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

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Abstract

Type 2 diabetes and cardiovascular disease are complex disorders involving metabolic and inflammatory mechanisms. Here we investigated whether sCD93, a group XIV c-type lectin of the endosialin family, plays a role in metabolic dysregulation or 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, $p<0.0001$). Genetic variants associated with diabetes risk (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) demonstrated no difference in atherosclerotic lesion development compared to *apoe*$^{-/-}$ cd93-sufficient littermates. However, *cd93*-deficient mice showed impaired glucose clearance and insulin sensitivity (compared to littermate controls) 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 CD93 as 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. 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, a combination of metabolic dysregulation and inflammatory pathways are important contributors. 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 in innate immune cells such as neutrophils and monocytes as well as in 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. Described as a factor involved in removal of apoptotic bodies, CD93 has also been involved in B cell maturation and Natural Killer T cell (iNKT cell) survival (6). 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 metabolism and CVD, *CD93* is a plausible candidate in the mouse non-obese diabetes *Idd13* locus (10), and we have previously shown that reduced levels of circulating sCD93 are associated with increased risk of myocardial infarction (MI) (11). 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; during the development of new but leaky blood vessels (14) as observed in retinopathy; during ischemia-related inflammation of cerebral vascular endothelium (15) thus reflecting MI.

Here we investigated sCD93 for effects on markers of metabolic dysregulation and early cardiovascular disease in human cohorts and in a mouse model with a genetic deficiency in sCD93. We further examined the mechanisms by which sCD93 acts.

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 without symptoms or history of coronary artery disease were enrolled from 7 European centres (at latitudes ranging from 43 to 62° North). 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 (IMT) phenotyping was performed by ultrasound at baseline, as well as 15 and 30 months after enrolment (16; 17). Approval was granted by the regional ethics committee for each recruitment centre and written informed consent was provided by all participants. Type 2 diabetes was defined as diagnosis, anti-diabetic medication or fasting glucose ≥7mmol/L. Soluble CD93 was measured using the Mesoscale platform, using the previously validated ELISA antibodies (11) and SECTOR Imager 2400. Characteristics of the cohort are presented in Table 1.

IMPROVE Genotyping

Reported type 2 diabetes risk-associated SNPs (18) were genotyped in the IMPROVE cohort using the Illumina Metabochip (19) and Immunochip (20) platforms. Genotyping was conducted at the SNP&SEQ Technology Platform, Uppsala University, Sweden and standard quality control was conducted; Subject exclusions: low call rate (<95%), cryptic relatedness or ambiguous sex. SNPs 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 (21) with default settings. The first MDS component demonstrates strong correlations with latitude of recruitment centre (Spearmans rank Rho 0.935 $p<0.0001$ and Rho 0.946 $p<0.0001$ for subjects without and with type 2 diabetes respectively).

Statistical analyses: IMPROVE Epidemiology

The trend test for ordered groups was used to assess an effect of recruitment centre latitude. 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 transformed for further statistical analyses. Multivariable regression analysis was used to identify 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) (22), was used to assess the effect of sCD93 levels on measures of IMT. Analyses were conducted using STATA 11.2 (STATCorp, College Station, TX, USA).

Statistical analyses: Genetics

Linear regression analyses assuming an additive genetic model were conducted in PLINK (21) to assess the influence of type 2 diabetes risk-associated SNPs on sCD93 levels, adjusting for age, sex and population structure (MDS1-3). Genotypes of 52 (of 62 known (18)) type 2 diabetes-risk associated SNPs were combined in an unweighted SNP score by summing the reported (18) type 2 diabetes risk-increasing alleles for each subject (thus representing the total burden of genetically determined type 2 diabetes risk). Only subjects without type 2 diabetes and with complete genotyping were included in this analysis. 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 study of subjects from the Stockholm area, aged 35-55 years at baseline (23). 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 were measured by Mesoscale in baseline samples and in a subset of follow-up samples (Online Supplemental Figure 1). Baseline samples were from subjects newly diagnosed with normal glucose tolerance (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 NGT subjects at baseline were also analysed. Some subjects remained NGT (n=370), whilst others had progressed to pre-diabetes (n=314) or type 2 diabetes (158). Karolinska Institutets Ethics committee approved the study and all subject gave their informed consent. ANOVA (adjusted for age and sex) was used to compare levels of sCD93 between glucose tolerance groups at baseline or after follow-up. T-tests were used to compare baseline levels of sCD93 from subjects diagnosed as NGT and prediabetes or T2D at follow-up. The effect of baseline sCD93 levels on risk of developing prediabetes or T2D was assessed using logistic regression, adjusting for age and sex, or age, sex, current smoking, BMI and blood pressure medication. Analyses were conducted in STATA 11.2 (STATCorp,Texas, USA).

