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Glucose-Dependent Insulinotropic Polypeptide (GIP) Stimulates Osteopontin Expression in the Vasculature via Endothelin-1 and CREB

Running title: GIP/GIP-receptor signaling in the vasculature.

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Abstract:

Glucose-dependent insulintropic polypeptide (GIP) is an incretin hormone with extrapancreatic effects beyond glycemic control. Here we demonstrate unexpected effects of GIP signaling in the vasculature. GIP induces the expression of the pro-atherogenic cytokine osteopontin (OPN) in mouse arteries, via local release of endothelin-1 (ET-1) and activation of cAMP response element binding protein (CREB). Infusion of GIP increases plasma OPN levels in healthy individuals. Plasma ET-1 and OPN levels are positively correlated in patients with critical limb ischemia. Fasting GIP levels are higher in individuals with a history of cardiovascular disease (myocardial infarction, stroke) when compared to controls. GIP receptor (GIPR) and OPN mRNA levels are higher in carotid endarterectomies from patients with symptoms (stroke, transient ischemic attacks, amaurosis fugax) than in asymptomatic patients; and expression associates to parameters characteristic of unstable and inflammatory plaques (increased lipid accumulation, macrophage infiltration and reduced smooth muscle cell content). While GIPR expression is predominantly endothelial in healthy arteries from human, mouse, rat and pig; remarkable up-regulation is observed in endothelial and smooth muscle cells upon culture conditions yielding a “vascular disease-like” phenotype. Moreover, a common variant rs10423928 in the GIPR gene associated with increased risk of stroke in type 2 diabetes patients.

Glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are the main incretin hormones secreted by the intestine after a meal to stimulate insulin secretion (1). Both hormones are rapidly degraded by the enzyme dipeptidyl peptidase IV (DPP IV), inhibition of which is a novel approach to enhance incretin levels in the treatment of type 2 diabetes (2). Apart from their insulintropic activity, a plethora of actions have been described in other tissues, including effects on the cardiovascular system (1; 3). While a cardioprotective role of GLP-1 has been suggested (3), less is known about GIP in this context. Early studies in cats (4) and dogs (5) showed that GIP infusion increased blood flow into the superior mesenteric artery and portal vein, while decreasing it in the pancreatic and hepatic arteries, optimizing nutrient delivery to the liver after a meal. GIP has also been suggested to promote the redistribution of blood from the periphery to the gut after a meal (6). Vasoconstriction and vasodilation seems achieved by differential production of endothelin-1 (ET-1) and nitric oxide, as suggested from studies using cultured endothelial cells (7).

ET-1 is not only a potent regulator of cardiovascular homeostasis, but also a stimulator of vascular smooth muscle cell (VSMC) proliferation, migration and synthesis of extracellular matrix (8), all features of a transition from a differentiated, contractile state, to a more dedifferentiated, proliferative and synthetic phenotype. Circulating ET-1 levels are increased in most cardiovascular diseases, playing a critical role in the structural and functional alterations associated with the development of diabetic vascular complications and hypertension (9). ET-1 is also elevated in atherosclerotic plaques and promotes the expression of inflammatory genes in VSMCs (8).

One emerging key player in the context of vascular disease is the matrix cytokine osteopontin (OPN), which is not only a marker of inflammation but also an active player in disease progression. Several growth factors, hormones and vasoactive agonists, including ET-1, have been shown to increase OPN expression in the vasculature (10; 11). While OPN deficiency results in reduced atherosclerotic lesions, OPN overexpression leads to enhanced lesion size (12; 13). This cytokine regulates proliferation and migration of VSMCs and endothelial cells during vascular repair and remodeling, and promotes leukocyte recruitment to the vessel wall and macrophage retention (12; 14). Clinically, plasma OPN levels are associated to the presence and extent of coronary artery disease independently of traditional risk factors (15) and to restenosis after balloon angioplasty (16). Hyperglycemia is another potent stimulus for the induction of OPN in the vascular wall (17) and increased plasma levels and arterial expression of OPN have been demonstrated in diabetic patients and mice (17; 18), suggesting a role for OPN in the development of diabetic vascular complications.

We recently demonstrated that GIP increases OPN expression in pancreatic β -cells and hence, also has a proliferative and anti-apoptotic role in this tissue (19). Similarly, GIP stimulates OPN expression in adipocytes, which was associated to insulin resistance (20; 21). We also showed that a variant (rs10423928) in the GIP receptor (*GIPR*) gene is associated with impaired glucose- and GIP-stimulated insulin secretion, and decreased BMI (19). Interestingly, another SNP (rs1800437) in the *GIPR* gene has been associated with features of the metabolic syndrome and cardiovascular disease (22). Here we explore the impact of GIP-signaling in the vasculature and its potential link to OPN.

Research Design and Methods

Cells, tissue, animals and human samples

Primary human coronary artery smooth muscle cells (HCASMC, Cascade Biologics), human microvascular endothelial cells (HMEC-1, CDC/Emory University), mouse aortic vascular smooth muscle cells (VSMC) and human myometrial resistance arteries (as described in (23)), carotid arteries from Wistar Kyoto rats, coronary arteries from healthy domestic pigs, and mouse aortas and carotid arteries were used. The following mouse strains were used: NMRI (Taconic Europe), FVBN Nuclear Factor of Activated T-cells (NFAT) luciferase transgenic mice (NFAT-luc (24)), NFATc3^{-/-} and control NFATc3^{+/+} littermates (25), Akita^{+/-} LDLr^{-/-} (B6.Cg-*Ins2^{Akita}LDLr^{tm1Her}/J*) and control LDLr^{-/-} littermates (Jackson Laboratories).

Plasma from patients with confirmed diagnosis of critical limb ischemia and healthy controls was analyzed for OPN, ET-1 and GIP levels. Human carotid plaques and plasma were collected at carotid endarterectomies. Clinical materials have been described in (26) and (27). Characteristics of the individuals included are described in **Tables S1-S2**. Cross-sectional fragments of 1mm from the most stenotic region of the carotid plaques were taken for histology and adjacent fragments for RNA isolation. The remaining of the plaques was homogenized for protein and cytokine analyses (27). GIP levels were measured in plasma from patients with cardiovascular disease or type 2 diabetes and healthy control subjects. The effect of GIP on plasma OPN levels was explored in individuals subjected to hyperglycemic clamp with infusion of GIP, performed as previously described (28). All participants gave their informed consent. The study protocols conformed to the Declaration of Helsinki and were approved by local Human Ethics Committee. Experiments involving animals were approved by the Animal Ethical

Committee in Lund/Malmö. More detailed protocols and description of the cohorts investigated are available in the Online Supplemental Data file.

Confocal immunofluorescence

GIPR and OPN were detected in HCASMCs, HMECs and/or arterial sections as previously described (17) using primary antibodies for GIPR and OPN. Von Willebrand Factor (vWF) and α -smooth muscle actin (α -SMA) were used to identify endothelial and smooth muscle cells, respectively. Expression was quantified in sections and cells using a Zeiss LSM 5 Pascal laser scanning confocal microscope.

Western blotting

OPN, GIPR, p-CREB and total CREB were detected in cells, intact aortas and pancreas homogenates as previously described (17) using β -actin as loading control. Band intensity was measured using the Quantity One Analysis software.

Luciferase reporter assay

Aortas from NFAT-luc transgenic mice (24) were stimulated *ex vivo* and luciferase activity measured as previously described (17). Optical density was normalized to protein content.

LDH activity

Cell death was measured by quantification of lactate dehydrogenase (LDH) activity in the culture medium using a Cytotoxicity Detection Kit (Roche Applied Science) according to the manufacturer's instructions.

ELISA assays

Plasma OPN (Quantikine human OPN ELISA kit, R&D Systems, Abingdon, UK), plasma ET-1 (Human Endothelin-1 Immunoassay, R&D Systems) and serum GIP (Human GIP (Total) ELISA Kit, #EZHGIP-54K, Millipore, St. Charles, Missouri) were analyzed according to the manufacturer's instructions. Direct cAMP ELISA (Enzo Life Sciences) was used for cAMP measurements in cells.

Proliferation

DNA synthesis was measured by thymidine incorporation where cells were pulsed with 1 mCi [methyl-3H]thymidine (Amersham Biosciences, Uppsala, Sweden) during the last 24 hours.

Cytokine assessment

Cytokine levels were measured in aliquots of human carotid plaque homogenates and plasma (Milliplex Kit-Human Cytokine/Chemokine Immunoassay) and analyzed with Luminex 100 IS 2.3 as previously described (29).

Plaque immunohistochemistry

Immunohistochemistry was performed on sections of carotid atherosclerotic plaques as previously described (29), using primary antibodies detecting GIPR, OPN, CD68 and α -actin.

Briefly, Oil Red O was used to detect lipids and Masson's trichrome with Ponceau-acid fuchsin and aniline blue for plaque collagen content. Slides were scanned with ScanScope Console Version 8.2 and photographed with Aperio image scope v.8.0. The % area of the plaque constituted by the different components was quantified blindly using Biopix iQ 2.1.8.

RNA isolation, cDNA synthesis and quantitative PCR

Total RNA was isolated from cultured cells, mouse arteries and human carotid plaques as before(29); reverse transcribed, thereafter *GIPR* and *OPN* mRNA levels were quantified by real-time PCR using TaqMan Gene Expression assays and normalized to the expression of housekeeping genes.

