{mean}	-0.004	0.005	0.438	-0.005	0.013	0.698
	IMT_{mean}	-0.001	0.002	0.547	-0.002	0.005	0.741
	CC-IMT_{max}	0.004	0.007	0.573	-0.015	0.016	0.351
	BIF-IMT_{max}	-0.008	0.012	0.463	-0.014	0.025	0.560
	IMT_{max}	0.006	0.012	0.621	0.002	0.025	0.937
	$IMT_{mean-max}$	0.002	0.004	0.642	-0.004	0.008	0.586
	fastest_progression	0.001	0.015	0.933	-0.045	0.030	0.136

Where: all multiple regression analyses were adjusted for age and sex; Progression variables also adjusted for their baseline counterparts; all IMT measurements in mm.

Online Supplemental Table 3: SDPP baseline characteristics by baseline glucose regulation status

	without diabetes	prediabetes	type 2 diabetes
n*	843	326	113
male (%)	489 (58)	180 (55)	63 (56)
Age (years)	47.8 (4.6)	48.5 (4.4)	49.5 (4.0)
Height (m)	1.73 (0.09)	172 (0.10)	1.71 (0.09)
Weight (kg)	79.5 (14.3)	85.7 (17.6)	88.2 (18.3)
BMI (kg/m2)	26.6 (4.12)	29.1 (5.4)	30.0 (5.8)
WHR	0.87 (0.07)	0.89 (0.08)	0.91 (0.07)
SBP	125 (15)	134 (19)	135 (17)
DBP	79 (10)	83 (11)	83 (9)
Fasting glucose (mmol/L)	4.82 (0.54)	5.54 (0.75)	7.71 (2.69)
Fasting insulin (mU/L)	16.8 (8.6)	21.7 (11.9)	29.9 (20.1)
sCD93 (ng/mL)	163 (44)	158 (44)	158 (41)
scurrent smokers (%)	257 (27.6)	85 (23.6)	46 (35.9)
BP treatment (%)	75 (8.1)	60 (16.7)	28 (21.9)

Where:* smallest n for any variable; values are presented as mean (standard deviation) for continuous measures and n (%) for categorical measures

Online Supplemental Table 4: SNPs associated with type 2 diabetes have little effect on sCD93 levels in subjects without type 2 diabetes

CHR	SNP	BP	Locus	proxy used	EAF	N	BETA	SE	L95	U95	P
1	rs17106184	50909985	*FAF1*	na	A	2470	0.000	0.014	-0.027	0.027	0.9753
1	rs2820446	219750717	*LYPLAL1*	rs4846567	T	2470	0.010	0.009	-0.007	0.027	0.2604
2	rs10190052	647760	*TMEM18*	rs4854348	A	2470	0.001	0.010	-0.018	0.021	0.9012
2	rs243088	60573870	*BCL11A*	rs243083	G	2470	-0.001	0.008	-0.016	0.014	0.8796
2	rs3923113	165555207	*GRB14*	rs7609045	A	2470	-0.002	0.008	-0.018	0.013	0.7622
2	rs2943640	227093585	*IRS1*	na	A	2470	0.007	0.008	-0.009	0.023	0.3904
3	rs1801282	12391583	*PPARG*	rs2197423	A	2470	0.009	0.012	-0.014	0.033	0.4366
3	rs11717195	123082398	*ADCY5*	na	C	2470	0.017	0.010	-0.002	0.036	0.0848
3	rs4402960	185511687	*IGF2BP2*	na	T	2470	0.001	0.008	-0.015	0.017	0.9104
3	rs6808574	187740523	*LPP*	na	T	2418	0.006	0.008	-0.009	0.022	0.4215
4	rs4458523	6293350	*WFS1*	rs10012946	T	2470	-0.001	0.008	-0.016	0.015	0.9313
4	rs6813195	153520475	*TMEM154*	na	T	2470	0.001	0.008	-0.015	0.017	0.8918
5	rs702634	53271420	*ARL15*	na	G	2470	0.015	0.008	-0.002	0.031	0.0769
5	rs319598	134240235	*PCBD2*	na	T	2470	-0.004	0.008	-0.020	0.011	0.5663