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 concentration of sCD93 in the periphery compared to their wild-type ($cd93^{+/+}$) littermates (Supplemental Table 1), rendering this a relevant model to be used in comparisons to human studies, as humans have

varying levels of sCD93 (11), rather than complete absence of sCD93. Therefore, this study focuses on *cd93*$^{+/+}$ 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.

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 (Online Supplemental Table 1) 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. The percent of iNKT cells in the liver was determined using the previously published method (10) with the exception that a violet viability dye (Live/Dead Life technologies) was included, using the Beckman Coulter Gallios™ flow cytometer.

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

For atherosclerosis studies, mice were crossed into *apoe*-deficient (*apoe*$^{-/-}$) mice (originally from Jackson Laboratory) and backcrossed 6 generations to C57BL/6N. Homozygous for *apoe* deficiency mice, with 2 or 1 copies of the *cd93* gene (*apoe*$^{-/-}$*cd93*$^{+/+}$ and *apoe*$^{-/-}$*cd93*$^{+/-}$

respectively) were fed a normal rodent diet for 32 weeks, at which point blood was 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 area of all the plaques in a given aortic arch were summed and expressed as the percentage of the total surface area of the aorta using ImageJ software (NIH).

Metabolic studies of mice (cd93$^{+/+}$ 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 15, 30, 60, 120 minutes.

Pancreatic morphology in the cd93-deficient mouse model (cd93$^{+/+}$ 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; and rabbit anti-rat, Vector Laboratories respectively) for 1hr at room temperature. Peroxidase-avidin/biotin complex was achieved using Vectastain 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 was measured using ImageJ software (NIH). The number of vWF-positive and total islets was 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*$^{-/-}$ mouse also fed on western diet for 16 weeks was included to confirm specificity of anti-cd93 staining.

Blood vessel integrity (*cd93*$^{+/+}$ *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 30min, 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 (24) and detected using GlowMax Multi with fluorescence 625/660-720 (Promega).

Peripheral markers of endothelial damage (*cd93*$^{+/+}$ *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 Mesoscale and the DuoKit for E-selectin (R&D Systems, Minneapolis, MN) with addition of SULFO-TAG labelled Streptavidin. vWF A2 was measured using SimpleStep Elisa kit from Abcam as per manufacturer's directions.

Statistical analysis of murine data

Students T-test was used to determine statistical significance between two groups. Differences between more than two groups were assessed by one-way ANOVA with post-hoc analysis using Tukey's multiple comparison test. When analysing repeated glucose metabolism measurements, a 2-way ANOVA repeated measurements test was used to establish significance with post-hoc analysis using Sidak's multiple comparisons test. PRISM (Graphpad Software, San Diego, USA) was used for ANOVA procedures.

Analysis of sCD93 release from endothelial cells

To assess the impact of diabetes-relevant conditions on sCD93 release, the human carotid endothelial cells (HCtAEC, 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 (Sigma-Aldrich)) were expanded in flasks coated with gelatin (Sigma-Aldrich). During passage 5, cells were seeded onto gelatin-coated 48 well plates. After overnight incubation with glucose-free DMEM (Sigma-Aldrich), HCtAEC were 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 as above.