Genetic studies

Associations of the *GIPR* rs10423928 SNP with cardiovascular events were explored in the following studies: Prevalence, Prediction and Prevention of Diabetes-Botnia (PPP-Botnia; (30)), the Malmo Diet and Cancer Study (MDC; (31)), METabolic Syndrome In Men (METSIM; (32)), Scania Diabetes Registry (SDR; (33)), Diabetes Genetic Initiative (DGI; (30)), Steno type 1 and type 2 diabetes studies (Steno T1D and T2D; (34; 35)), Malmö Preventive Project (MPP; (36)) and Finnish Diabetic Nephropathy study (FinnDiane, (37)). Genotyping of rs10423928 was performed as previously described (19). Detailed description of the cohorts investigated is available in the Online Supplemental Data file.

Statistical Analysis

For in vitro studies, results are expressed as means \pm SEM and GraphPad (Prism5.0) was used for the analyses. Significance was determined using two-tailed Student's t-test, or one-way analysis of variance followed by Bonferroni post-tests for normally distributed variables; Mann-Whitney or Kruskal-Wallis tests for not normally distributed variables. SPSS version 17.0 (SPSS Inc., Chicago) was used to analyze non-parametric, bivariate correlations in human carotid plaques. Associations between genotype and cardiovascular disease outcomes were investigated using logistic regressions and associations between genotype and blood pressure variables were tested using linear regression. Fixed effect meta-analyses were performed with the metan command in STATA/SE version 12.1 (StataCorp LP; College Station, TX). Non-normally distributed variables were logarithmically transformed before analysis. **P<0.05, **P<0.01 and ***P<0.001.*

Results

GIPR is expressed in native arteries

Using confocal immunofluorescence microscopy, we showed that in normal healthy mouse aorta, GIPR is predominantly expressed in the endothelium and to a little extent in smooth muscle cells of the media (**Figure 1A-C**). This was also true in intact arteries from different vascular beds and species (**Figure S1A**). Using RT-PCR, *GIPR* mRNA was detected by two different primer pairs in intact mouse aorta (**Figure 1D**). Western blotting revealed a band at the expected molecular weight (~65 kDa) in endothelial cells (**Figure 1E, left panel**) and in pancreas homogenate from GIPR competent mice but not from GIPR KO mice (**Figure 1E, right panel**).

GIP stimulation increases OPN expression in the vascular wall via local release of endothelin-1

Culture of intact mouse aortas for 3 days with various concentrations of GIP resulted in up-regulation of OPN in the media of the arteries, as determined by confocal immunofluorescence microscopy (**Figures 2A-B**) and western blotting (**Figure 2C**), with a significant effect at physiological nanomolar concentrations. Even though a tendency to increased OPN expression was observed after 24h of GIP stimulation (**Figure S2A**), the effects became significant only after 2 (**Figure 3C-D**) and 3 days (**Figure 2**).

In agreement with previous studies showing that GIP stimulates ET-1 release from cultured endothelial cells (38), stimulation of intact mouse aortas with GIP resulted in dose-dependent ET-1 release to the culture media (**Figure 3A**). ET-1 stimulation of intact mouse aorta resulted in turn, in increased OPN protein (**Figure 3B**). Culture of mouse aorta with GIP in the

presence of the endothelin-1 receptor blockers BQ788 and BQ123 completely abolished the induction of OPN (**Figures 3C-E**), while the blockers alone had no significant effect on OPN expression (**Figure 3E**). To better elucidate the contribution of endothelial and VSMCs to the GIP responses observed in intact arteries, experiments using isolated cells were performed (**Figure 4**; upper panels A-C for endothelial cells; lower panels E-H for VSMCs; panel D for intact aorta). Data show that GIP stimulates ET-1 release from endothelial cells (**Figure 4A**), but not from VSMCs (**Figure 4E**). Further, ET-1 dose-dependently increases OPN expression in VSMCs (**Figure 4F**) but has no effect on OPN in endothelial cells (**Figure S3A**). No direct effects of GIP on OPN expression in either endothelial cells or VSMCs were detected (**Figures S3B-C**); suggesting that both cell types must be present for arterial GIP-induced OPN expression to occur. **Figure 4** also includes a cartoon summarizing the proposed mechanism underlying GIP-induced OPN expression in intact arteries.

CREB rather than NFAT mediates GIP-induced OPN expression

Recently, we reported that the calcium-dependent transcription factor NFAT regulates the expression of arterial OPN in response to hyperglycemia (17). Moreover, we recently demonstrated that GIP-induced OPN expression in adipocytes is mediated by NFAT (20). Therefore, we speculated whether the effect of GIP on arterial OPN could be mediated via NFAT activation. However, we found that stimulation of aortas from NFAT-luciferase reporter mice for 12 hours with various concentrations of GIP had no effect on NFAT-dependent transcriptional activity (**Figure S4A**). Also, incubation of aortas with the NFAT inhibitor A-285222 (17) or lack of NFATc3 protein in aortas from NFATc3 deficient mice did not prevent GIP-induced OPN

expression (**Figure S4B-C**), ruling out a role for NFAT in GIP-induced OPN expression in intact arteries.

In VSMCs, several transcription factors in addition to NFAT have been shown to participate in the regulation of OPN expression, including CREB, NF- κ B, AP-1 and upstream stimulatory factors 1 and 2 (USF-1/-2) (17). In both β -INS-1 cells and adipocytes, GIP stimulation was shown to increase CREB phosphorylation (39; 40). Here we found that stimulation with GIP increases CREB phosphorylation in ECs but not in VSMCs, as assessed by western blot (**Figure 4B and S3D**). Moreover, GIP-induced ET-1 release from ECs was prevented by the small molecule CREB antagonist KG-501 (2-naphthol-AS-E-phosphate; **Figure 4C**). CREB phosphorylation was also increased in VSMCs in response to ET-1 stimulation (**Figure 4G**), and ET-1 induced OPN expression was prevented by KG-501 (**Figure 4H**) whereas the blocker alone had no effect (**Figure S3E**). No toxic effects of KG-501 were observed at the concentrations used here (10 μ mol/l and below), as assessed by measurements of LDH activity in ECs and VSMCs (**Figure S3F-G**). Taken together, this data suggest that CREB plays a role at two levels, being involved in 1) the regulation of GIP-stimulated ET-1 production in ECs and in 2) the regulation of ET-1-stimulated OPN expression in VSMCs. This is in agreement with previous studies showing involvement of CREB in the regulation of both ET-1 (41) and OPN (42; 43). The involvement of CREB at these two levels may explain the dramatic effect of KG-501 on OPN expression observed in experiments using intact arteries when both cell types are present (**Figure 4D**).

In line with previous work demonstrating lack of effect of GIP on cAMP levels in ECs isolated from hepatic artery or portal vein (7), here we found no effect of GIP on cAMP levels in

cultured ECs at a GIP dose that effectively increases ET-1 levels (**Figure S5A**). Moreover, GIP was able to induce ET-1 release even in the presence of the PKA inhibitors Rp-cAMPS and H-89 (**Figure S5B**). Thus, data suggest that GIP does not engage the traditional cAMP-PKA pathway in ECs. Intriguingly, the two PKA inhibitors had opposite effects on basal ET-1 release (**Figure S5B**). While Rp-cAMPS increased ET-1 release, suggesting an inhibitory effect of constitutively active PKA under basal non-stimulated conditions; H-89 reduced ET-1 release (**Figure S5B**). A relatively large number of PKA-independent effects described for H-89, including inhibition of at least 8 other kinases could account for the discrepancies between the effects of Rp-cAMPS and H89 (44).

Plasma ET-1 positively correlates to plasma OPN in patients with critical limb ischemia

Having established a link between GIP, ET-1 and OPN in the vasculature, we next examined the level of these in individuals suffering from vascular disease. Significantly higher plasma ET-1 and OPN were measured in patients with critical limb ischemia when compared to control individuals (**Figures 5A-B**). Several clinical characteristics of the patients with critical limb ischemia (i.e. age, smoking) could contribute to these increased levels of ET-1 (Table S1). A positive correlation was found between plasma ET-1 and OPN ($r=0.424$, $P<0.0001$; **Figure 5D**). Non-fasted plasma levels of GIP were not different between ischemic patients and controls (**Figure 5C**), and were consistent with previously reported levels (45).

GIPR and OPN mRNA are increased in carotid atherosclerotic plaques from symptomatic patients

Expression of GIPR and OPN were demonstrated in human carotid endarterectomy sections by immunohistochemistry (**Figure 5H and S6**). Significantly higher *GIPR* mRNA levels were found in plaques from patients with symptoms (stroke, transient ischemic attacks, amaurosis fugax) when compared to those from patients without symptoms ($P=0.0177$, **Figure 5E**). Plaque *OPN* mRNA and plasma OPN concentration were also higher in symptomatic patients compared to asymptomatic patients ($P=0.0022$ and $P=0.044$, respectively; **Figures 5F-G**), and there was a significant, positive correlation between plaque *GIPR* and *OPN* mRNA levels ($r=0.566$, $P<0.0001$; **Figure 5I**). Moreover, both *GIPR* and *OPN* mRNA levels correlated with the number of clinical events (**Table 1**). As opposed to what we found in patients with critical limb ischemia when blood samples were collected under non-fasting conditions, fasting GIP levels were significantly higher in individuals with a history of cardiovascular disease (CVD; myocardial infarction or stroke) when compared to individuals with no history of CVD in the PPP-Botnia study ($P=0.002$; **Figure 5J**).

Plaque histology revealed that *GIPR* and *OPN* mRNA were positively correlated to the extent of lipid accumulation (Oil Red O staining), macrophage infiltration (CD68 staining) and elastin contents; and negatively correlated to α -actin contents (**Table 1**). Positive correlations between *GIPR* and *OPN* mRNA levels and plaque IL-10, IL-1 β , IL-6, MCP-1, MIP-1 β and RANTES were found. Also, *GIPR* mRNA was positively correlated to plaque PDGF AB/BB whereas *OPN* mRNA correlated with plaque eotaxin, fractalkine, IL-12p70 and VEGF. A complete list of the parameters analyzed, including the above mentioned histological and plaque cytokines, is shown in **Table 1**. Taken together, data provide evidence of a link between the

expression of *GIPR* and *OPN* and parameters characteristic of more unstable and inflammatory plaques.