6	rs7756992	20679709	*CDKAL1*	na	G	2470	0.005	0.009	-0.012	0.022	0.5535
6	rs9472138	43811762	*VEGFA*	na	T	2470	-0.012	0.009	-0.029	0.005	0.1677
6	rs4273712	126964510	*C6orf173*	na	G	2470	0.000	0.008	-0.016	0.017	0.9881
6	rs6937795	137299152	*IL20RA*	rs4407733	A	2470	0.006	0.008	-0.009	0.021	0.4567
7	rs7795991	13900731	*ETV1*	na	G	2470	-0.003	0.008	-0.019	0.012	0.6565
7	rs17168486	14898282	*DGKB*	na	T	2470	-0.011	0.010	-0.031	0.009	0.2819
7	rs849135	28196413	*JAZF1*	na	A	2470	-0.009	0.008	-0.024	0.007	0.2617
8	rs516946	41519248	*ANK1*	na	T	2470	-0.021	0.009	-0.039	-0.003	**0.0211**
8	rs7845219	95937502	*TP53INP1*	na	C	2470	-0.006	0.008	-0.021	0.009	0.4660
8	rs3802177	118185025	*SLC30A8*	na	A	2470	-0.009	0.008	-0.026	0.007	0.2682
8	rs1561927	129568078	*TMEM75*	na	C	2469	-0.007	0.009	-0.024	0.010	0.4307
9	rs7041847	4293150	*GLIS3*	rs10814916	A	2470	0.005	0.008	-0.011	0.020	0.5529
9	rs10811661	22132076	*CDKN2A,CDKN2B*	rs2383208	G	2470	-0.006	0.010	-0.025	0.013	0.5390
9	rs17791513	81905590	*TLE4*	na	G	2470	-0.006	0.014	-0.034	0.022	0.6781
9	rs2796441	84308948	*TLE1*	na	A	2470	0.015	0.008	-0.001	0.030	0.0636
10	rs11257655	12307894	*CDC123*	na	T	2470	-0.016	0.009	-0.035	0.002	0.0759

10	rs12571751	80942631	*ZMIZ1*	na	G	2470	0.006	0.008	-0.010	0.021	0.4758
10	rs10788575	89768584	*PTEN*	na	A	2470	0.027	0.012	0.004	0.049	**0.0213**
10	rs1111875	94462882	*HHEX,IDE*	na	T	2470	0.006	0.008	-0.009	0.021	0.4612
10	rs7903146	114758349	*TCF7L2*	na	T	2470	0.012	0.009	-0.005	0.029	0.1699
10	rs10510110	124193181	*PLEKHA1*	rs2280141	T	2470	-0.002	0.008	-0.017	0.014	0.8177
11	rs163184	2847069	*KCNQ1*	na	T	2469	-0.003	0.008	-0.018	0.013	0.7320
11	rs5215	17408630	*KCNJ11*	na	C	2469	0.004	0.008	-0.012	0.020	0.6120
11	rs1552224	72433098	*ARAP1,CENTD2*	na	C	2469	-0.004	0.010	-0.025	0.016	0.6800
11	rs10830963	92708710	*MTNR1B*	na	G	2470	-0.004	0.008	-0.021	0.012	0.5925
12	rs10842994	27965150	*KLHDC5*	na	T	2470	-0.011	0.010	-0.032	0.009	0.2689
12	rs2261181	66212318	*HMGA2*	na	T	2470	-0.003	0.012	-0.028	0.021	0.7921
12	rs1727313	123616514	*MPHOSPH9*	rs1727294	A	2470	0.000	0.009	-0.018	0.018	0.9864
13	rs10507349	26781528	*RNF6*	na	A	2470	0.003	0.010	-0.016	0.022	0.7688
13	rs1359790	80717156	*SPRY2*	na	A	2470	-0.007	0.009	-0.024	0.010	0.4016
15	rs7163757	62383155	*C2CD4A*	rs4502156	C	2470	-0.009	0.008	-0.025	0.006	0.2485
15	rs7178572	77747190	*HMG20A*	na	A	2470	0.013	0.008	-0.003	0.030	0.1143

15	rs12899811	91544076	*PRC1*	na	G	2469	0.005	0.008	-0.011	0.021	0.5474
16	rs9936385	53819198	*FTO*	rs9923233	C	2470	0.001	0.008	-0.014	0.017	0.8634
17	rs11651755	36099840	*HNF1B*	na	C	2470	-0.016	0.008	-0.032	-0.001	**0.0423**
18	rs12970134	57876227	*MC4R*	rs11663816	C	2470	-0.003	0.009	-0.021	0.015	0.7217
19	rs8108269	46158513	*GIPR*	na	G	2470	0.005	0.008	-0.012	0.021	0.5838
20	rs4812829	42989267	*HNF4A*	na	A	2470	-0.011	0.010	-0.030	0.009	0.2971

Where: analyses adjusted for age, sex and population structure; EAF, type 2 diabetes risk-increasing allele; na, not applicable.

Online Supplemental Table 5: Effect of sCD93-associated SNPs (Mälarstig *et al* 2012) on sCD93 and HOMA indices.

		without diabetes (n=2474)												type 2 diabetes (n=900)		
		sCD93			HOMA B			HOMA S			HOMA IR			sCD93		
SNP	EA	Beta	Se	P	Beta	Se	P	Beta	Se	P	Beta	Se	P	Beta	Se	P
rs2749812	A	-0.018	0.010	0.0754	-0.003	0.022	0.8926	0.002	0.033	0.9612	-0.002	0.033	0.9612	0.003	0.015	0.8621
rs3746731	G	-0.006	0.008	0.4815	-0.005	0.017	0.7514	-0.014	0.026	0.5900	0.014	0.026	0.5900	0.009	0.012	0.4364

Where: analyses adjusted for age, sex and population structure; EA, effect allele; Homeostasis Model Assessment (HOMA) indices were computed using the HOMA2 calculator (https://www.dtu.ox.ac.uk/homacalculator). Effect allele frequency of rs2749812 =0.17 and rs3746731 =0.48.