Results

Plasma levels of sCD93 in IMPROVE

In IMPROVE, latitude was the strongest independent predictor of IMT (17). No significant association was observed between sCD93 and latitude ($p=0.942$). Consistent with previous reports (11), there was no significant difference between men and women (mean±sd: 162±42ng/mL vs 163±45ng/mL, $p=0.3833$). 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 IMT or other CVD risk factors.

sCD93 levels and metabolic or cardiovascular risk markers

In the subjects without diabetes, sCD93 correlated with age, height, and metabolic markers (BMI, insulin, HOMA indices, vitamin D and adiponectin; Table 2). Consistent with lower levels being associated with poor metabolic control, sCD93 was positively correlated with adiponectin and vitamin D, but inversely with BMI, insulin and HOMA. The association between sCD93 and lipids was confounded by lipid-lowering medication (Table 2). In lipid-lowering-naïve subjects, sCD93 levels were associated with an advantageous metabolic profile, i.e positively with HDL levels and negatively with TGs. A negative correlation was observed between sCD93 levels and SBP, however this association was lost when analysing subjects without anti-hypertensive medication. Associations with metabolic variables remained significant after adjustment for age and sex (Online Supplemental Table 2).

sCD93 levels and IMT in IMPROVE

As cardiovascular risk factors have a large impact on IMT measures (16; 17), these parameters were considered for inclusion in multiple regression models. Proinsulin and insulin measurements were omitted as they are not informative in subjects with diabetes (due to

influence of medication and pathology). 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). sCD93 were not associated with any baseline or progression measures of IMT in subjects with or without type 2 diabetes, when adjusting for age and sex (Supplemental Table 3), 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 (assuming an effect size of $\geq$0.009 gave 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 IMT.

Type 2 diabetes risk-associated SNPs and sCD93 levels

A Mendelian randomisation experiment was conducted to assess whether reduced sCD93 levels are a consequence or possible cause of type 2 diabetes. If reduced sCD93 levels are a consequence 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 (18)) 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 (Spearmans rank rho=0.0045, p=0.8248). These findings indicate that genetic susceptibility to type 2 diabetes is unlikely to be 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

In order to assess whether the multiple metabolic aberrations which characterize IMPROVE affect the results presented, the prospective SDPP cohort, specifically designed to assess potential biomarkers of type 2 diabetes, was investigated. Baseline and follow-up features are presented in Supplemental Table 5 and Table 3, respectively. Baseline levels of sCD93 did not differ across glucose tolerance groups: NGT 163±44ng/mL, prediabetes 158±44ng/mL and type 2 diabetes 158±41ng/mL (ANOVA p=0.23, adjustment for age and sex). Similarly, no significant difference was found between follow-up levels of NGT, pre-diabetes or type 2 diabetes (153±42, 154±51 and 154±48 ng/mL, respectively). To assess whether baseline sCD93 levels influenced progression to prediabetes or to type 2 diabetes over the time, baseline levels were compared between subjects (all NGT at baseline) who were diagnosed as NGT, prediabetes or type 2 diabetes at follow-up. Subjects who remained NGT at follow-up had significantly higher baseline 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 baseline sCD93 levels was observed in subjects who remained NGT at follow-up compared to those who progressed to pre-diabetes during follow-up (166±44ng/mL vs 161±44ng/mL, respectively, p=0.058). Logistic regression demonstrated that baseline sCD93 levels were significantly associated with progression to poor metabolic control (prediabetes or T2D, beta -0.612, se 0.272, p=0.024) but not to T2D specifically (beta -0.573, se 0.346, p=0.098), and this was independent of current smoking, blood pressure medication and BMI (beta -0.894, se 0.298, p=0.003). However, inclusion of sCD93 levels in the model did not provide additional benefits (area under ROC curve 0.73 irrespective of inclusion of sCD93).