Plasticity of GIPR expression relates to vascular phenotype

The elevated *GIPR* mRNA expression in plaques from symptomatic patients suggested plasticity of GIPR expression of potential relevance for GIP-signaling in the context of vascular disease. Considering also the positive correlation observed between the expression of GIPR and *OPN* mRNA in the plaque (**Figure 5I**), we wanted to explore what could drive GIPR expression in vascular cells. GIPR was primarily detected in the endothelium of freshly dissected healthy arteries (**Figure 1A & C**), but also outside the endothelium in atherosclerotic vessels (**Figure 5H**). Interestingly, GIPR protein was detected in arterial smooth muscle cells when cultured under growth stimulatory conditions (**Figure 6A**), which is known to result in a proliferative VSMC phenotype, as shown by numerous publications in the past (reviewed in (46)) and here confirmed by significantly increased thymidine incorporation (**Fig. 6B**, right panel). It is widely accepted that while VSMCs in the healthy vessel wall are contractile, dispersed cells in culture rapidly modulate from a contractile differentiated phenotype to a synthetic phenotype (47). This phenotypic switch has been shown to take place and contribute to vascular disease states (i.e. atherosclerosis, hypertensive microvessels, restenosis)(48). A time-dependent decrease in *GIPR* expression was found instead when smooth muscle cells were cultured in differentiating medium (**Figure 6B**, left panel). Corresponding experiments were also performed using endothelial cells, showing up regulation of GIPR mRNA when ECs are stressed by the removal of serum from the culture media (**Figure 6C**). Interestingly, a dramatic up regulation of GIPR mRNA was observed

when intact aorta was cultured (**Figure 6D**), a procedure known to result in rapid phenotypic switch due to loss of tensile stress (49; 50).

GIPR expression was also modulated by high glucose and insulin. As shown in **Figure 6E**, stimulation of arterial smooth muscle cells with 20 mmol/l glucose increased *GIPR* expression, and this was prevented by the addition of insulin to the culture medium. In contrast, glucose and insulin had no effect on *GIPR* mRNA expression in ECs (**Figure 6F**). Along these lines, a trend towards elevated *GIPR* expression was found in carotid arteries from Akita^{+/-}LDLr^{-/-} mice when compared to non-diabetic LDLr^{-/-} controls (P=0.0503, **Figure 6G**). In these mice, *GIPR* mRNA expression correlated significantly to blood glucose levels (r=0.470, P=0.027, N=22). Fasting GIP levels were significantly higher in patients with diabetes than in normal glucose tolerant individuals (57.3±51.2 pg/ml vs. 37.2±25.6 pg/ml, P=4.08e-11, N=310 vs. 4009 from the PPP-Botnia study, **Figure 6H**).

A common variant in the GIPR gene associates with increased risk of stroke

Combining data from several studies, we analyzed whether the SNP rs10423928 in the *GIPR* gene would influence the risk of vascular disease. Type 2 diabetes patients carrying the A-allele of this SNP had an increased risk of stroke (Odds ratio 1.22, P_{meta}=0.00799, **Figure 7A** and **Tables S3-S4**), but this association was not observed in non-diabetic individuals, nor in patients with type 1 diabetes (**Figure 7B** and **Tables S3-S4 and S7**). Genotype had no effect on the risk of myocardial infarction or of retinopathy, nor did it affect blood pressure (**Tables S3 and S5-S8**).

In order to explore if GIP stimulation could induce OPN *in vivo*, we performed GIP infusions in 47 healthy individuals and measured the levels of OPN in plasma before and 105 minutes after the GIP infusion. As shown in **Figure 7C**, GIP infusion increased plasma OPN levels in a genotype-dependent fashion, since only carriers of TA/AA responded with increased OPN, while carriers of TT did not.

The impact of the SNP rs10423928 in the *GIPR* gene was also examined in the patients undergoing carotid endarterectomy. Significant associations were observed between mRNA levels of *GIPR* or *OPN* and the number of events in TA/AA but not in TT genotype carriers (**Table S9**). Other parameters showing association to *GIPR* and/or *OPN* mRNA in TA/AA genotype carriers included plaque CD68, eotaxin, IL-10 and VEGF as well as plasma IL1 β (**Table S9**). Also of interest, we found a significant association between *GIPR* mRNA and HbA1c in TA/AA carriers only.

Discussion

The major findings in this study were that: 1) GIP stimulation increased the expression of OPN in mouse native arteries *ex vivo* by a mechanism involving the release of ET-1 and activation of CREB; 2) infusion of GIP increased plasma levels of OPN; and plasma ET-1 and OPN levels were positively correlated in patients with critical limb ischemia; 3) fasting GIP levels were significantly higher in individuals with a history of cardiovascular disease (myocardial infarction or stroke) when compared to controls; 4) patients with symptoms (stroke, transient ischemic attacks, amaurosis fugax) exhibited higher plaque *GIPR* and *OPN* mRNA levels and higher plasma OPN than asymptomatic patients; mRNA expression levels associated to parameters characteristic of more unstable and inflammatory plaques; 5) plasticity in *GIPR* expression was observed in vascular cells, with increased expression when cells were cultured under conditions leading to a more “vascular disease-like” phenotype, or upon changes in glucose and insulin that would mimic the diabetic phenotype, or in arteries from diabetic mice; and 6) a common variant (rs10423928) in the *GIPR* gene associated with increased plasma OPN after GIP infusions and with increased risk of stroke in patients with type 2 diabetes.

We here extend our previous findings that GIP can stimulate OPN expression in pancreatic islets (19) and adipose tissue (20) to the vascular system. Plenty of evidence support a role for OPN in the initiation and progression of vascular disease and diabetic vascular complications, which has led to the view that OPN could serve as a potential biomarker for cardiovascular disease (51). In human atherosclerotic plaques, *OPN* mRNA correlates with the stage of the disease (52) and OPN protein expression in carotid lesions has been ascribed prognostic value for future cardiovascular events (53). Here we find significant up regulation of

OPN expression in intact arteries stimulated with physiological GIP concentrations comparable to levels reached after a mixed meal (45). The *in vivo* half-life of GIP in serum is approximately 3-5 minutes due to rapid degradation by DPP IV (54). In our *in vitro* studies, some endothelial DPP IV activity can be predicted since arteries were cultured intact, therefore GIP was supplemented every 24 h to mimic the concentrations observed in humans (55). The functional outcome of the effect of GIP on OPN expression seems very different depending on the tissue. While OPN seems protective of pancreatic β -cells by increasing cell proliferation and reducing cytokine-induced apoptosis (19), it promotes lipogenesis, inflammation and insulin resistance in adipose tissue (20). Therefore, inhibition of GIP-induced OPN expression might be desirable in vessels and fat but not in islets.

GIP-induced OPN expression in intact vessels was dependent on local release of ET-1 since it was inhibited by blockers of ET-1 receptors. This is in line with previous work showing dose-dependent GIP-induced ET-1 release from cultured human endothelial cells (38). The effect was GIPR-specific and limited to cells from certain vascular beds, which was thought to be due to differential expression of GIPR splice variants (7). ET-1 is thought to bind to receptors close to the site of release, acting in a paracrine or autocrine fashion (56). To our knowledge, no dynamic ET-1 biosensor has yet been developed; so a limitation of this study is that we could not determine the concentrations of ET-1 that vascular cells face *in situ* upon stimulation with GIP. Nevertheless, GIP doses capable of inducing OPN in intact arteries (i.e. 0.1 nmol/l; Fig. 2C), resulted in a ~2.5 pg/ml change in ET-1 measured in the culture media; which would equate ~0.1 nmol/l ET-1, but presumably higher levels in the vicinity of the site of release. A long-lasting activation of the GIP-ET-1 axis could potentially lead to vasoconstriction, increased mitogenic

and pro-inflammatory status, formation of free radicals and platelet activation; all known actions of ET-1 at nanomolar levels, and determinants of cardiovascular disease.

Under normal physiological conditions, the effects of GIP on the vascular wall may be limited by the readily available degrading enzymes (DPP IV and neutral endopeptidases), by the sparse and restricted expression of GIPR to the endothelium (**Figure 1A**) and by the many well-functioning systems of damage control (i.e. endothelium derived relaxing factors). However, under pathological conditions as well as during inhibition of degrading enzymes, the scenario may be different. Our *in vitro* data demonstrate an effect of GIP on vascular OPN, hence the here reported elevated circulating GIP levels *in vivo* in patients with cardiovascular disease and in patients with type 2 diabetes, may be anticipated to exacerbate this effect. GIP levels have been shown to be increased, decreased or unaffected in adults with T2D or IGT when compared to normoglycemic individuals. Much of the early discrepancies can be explained by differences in the assays used (57), but also by indistinctly referring to incremental (i.e. secreted after a standard OGTT or meal test), non-fasting unstimulated and fasting GIP levels. In a recent meta-analysis of 22 studies including 688 patients, it was concluded that patients with T2D (N=363), in general, exhibit normal GIP secretion in response to OGTTs or meal tests, but have elevated fasting plasma GIP levels compared to healthy controls (58). This last is in agreement with our data in Figure 6H. Altogether, this underscores the potential relevance of monitoring fasting and not only secreted GIP levels. Along these lines, 3 times higher serum GIP concentration was reported in patients with the metabolic syndrome when compared to pre-metabolic syndrome subjects (19.0 ± 45.7 pg/ml vs. 6.5 ± 10.2 pg/ml; $P=0.034$) (59).