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), defective maturation of B cells and iNKT cells (23; 25) and altered vascular permeability in glioma (9). These mice lack only exon 1 of the *cd93* gene and had a mixed genetic background, (129/sv embryonic stem cells crossed to C57BL/6J). 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, mice carrying one *cd93* gene ($cd93^{+/-}$) had approximately half the concentration of circulating scd93 compared to wild type mice ($cd93^{+/+}$, 104±18 vs 254±63 ng/mL respectively, p=0.008, Supplemental Table 1). Compared to $cd93^{+/+}$, $cd93^{+/-}$ mice showed no difference in mature B cell populations (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 $apoe^{-/-}cd93^{+/+}$ vs $apoe^{-/-}cd93^{+/-}$ mice

To investigate the impact of CD93 on atherosclerosis, the *cd93*-deficient mouse model was crossed with the *apoe*-deficient ($apoe^{-/-}$) mice, commonly used to study atherosclerosis. $Apoe^{-/-}cd93^{+/+}$ 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 $apoe^{-/-}cd93^{+/+}$ 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 IMT.

Metabolic characteristics of $cd93^{+/+}$ vs $cd93^{+/}$ mice

To mirror the human metabolic findings, we investigated whether mice with reduced sCD93 levels had impaired glucose metabolism. When fed a chow diet, both genotypes demonstrated a similar rate of glucose clearance, however $cd93^{+/-}$ male mice had higher basal level of glucose

compared with *cd93*$^{+/+}$ (mean ± sem: 187.4 ± 13.6 mg/dL vs 161.9 ± 4.2 mg/dL respectively, after 4 hour fasting, Figure 2~~1~~). Female mice demonstrated no significant difference (mean ± sem: 137.5 ± 4.7 mg/dL vs 137.4 ± 5.7 mg/dL, after 4 hour fasting, 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*$^{+/+}$ mice, which was not due to a difference in weight (Figure 2). This was not seen in female mice (Online Supplement Figure 2). Levels of fasting insulin and biomarkers of metabolic dysregulation (leptin, glucagon, resistin and GLP-1) were measured and compared between *cd93*$^{+/-}$ and *cd93*$^{+/+}$ 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*$^{+/+}$ mice and were more insulin resistant (as measured by HOMA-IR).

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

The number and the average size of islets did not differ between *cd93*$^{+/+}$ and *cd93*$^{+/-}$ mice (21.7 vs 24.1, p=0.34 and 595 vs 622 pixels, p=0.42, respectively). Insulin staining was visible in islets in all genotypes, however some interstitial insulin staining was apparent in sections from the *cd93*$^{+/-}$ mice (Figure 3, top panel). As expected, vWF staining was restricted to endothelium in all genotypes (Figure 3, middle panel). In *cd93*$^{+/+}$ mice, cd93 demonstrated endothelial staining (as expected with cell surface-attached cd93; Figure 3, bottom 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 cd93 staining was less obvious, but the diffuse cd93 staining was clearly visible. However, in the pancreas obtained from a *cd93*$^{-/-}$ mouse, no cd93 staining was observed. Interestingly, *cd93*$^{+/-}$ mice had a trend (p=0.08) of decreased percentage of vWF positive islets

compared to *cd93*$^{+/+}$ mice, indicating the presence of endothelial disturbances in western diet fed *cd93*$^{+/-}$ mice (Figure 4A).

Pancreatic blood vessel integrity

Given the role of cd93 in vessel leakage (9), and the interstitial insulin staining in *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 confined 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 compared to *cd93*$^{+/+}$ littermates (Figure 4B). The finding that higher levels of Evans blue were detected in *cd93*$^{+/-}$ than *cd93*$^{+/+}$ 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 animals 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*$^{+/+}$ (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 as 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 did not influence the release of sCD93 from primary HCtAEC under basal (media) or LPS-stimulated conditions (Online Supplemental Figure 3~~2~~A), however hyper-glycaemia (30mM glucose) reduced PMA-stimulated release of sCD93 compared to normo-glycaemia (5mM

glucose). This experiment was repeated with the EA.Hy 926 cell line, with comparable results (Online Supplemental Figure 3B).

Discussion

The main objective of this study was to elucidate whether sCD93 plays 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 the present study is the finding that reduced levels of sCD93 were associated with metabolic dysregulation. In addition, we show that: i) lower sCD93 levels were observed in high CVD risk subjects with type 2 diabetes than those without; 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 precede development of type 2 diabetes; v) dietary stress in a *cd93*-deficient mouse model caused impaired metabolic regulation and increased endothelial damage; vi) cd93 (both cell surface-bound and soluble) was 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 is consistent between human and mouse.

Thus, we propose that CD93 expression and sCD93 release in pancreatic islets are components of stress responses and are ~~is~~ important for endothelial integrity and thereby metabolic control. CD93-deficiency leads to leaky blood vessels, which under normal metabolic conditions 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 would permit 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 6) 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 (18)). Whilst IMPROVE is the largest cohort to date with data on sCD93 levels, this cohort is not metabolically uniform, in contrast to the myocardial infarction 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 (11) 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. Women of IMPROVE were all in 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 and angiogenic mediators. Our demonstration of clear cd93 staining in pancreatic islets is novel and might reflect a protective function, whereby a deficiency in cd93 results in morphological and physiological changes in the pancreas. Furthermore, the *in vitro* studies showing 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 are 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 CD93 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.

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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. CÖ, AB and RS conducted the immunohistochemistry. All authors edited and approved the manuscript. RJS and AB take full responsibility for this work. . 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.

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Table 1: IMPROVE cohort characteristics

	without diabetes	type 2 diabetes	P
N	2470	901	
Male [%]	1138 [44.7]	533 [57.4]	**<0.0001**
Age (years)	64.2 (5.4)	64.2 (5.6)	0.8246
Height (m)	1.67 (0.09)	1.69 (0.09)	**<0.0001**
BMI (kg/m2)	26.6 (3.9)	29.2 (4.6)	**<0.0001**
WHR	0.91 (0.08)	0.95 (0.09)	**<0.0001**
SBP	141 (19)	145 (18)	**<0.0001**
DBP	82 (10)	82 (10)	0.3524
LDL (mmol/L)	3.71 (0.97)	3.07 (0.95)	**<0.0001**
HDL (mmol/L)*	1.31 (0.36)	1.14 (0.33)	**<0.0001**
Triglycerides (mmol/L)*	1.47 (0.90)	1.91 (1.82)	**<0.0001**
Fasting glucose (mmol/L)*	5.29 (0.67)	7.71 (2.18)	**<0.0001**
C reactive protein (mmol/L)*	2.89 (6.16)	3.20 (4.22)	**0.0001**
CD93 (ng/mL)*	164 (45)	157 (40)	**<0.0001**
Fasting proinsulin (pmol/L)*	6.03 (6.26)	10.5 (8.88)	**<0.0001**
Fasting insulin (pmol/L)*	44.4 (61.5)	66.5 (88.4)	**<0.0001**
HOMA B*	68.9 (54.3)	50.8 (50.8)	**<0.0001**
HOMA IR*	0.83 (1.09)	1.33 (1.66)	**<0.0001**
Uric acid (mmol/L)*	309 (70)	333 (76)	**<0.0001**
Creatinine (mmol/L)*	80.3 (17.7)	82.8 (17.7)	**<0.0001**
Vitamin D (nmol/L)*	50.7 (21.5)	48.2 (20.2)	0.963
Adiponectin (ug/mL)*	14.2 (9.9)	9.43 (7.19)	**<0.0001**
Leptin (ng/mL)*	20.0 (17.0)	21.6 (17.4)	**0.0138**