Changes in vascular GIPR expression, such as those reported in this study may also contribute to the aggravation of GIP effects. Under pathological conditions, expression of GIPR was not restricted to the inner lining of the vessels but also detected in atherosclerotic lesions and in the media of the arteries (**Figure 5H**), with higher mRNA expression in samples from symptomatic patients. It is difficult to speculate about the functional impact of this increased mRNA expression and differences between symptomatic and asymptomatic patients may seem small, but yet, they were discernible despite that both groups of patients have severe atherosclerosis with a stenosis degree of >80%. Carotid endarterectomies from symptomatic patients have increased levels of inflammatory markers and increased macrophages and lipid infiltration (60), as well as increased OPN expression (53; 61) when compared to plaques from asymptomatic patients. Our data show that plaque *GIPR* and *OPN* mRNA levels were positively correlated to each other and associated to parameters characteristic of a more unstable and inflammatory plaque. This is in line with the *in vitro* data which clearly demonstrate increased GIPR expression under conditions leading to endothelial cell stress, dedifferentiation of smooth muscle cells towards a more proliferative state or when cultured in the presence of high glucose, while insulin prevented the effects of glucose. GIPR plasticity has been previously demonstrated in other tissues. Higher GIPR (and GIP) expression was reported in the retinas of STZ-induced diabetic rats (62). In pancreatic islets on the other hand, GIPR expression is down regulated by hyperglycemia in rats and humans (19; 63; 64). In type 2 diabetes, a considerable loss of GIP efficacy has been demonstrated (reviewed in Meier et al 2010 (65)), and this refers to the loss of incretin activity or pancreatic GIP effects on insulin secretion. The exact mechanisms behind this reduced insulintropic effect are not completely clear but are inherent to the ability of the pancreatic beta-cell to respond to GIP. Our data does not support a loss of GIP efficacy in

arteries of type 2 diabetic patients, highlighting the need of a distinction between pancreatic and extrapancreatic effects of GIP.

Finally, as the SNP rs10423928 in the *GIPR* gene had been associated with increased glycemia, we explored whether this variant in the *GIPR* gene would influence risk of vascular complications in patients with diabetes. Type 2 diabetes patients carrying the less common A allele had an increased risk of stroke, but we did not observe any increased risk of MI, nor of retinopathy in patients with type 1 and type 2 diabetes. There was a clear effect of this genotype on the OPN response to GIP stimulation, which was restricted to carriers of AA/AT genotypes. Only these carriers showed significant correlations between plaque *GIPR* and *OPN* expression and the number of clinical events, further supporting a functional role of the SNP. Given the stronger stimulatory effect of GIP on OPN in AA/AT carriers one could envision a stronger stimulatory effect on ET-1 and thereby increased blood pressure in these carriers, but we did not detect significant effects on blood or pulse pressure. One should keep in mind that blood pressure measurements were not sufficiently standardized in these studies.

At this point, we do not know how the variant influences the molecular function of the GIPR in the vasculature, whether it influences expression of a specific isoform, or if it is associated with a gain- or- reduction of function. Based upon information from the 1000 Genomes Project, there are 5 SNPs in the *GIPR* gene that are in perfect LD ($D' = 1$) with the rs10423928 SNP. Four out of these 5 SNPs are intronic, but one (rs1800437) results in non-synonymous coding, resulting in an residue substitution (E[Glu] \rightarrow Q[Gln]). Using PolyPhen-2, a tool to predict possible impact of an amino acid substitution on the structure and function of a

human protein (<http://genetics.bwh.harvard.edu/pph2/>), this amino acid substitution is predicted to be “probably damaging” with a score of 1.000. This variant, also designated E354Q, was recently shown to cause GIP-induced desensitization by increasing internalization of GIPR receptor (66). However, based on the impact of the variant studied here (rs10423928) on OPN levels after GIP infusion, it appears associated with a gain-of-function, but a final proof will require additional studies to elucidate the effects of GIP on different human vessels in relation to genotype. In cultured endothelial cells several splice isoforms with potentially divergent functions have been reported (6) and in human adipose tissue we have observed a large number of splice isoforms, some of which seem to be regulated by SNPs in the GIPR gene (21). Additional support for a vascular role for GIP comes from the CARDIoGRAM Consortium where a variant in the GIP gene was associated with myocardial infarction (67).

Adding complexity to the vascular effects of GIP, recent studies in dyslipidemic apoE knock-out mice suggested antiatherogenic effects of GIP or DPP IV inhibition, apparently via decreased CD36 expression in macrophages and decreased foam cell formation (68-70). In this mouse model, treatment with GIP significantly reduced plasma nonesterified fatty acid (NEFA) levels, which could in part explain the reduced CD36 and macrophage foam cell formation (71). Several other discrepancies were observed when compared to studies using other experimental animals or to clinical data, such as a decreased body weight and remarkable reduction in non-HDL-cholesterol. ApoE knock-out mice have high levels of very-low-density lipoprotein (VLDL) and contain ApoB-48 in their lipoproteins, not fully recapitulating human lipoproteins. These marked differences between mice and men emphasize the need to study GIP effects in humans.

Despite promising results in recently published metanalysis of DPP-4 inhibitors and CV risk (72), the short observation period of the included trials (mean follow up of 44.1 weeks), the extremely low CV event rate in the DPP-4 inhibitor arms and the fact that studies were not controlled for the use of cardioprotective drugs question the value of the results. Based on lessons from the United Kingdom Prospective Diabetes Study (UKPDS) longer follow-ups (i.e. 10 years) may be required to demonstrate potential long-term risks or benefits of therapy. Data from the more recent and superiorly designed studies SAVOR (73) and EXAMINE (74), showed neutral effects of saxagliptin and alogliptin, respectively, on a composite of death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke. Similar neutral results were more recently also reported for sitagliptin (75). All these results were reported in spite of predicted cardioprotective benefit (based on GLP-1 related experimental data and pooled data from phase 2b-3 studies of saxagliptin)(76). Also recently, data from 2 independent clinical trials demonstrated that DPP IV inhibition attenuated endothelial function (assessed by measurements of flow mediated dilatation) in type 2 diabetic patients (77). These findings may not be definitive but merit further investigation.

In conclusion, we demonstrate an unprecedented link between the incretin hormone GIP and the inflammatory cytokine OPN known to promote atherosclerotic disease in humans, an effect which seems partially influenced by variants in the *GIPR* gene (**Figure S7**). In conditions with increased GIP levels or GIPR expression, these untoward extrapancreatic effects of GIP should be taken into account. This study also highlights the need of hard endpoints from trials designed to evaluate long-term CVD benefits and side effects of therapies using DPP IV

inhibitors. Considering the results presented here, randomization or closer analysis of already collected data based on GIPR genotype may add an important dimension to future studies. In the context of DPP IV therapy, it is also possible that the effects of GIP may be counteracted by the so far described beneficial effects of GLP-1, shown in numerous publications to increase NO availability in a wide range of vascular beds (recently reviewed in (78)) and to inhibit endothelin-1 production (79). GIP and GLP-1 may behave as the *yin & yang* for systemic endothelin-1 and NO production. Simultaneous infusion of GIP and GLP-1 should be tested to determine whether one or the other incretin will dominate, whether the dominance would be vascular bed specific or affected by age, given that the balance between vasoconstriction/vasodilation is normally shifted with age due to reduced NO bioavailability and increased oxidative stress.

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Figure legends:

Figure 1. GIPR is expressed in native arteries. (A) Confocal images showing GIPR, α -SMA and vWF (red, upper panels) and overlap with nuclear staining (SYTOX Green, lower panels) in sections from mouse aorta. Two different antibodies targeting an internal region (amino acids 268-337) or the N-terminal region (-NH₂) of the GIPR protein were used. Bars=100 μ m. **(B)** Omitted primary control images for GIPR and vWF (anti-rabbit secondary antibody alone) and for α -SMA (anti-mouse secondary antibody alone) **(C)** Zoom-in of mouse aortic intima stained for GIPR as in 1A. Arrows point at individual endothelial cells. Bar=20 μ m. **(Ca-b)** Insets show GIPR positive staining in cultured HMECs (upper panel a) and overlap with SYTOX Green staining (lower panel b). Bar=25 μ m **(D)** RT-PCR showing expression of GIPR in mouse aorta, amplified by primer pairs recognizing exons 4-5 and 6-7. No amplification was observed in the absence of reverse transcriptase (-RT). **(E)** Western blot showing GIPR protein in HMECs (left panel), and in pancreas homogenate from GIPR WT and KO mice (right panel), at expected molecular weight (~65 kDa).

Figure 2. GIP stimulation increases OPN expression in mouse aorta. (A) Confocal images showing induction of OPN expression (red) in mouse aorta after GIP stimulation for 3 days. Nuclei were stained with SYTOX Green. Bars=20 μ m. **(B)** Quantified data from experiments in A (N=4-7 in each bar). **(C)** Summarized western blot data and representative immunoblot showing OPN expression in mouse aorta after GIP stimulation for 3 days; normalized to β -actin and expressed as percentage of untreated control. Data is from at least 5 mice for each condition.

Figure 3. ET-1 receptor inhibition prevents GIP-induced OPN expression. (A) ET-1 levels measured in the medium after culture of intact mouse aorta in the presence and absence of GIP for 3 days. (B) Summarized western blot data and representative immunoblot showing OPN expression in aorta after 3 days stimulation with ET-1. (C) Confocal images showing OPN expression (red) in mouse aorta cultured for 2 days with or without GIP (100 nmol/l) and ET-1 receptor inhibitors BQ788 and BQ123 (10 μ mol/l each). Nuclei were stained with SYTOX Green. Bars=20 μ m. (D) Quantified data from experiments in C (N=4). (E) Summarized western blot data and representative immunoblots showing OPN expression in aorta after 3 days treatment with or without GIP (1.0 nmol/l) and BQ788/BQ123 (10 μ mol/l each). For (B) and (E), OPN was normalized to β -actin and expressed as percentage of untreated control. Data is from at least 3 mice for each condition.

Figure 4. GIP stimulation increases OPN expression via ET-1 release and CREB activation. (A) ET-1 levels measured in medium from endothelial cells after stimulation with GIP for 24h. N=3 per dose. (B) Summarized data and representative immunoblot showing p-CREB/total CREB ratio in ECs after 30 min stimulation with GIP. N=8-9 per condition. (C) ET-1 levels measured in medium from ECs after stimulation with GIP in the presence or absence of the CREB inhibitor KG-501 (KG) for 24h. N=3-7 per condition. (D) Summarized data and representative immunoblot showing OPN expression in mouse aorta treated with GIP (1 nmol/l) for 3 days, with or without KG (10 μ mol/l). Experiments include aorta samples from at least 5 mice for each condition. (E) ET-1 levels measured in medium from VSMCs after stimulation with GIP for 24h. N=3 per dose. (F) Summarized data and representative immunoblot showing OPN expression in VSMCs after stimulation with ET-1 for 24h. N=6 per condition. (G) Summarized data and representative immunoblot showing p-CREB/total CREB ratio in VSMCs

after stimulation with ET-1 for 30 min. N=8-13 per condition. **(H)** Summarized data and representative immunoblot showing OPN expression in mouse aorta treated with ET-1 for 24h, with or without KG. N=7-11 per condition. Data is expressed as percentage of untreated control in all panels except A and E; OPN expression is normalized to β -actin. In the center, a schematic view of the proposed GIP-ET-1-OPN axis in endothelial and smooth muscle cells of the vascular wall is shown.

Figure 5. Plasma OPN and ET-1 are elevated in patients with critical limb ischemia and *GIPR* and *OPN* mRNA are higher in carotid atherosclerotic plaques from symptomatic patients. Plasma levels of **(A)** ET-1, **(B)** OPN and **(C)** total GIP, measured using ELISA in non-fasted patients with critical limb ischemia (N=84 for ET-1, N=85 for OPN and GIP) and control individuals (N=101). **(D)** Correlation between plasma OPN and ET-1. **(E)** *GIPR* and **(F)** *OPN* mRNA levels in plaques from asymptomatic (AS, N=63) and symptomatic (S, N=87) patients, normalized to *HPRT*, *cyclophilin A* and *POLR2A*. **(G)** Plasma OPN levels in asymptomatic (AS, N=54) and symptomatic (S, N=81) patients. **(H)** *GIPR* and OPN expression in carotid atherosclerotic plaque sections from asymptomatic and symptomatic patients. Bars=1 mm, inset=100 μ m. **(I)** Correlation between plaque *OPN* and *GIPR* mRNA levels. **(J)** Plasma GIP levels measured in patients with cardiovascular disease (CVD; myocardial infarction and/or stroke, N=201) and control subjects (N=4,726) in PPP-Botnia. $**P=0.002$ from logistic regression; GIP values adjusted for BMI, age and gender.

Figure 6. Plasticity of *GIPR* expression in the vasculature. **(A)** and **(D)** Confocal immunofluorescence image showing *GIPR* (red) and nuclei (SYTOX Green) in growth stimulated HCASMCs. Bar=20 μ m **(B)** and **(C)** Measurements of *GIPR* mRNA (left panels) and thymidine incorporation (right panels) in HCASMCs (B) cultured with smooth muscle growth

supplement (+GS) or differentiating supplement (-GS) and HMEC-1 cells (C) with or without endothelial cell growth supplement (GS) for 1 or 2 days. *GIPR* expression is normalized to *cyclophilin A* and data is expressed as percentage of 0d. **(D)** *GIPR* mRNA in mouse aorta, freshly isolated (0 days) and after 1-3 days in culture; normalized to *cyclophilin B* and *18S* and expressed as percentage of 0d (N=8-16 per group). **(E) and (F)** *GIPR* mRNA expression in HCASMCs (E) and HMECs (F) upon 2 days stimulation with 20 mmol/l glucose with or without insulin (100 nmol/l); normalized to *HPRT*, *cyclophilin A* and *POLR2A* and expressed as percentage of control cells (4.6 mmol/l glucose, N=5). **(G)** *GIPR* mRNA in carotid arteries of diabetic Akita^{+/-}LDLr^{-/-} (Akita) and non-diabetic LDLr^{-/-} control mice; normalized to *cyclophilin B* and *18S* and expressed as percentage of control mice (N=9 per group). **(H)** Plasma GIP levels in patients with type 2 diabetes (T2D, N=310) and control subjects (N=4,009) in PPP-Botnia.

Figure 7. *GIPR* rs10423928 associates with increased risk of stroke and plasma OPN in response to GIP infusion. **(A)** Meta-analysis of the effect of *GIPR* rs10423928 on ischemic stroke in patients with T2D, including 7,471 individuals without stroke and 523 individuals with stroke ($P=0.00799$). **(B)** Meta-analysis of the effect of *GIPR* rs10423928 on ischemic stroke in all individuals irrespective of diabetes status, including 48,596 individuals without stroke and 2,147 individuals with stroke ($P=0.133$). **(C)** Plasma OPN levels were increased upon GIP infusion in healthy individuals (N=42), an effect evident in TA/AA carriers, but not in TT.