IL-5 (pg/mL)*		0.67 (1.82)	0.86 (3.50)	**<0.0001**
Pack years		9.84 (16.3)	14.1 (18.6)	**<0.0001**
Current smoking [%]		381 [15.0]	143 [15.4]	0.7483
Lipid-lowering medication [%]		1268 [49.8]	449 [48.6]	0.542
Anti-hypertensive medication [%]		1397 [54.8]	604 [65.0]	**<0.0001**
Baseline	CC-IMT_{mean}*	0.738 (0.141)	0.758 (0.145)	**0.0001**
	BIF-IMT_{mean}*	1.131 (0.396)	1.190 (0.429)	**0.0002**
	IMT_{mean}*	0.880 (0.196)	0.918 (0.206)	**<0.0001**
	CC-IMT_{max}*	1.185 (0.196)	1.225 (0.412)	**0.0035**
	BIF-IMT_{max}*	1.840 (0.750)	1.954 (0.829)	**0.0004**
	IMT_{max}*	1.998 (0.792)	2.140 (0.862)	**<0.0001**
	$IMT_{mean-max}$*	1.239 (0.292)	1.290 (0.312)	**<0.0001**
Progression	CC-IMT_{mean}	0.008 (0.025)	0.011 (0.034)	**0.0031**
	BIF-IMT_{mean}	0.032 (0.070)	0.040 (0.087)	**0.0134**
	IMT_{mean}	0.018 (0.030)	0.022 (0.035)	**0.0007**
	CC-IMT_{max}	0.013 (0.087)	0.019 (0.113)	0.1385
	BIF-IMT_{max}	0.047 (0.153)	0.058 (0.178)	0.0700
	IMT_{max}	0.040 (0.157)	0.056 (0.178)	**0.0145**
	$IMT_{mean-max}$	0.162 (0.140)	0.188 (0.155)	**0.0482**
	fastest_progression	0.024 (0.051)	0.028 (0.054)	**<0.0001**

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. P-value as determined by T-test. * log10 transformed prior to analysis.

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	-0.020	-0.5704
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	-0.024	0.4826
fasting insulin (pmol/L)	-0.078	**0.0001**	-0.007	0.8293
HOMA B	-0.054	**0.0076**	0.021	0.5333
HOMA IR	-0.080	**0.0001**	-0.011	0.7263
Uric Acid (micromol/L)	0.019	0.3567	0.023	0.5073
Creatinine (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.

Table 3: Characteristics of the SDPP subjects diagnosed at baseline as NGT

	Follow up diagnosis	without diabetes	prediabetes	type 2 diabetes	ANOVA p
	N~~n~~*	370	314	158	
	M~~m~~ale [%]	200 [54]	179 [57]	110 [69]	**0.0019**
Baseline	Age (years)	47.3 (4.7)	48.2 (4.4)	48.2 (4.6)	**0.0102**
	Height (m)	1.73 (0.09)	1.72 (0.09)	1.74 (0.09)	0.1294
	Weight (kg)	74.9 (12.4)	81.8 (14.1)	85.6 (15.2)	**<0.0001**
	BMI (kg/m2)	24.9 (3.2)	27.6 (4.1)	28.4 (4.7)	**<0.0001**
	WHR	0.84 (0.07)	0.87 (0.07)	0.90 (0.06)	**<0.0001**
	SBP	121 (14)	128 (15)	130 (15)	**<0.0001**
	DBP	76 (9)	80 (9)	81 (9)	**<0.0001**
	Fasting glucose (mmol/L)	4.6 0 (0.49)	4.94 (0.50)	5.06 (0.56)	**<0.0001**
	Fasting insulin (mU/L)	14.2 (6.3)	17.5 (9.0)	21.2 (10.2)	**<0.0001**
	sCD93 (ng/mL)	166 (44)	161 (44)	158 (45)	0.0700
	~~sc~~Current smokers [%]	89 [22.2]	105 [29.2]	63 [36.8]	**0.0012**
	BP treatment [%]	19 [4.8]	38 [10.6]	18 [10.6]	**0.0055**
Followup	Follow-up time	9.1 (1.3)	9.2 (1.2)	9.5 (1.2)	**0.0025**
	Age (years)	56.5 (4.8)	57.4 (4.5)	57.7 (4.7)	**0.0021**
	Height (m)	1.72 (0.09)	1.71 (0.09)	1.73 (0.09)	0.0727
	Weight (kg)	77.1 (13.3)	86.5 (15.9)	91.0 (18.2)	**<0.0001**
	BMI (kg/m2)	25.9 (3.4)	29.4 (4.8)	30.3 (5.8)	**<0.0001**
	WHR	0.88 (0.06)	0.91 (0.06)	0.94 (0.07)	**<0.0001**