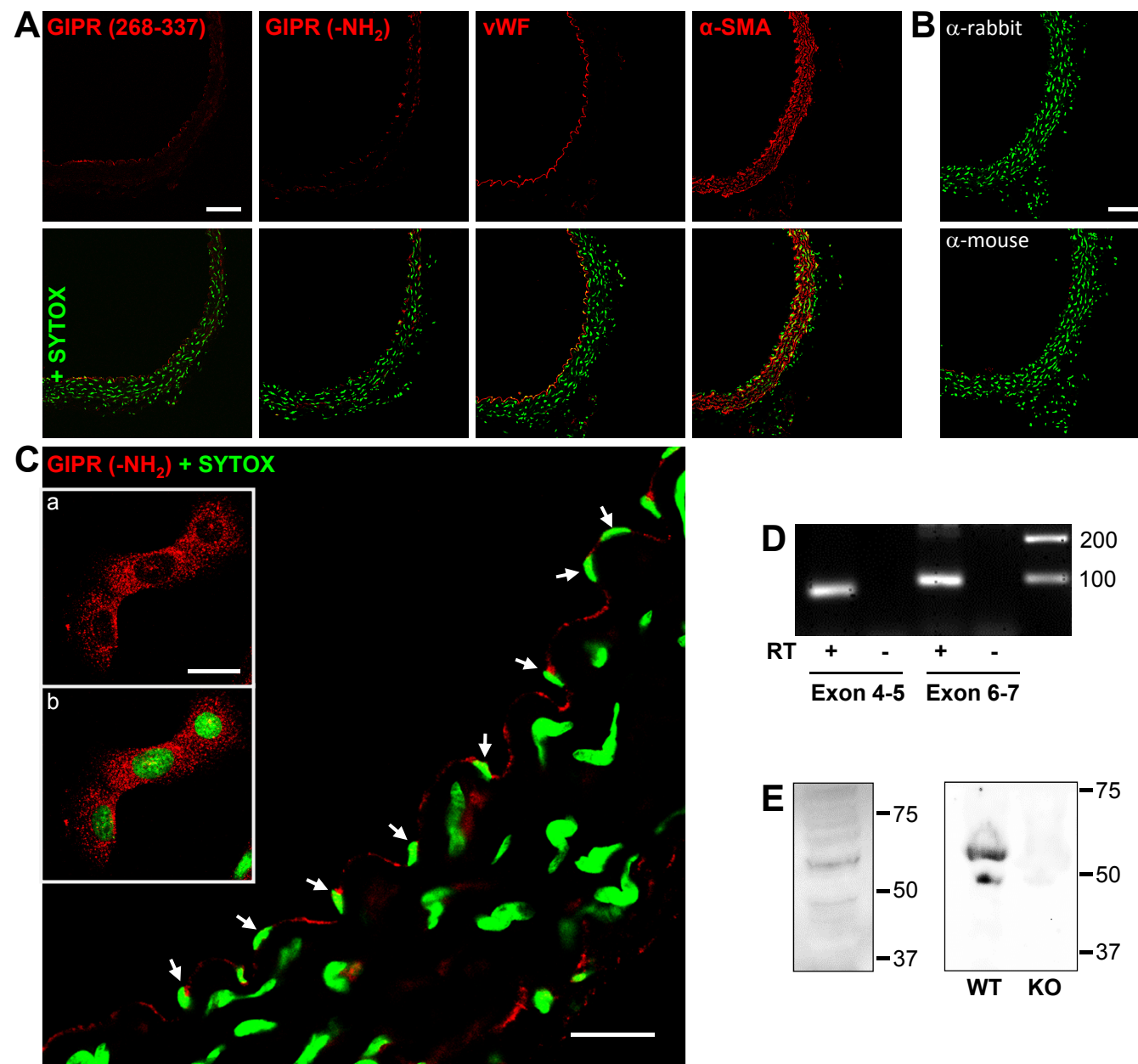


Figure 1

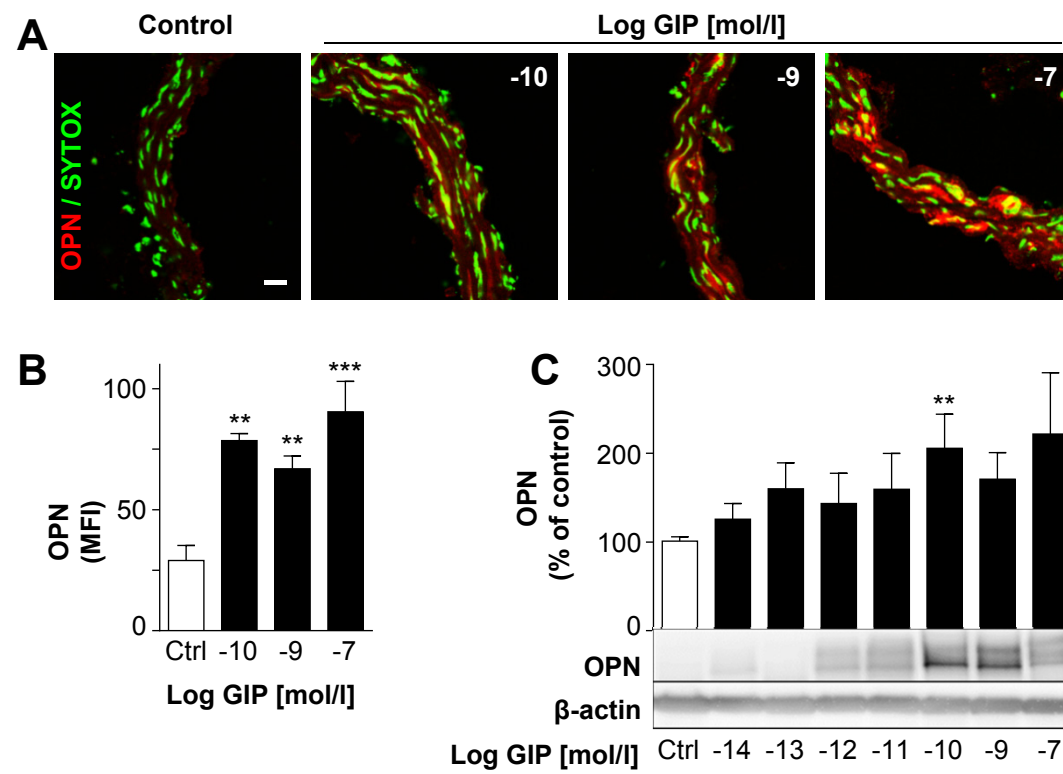


Figure 2

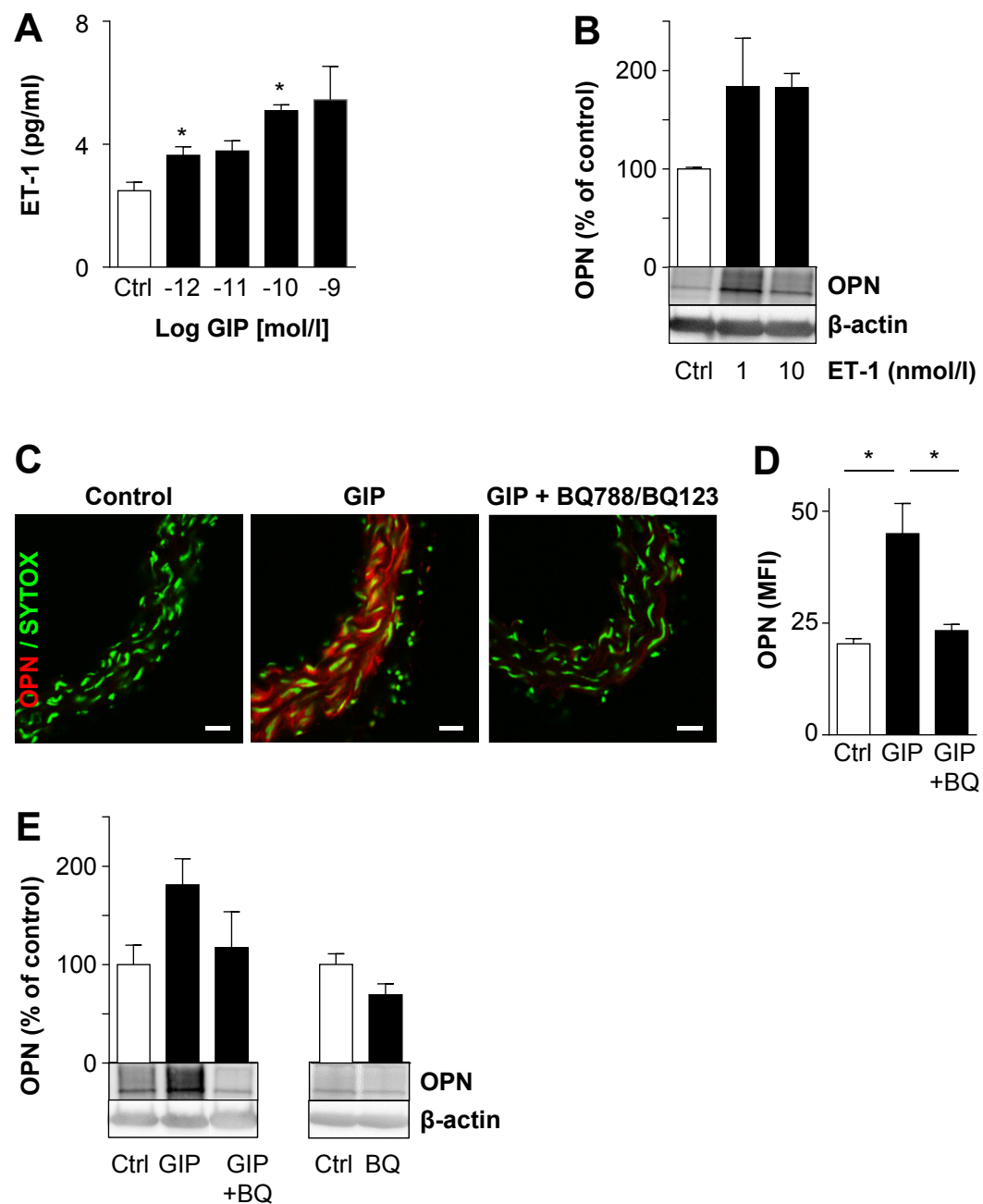


Figure 3

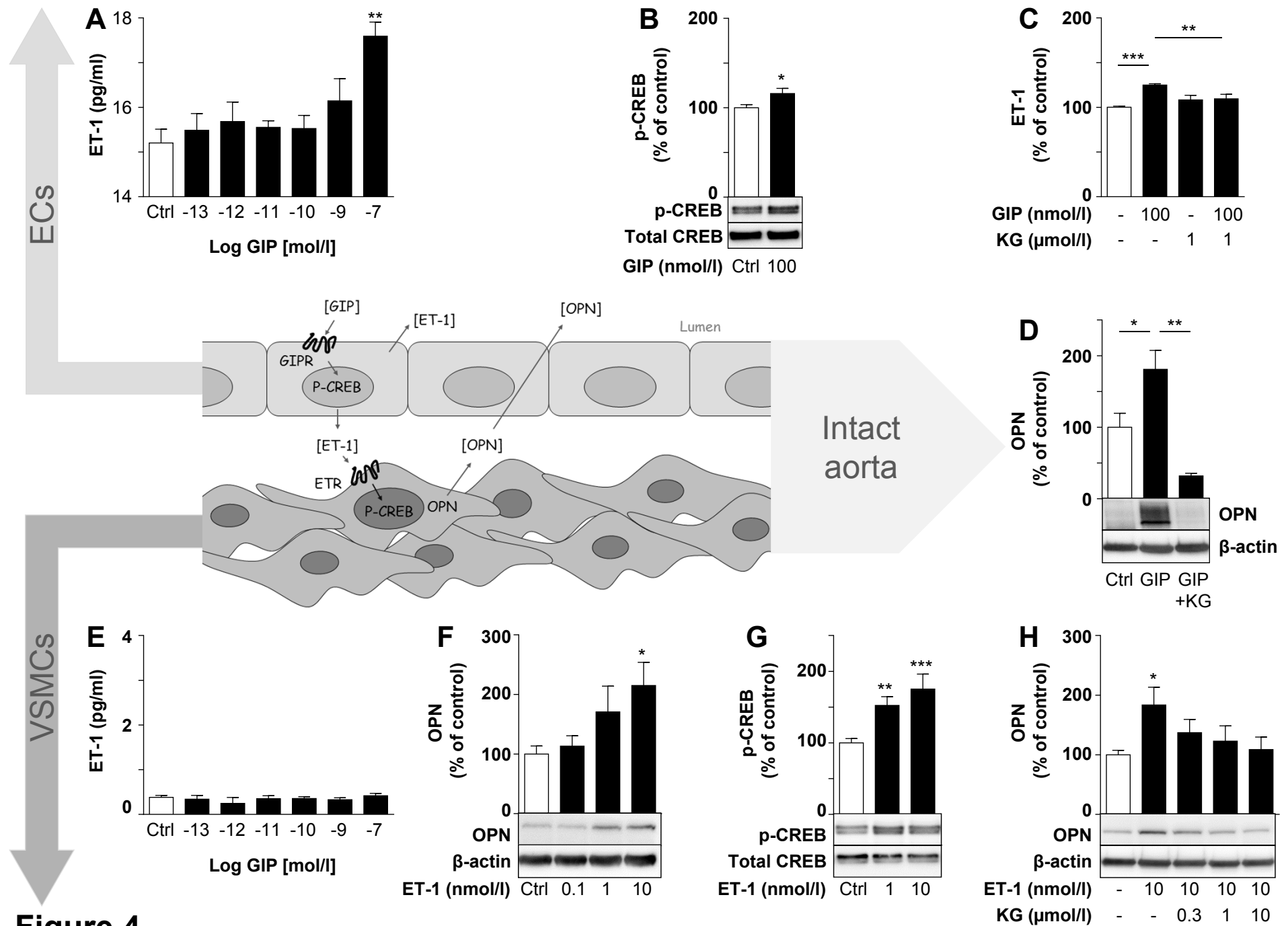


Figure 4

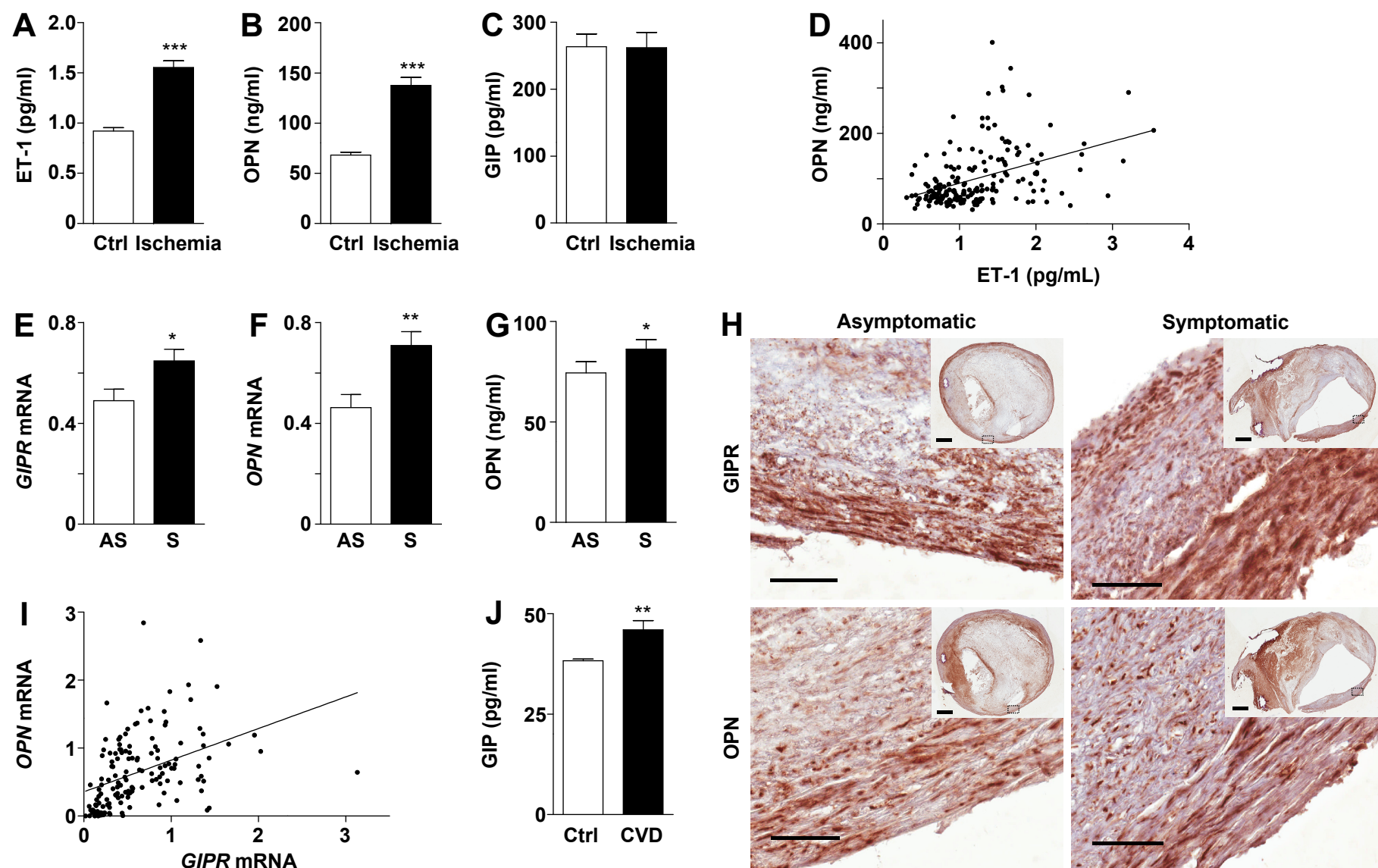


Figure 5

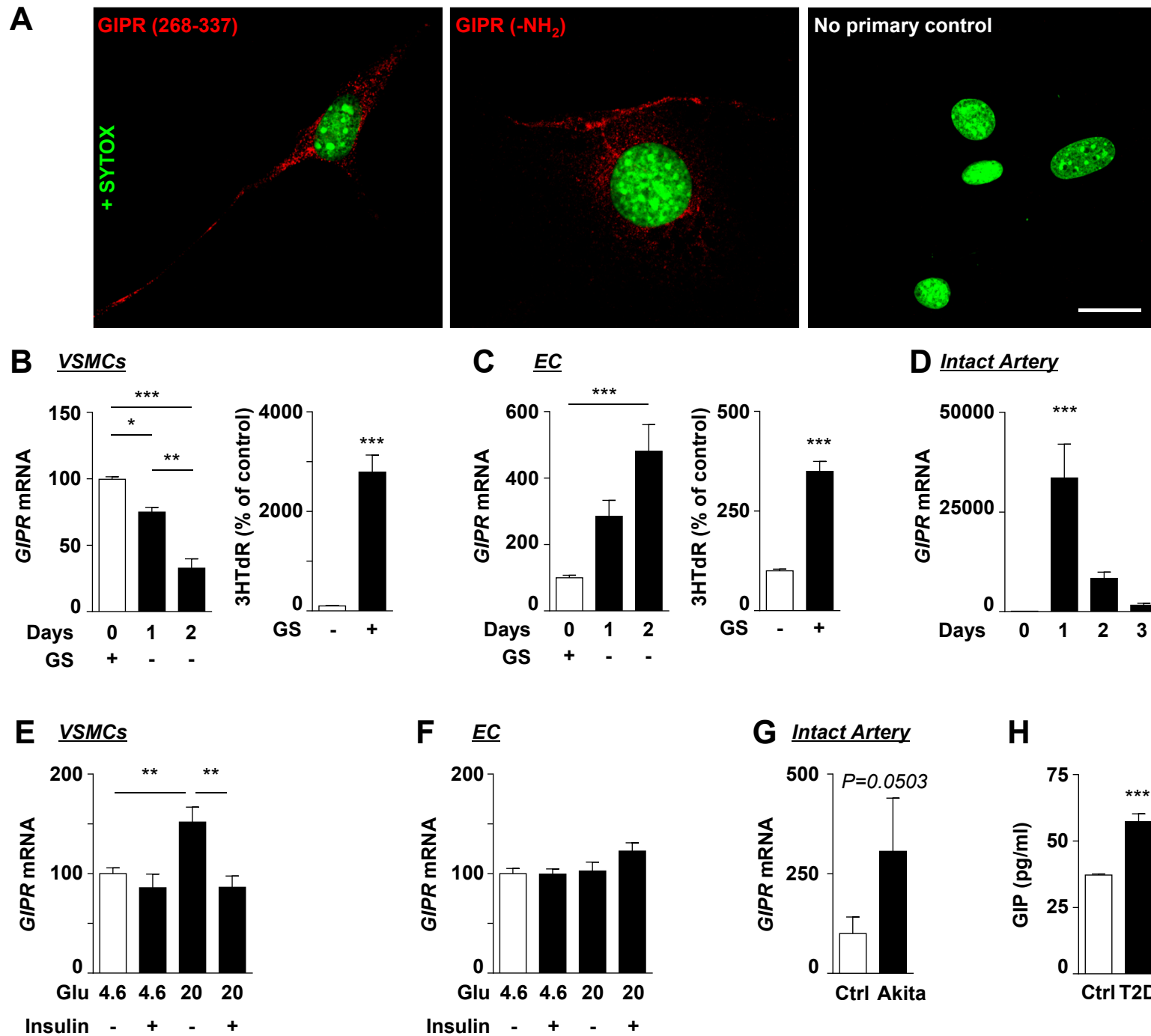
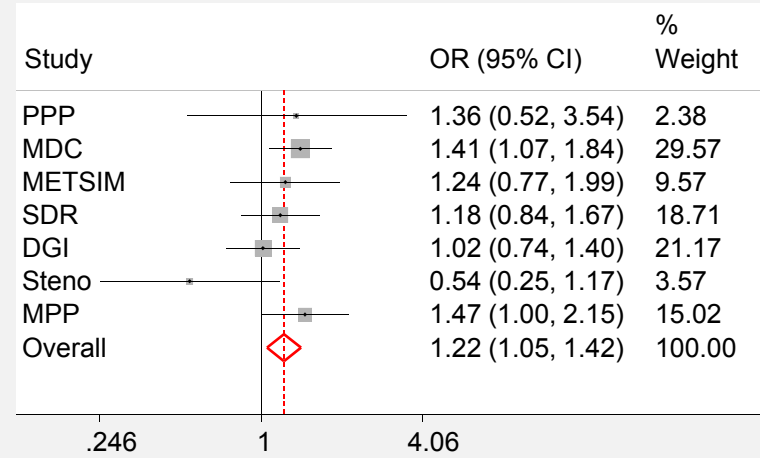