	SBP	133 (17)	143 (17)	144 (18)	**<0.0001**
	DBP	82 (10)	87 (10)	87 (11)	**<0.0001**
	Fasting glucose (mmol/L)	4.86 (0.46)	5.72 (0.68)	7.35 (2.18)	**<0.0001**
	Fasting insulin (mU/L)	14.6 (6.0)	21.2 (11.9)	26.6 (13.2)	**<0.0001**
	sCD93 (ng/mL)	153 (42)	154 (51)	154 (48)	0.9654
	delta sCD93	13 (46)	7 (52)	4 (55)	0.0814
	C~~s~~current smokers [%]	61 [15.3]	73 [20.3]	34 [20.0]	0.1486
	T2D treatment [%]	0	0	39 [22.8]	**<0.0001**
	BP treatment [%]	64 [16.0]	133 [36.9]	72 [42.1]	**<0.0001**

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 relevant analytes

	cd93+/-	*cd93*+/+	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 the descending aorta stained with sudan IV, from A) *apoe*$^{-/-}$ *cd93*$^{+/-}$ and B) *apoe*$^{-/-}$*cd93*$^{+/+}$ mice. C) Quantification of lesions in the descending aorta of female and male *apoe*$^{-/-}$*cd93*$^{+/-}$ (black dots, n=11 (6 male, 5 female)) or *apoe*$^{-/-}$*cd93*$^{+/+}$ (black squares, n=9 (5 male, 4 female)) mice.

Figure 2: Glucose metabolism of *cd93*$^{+/-}$ male mice compared to *cd93*$^{+/+}$ male mice (black dots and black squares respectively). A) Glucose tolerance test of *cd93*$^{+/-}$ and *cd93*$^{+/+}$ male mice (n=10-13), aged 4 months, before given a western diet. B) Weight of *cd93*$^{+/-}$ and *cd93*$^{+/+}$ male mice (n=9-12 respectively), after 16 weeks of western diet. C) Glucose tolerance test of *cd93*$^{+/-}$ and *cd93*$^{+/+}$ male mice (n=9-12 respectively), after 16 weeks of western diet. D) Insulin tolerance test of *cd93*$^{+/-}$ and *cd93*$^{+/+}$ male mice (n=9-11 respectively), after 16 weeks of western diet. Repeated measures 2 way ANOVA showed statistical significance for C and D with ** indicating $p \leq 0.01$ or * indicating $p \leq 0.05$ statistical significance at a particular time point/s between genotypes using post-hoc analysis of Sidak's multiple comparisons test, 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*$^{+/+}$, *cd93*$^{+/-}$ *and cd93-/-* respectively).

Figure 4: Vascular integrity and endothelial damage in *cd93*$^{+/+}$ and *cd93*$^{+/-}$ mice. A) Percentage of vWF positive islets in pancreas from mice fed 16 weeks on western diet (4 of each genotype).

B) Quantification of Evans blue in pancreas in *cd93*$^{+/-}$ and *cd93*$^{+/+}$ mice (4 of each genotype, black and white bars respectively), * $p \leq 0.05$ Student's T-test. C) Plasma levels of soluble vWF A2 in *cd93*$^{+/-}$ and *cd93*$^{+/+}$ and mice and fed 16 weeks on western diet (black and white bars respectively) or on chow diet (dark and light hashed bars respectively), n=9-11 per genotype, * $p \leq 0.05$ one way ANOVA, using Tukey's multiple comparison test between genotypes and diet. D) Plasma levels of e-selectin in *cd93*$^{+/-}$ and *cd93*$^{+/+}$ and mice and fed 16 weeks on western diet (black and white bars respectively) or on chow diet (dark and light hashed bars respectively), n=9-11 per genotype, * $p \leq 0.05$ Student's T-test. All error bars indicate SEM.