Figure 6

A

Ischemic stroke in diabetic patients and GIPR rs10423928

**B**

Ischemic stroke and GIPR rs10423928

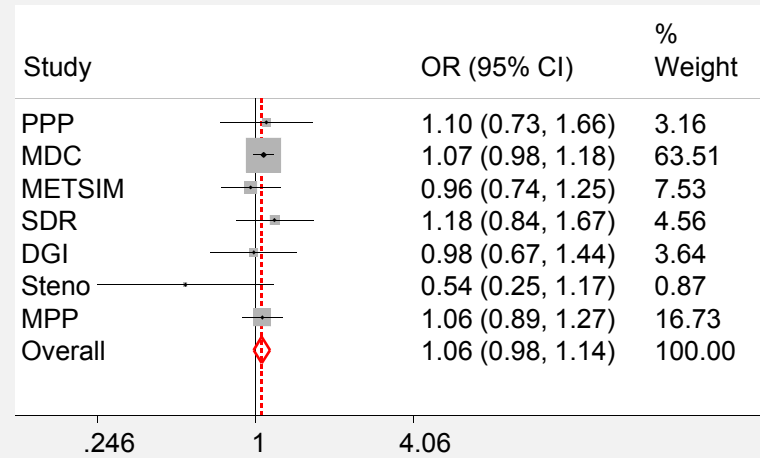
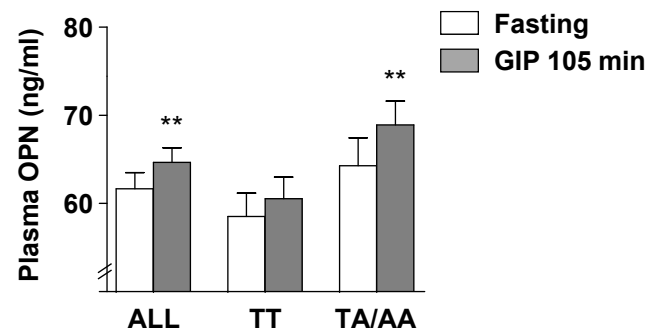
**C****Figure 7**

Table 1. Correlations between carotid artery plaque mRNA and clinical, biochemical and histological parameters.

	<i>GIPR</i> mRNA		<i>OPN</i> mRNA	
	r	N	r	N
<i>mRNA</i>				
<i>GIPR</i>	-	-	0.576***	150
<i>OPN</i>	0.576***	150	-	-
<i>Clinical data</i>				
Degree of stenosis (%)	0.035	150	-0.048	150
Number of events	0.189*	150	0.200*	150
Age (years)	-0.009	150	0.111	150
Body Mass Index (kg/m ²)	0.056	150	-0.059	150
<i>Histology (% area)</i>				
Oil-Red-O	0.290***	143	0.463***	143
CD68	0.264**	111	0.347***	111
Elastin	0.269**	91	0.465***	91
α -actin	-0.341***	122	-0.352***	122
Masson	-0.065	146	0.019	146
Calcium	0.055	123	0.062	123
<i>Extracellular matrix (mg/g)</i>				
GAG	-0.108	61	-0.070	61
Collagen	-0.076	61	-0.166	61
Elastin	0.030	61	-0.108	61
Hydroxyapatite	0.055	61	-0.088	61
<i>Plaque cytokines (pg/g)</i>				
Eotaxin	-0.059	112	-0.222*	112
Fractalkine	-0.055	124	-0.212*	124
IFN- γ	-0.027	122	-0.115	122
IL-10	0.227*	124	0.272**	124
IL-12p40	0.040	105	0.094	105
IL-12p70	-0.108	124	-0.182*	124
IL-1 β	0.255**	124	0.268**	124
IL-6	0.228*	124	0.276**	124
MCP-1	0.258**	124	0.349***	124
MIP-1 β	0.200*	124	0.307**	124
PDGF-AB/BB	0.181*	124	0.079	124
RANTES	0.289**	124	0.252**	124
sCD40L	0.175	122	-0.033	122
TNF- α	-0.005	124	0.050	124

VEGF	-0.074	117	-0.198*	117
<i>Plasma cytokines (pg/ml)</i>				
Eotaxin	0.100	123	0.084	123
Fractalkine	0.157	121	0.063	121
IFN- γ	0.118	124	0.125	124
IL-10	-0.102	124	-0.060	124
IL-12p40	0.077	119	0.011	119
IL-12p70	-0.012	124	0.001	124
IL-1 β	-0.061	124	-0.084	124
IL-6	0.042	124	0.020	124
MCP-1	-0.029	124	-0.040	124
MIP-1 β	-0.025	124	-0.005	124
PDGF-AB/BB	-0.208*	93	-0.160	93
RANTES	0.077	124	0.056	124
sCD40L	-0.075	102	-0.168	102
TNF- α	0.115	124	0.038	124
VEGF	0.086	121	0.100	121
<i>Blood samples</i>				
Hemoglobin (g/l)	0.069	150	0.081	150
White blood cell counts (*10 ⁹ /l)	-0.083	150	-0.112	150
Platelets count (*10 ⁹ /l)	-0.135	150	-0.053	150
International Normalized Ratio	-0.018	148	-0.005	148
Creatinin (mmol/l)	0.028	150	0.014	150
CRP (mg/l)	0.121	138	-0.051	138
Cholesterol (mmol/l)	0.141	136	0.114	136
Triglycerides (mmol/l)	0.171*	133	0.098	133
LDL (mmol/l)	0.080	133	0.085	133
HDL (mmol/l)	-0.032	135	0.021	135
HbA1c (mmol/mol)	0.094	50	-0.023	50

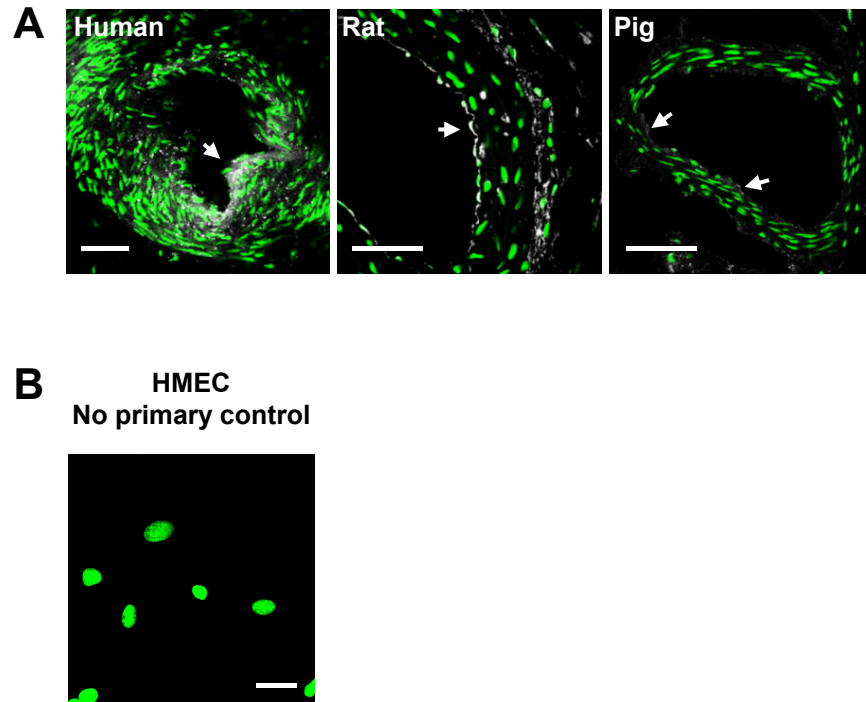


Figure S1. (A) Confocal images showing GIPR (white) and nuclei (SYTOX Green) in sections from human myometrial artery, rat carotid and pig coronary artery. Arrows point at endothelial GIPR expression. Bars=50 μ m. **(C)** Omitted primary control images for GIPR (anti-rabbit secondary antibody alone). Bar=20 μ m.

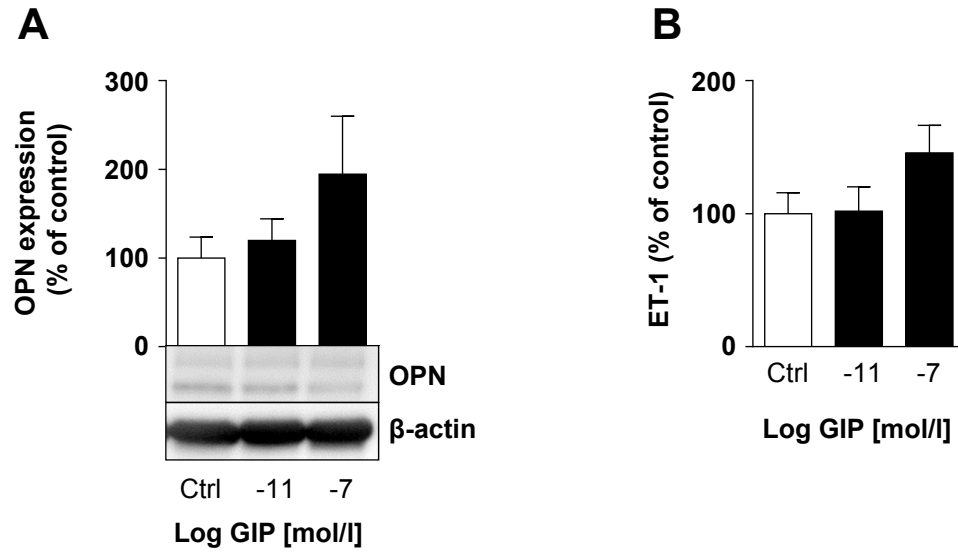


Figure S2. Effect of 24h stimulation with GIP on OPN and ET-1. (A) Summarized western blot data and representative immunoblot showing OPN expression in mouse aorta after GIP stimulation for 24h; normalized to β -actin. **(B)** ET-1 levels measured in the medium after culture of intact mouse aorta in the presence and absence of GIP for 24h as in (A). Data is from at least 3 mice for each condition and data is expressed as percentage of untreated control.

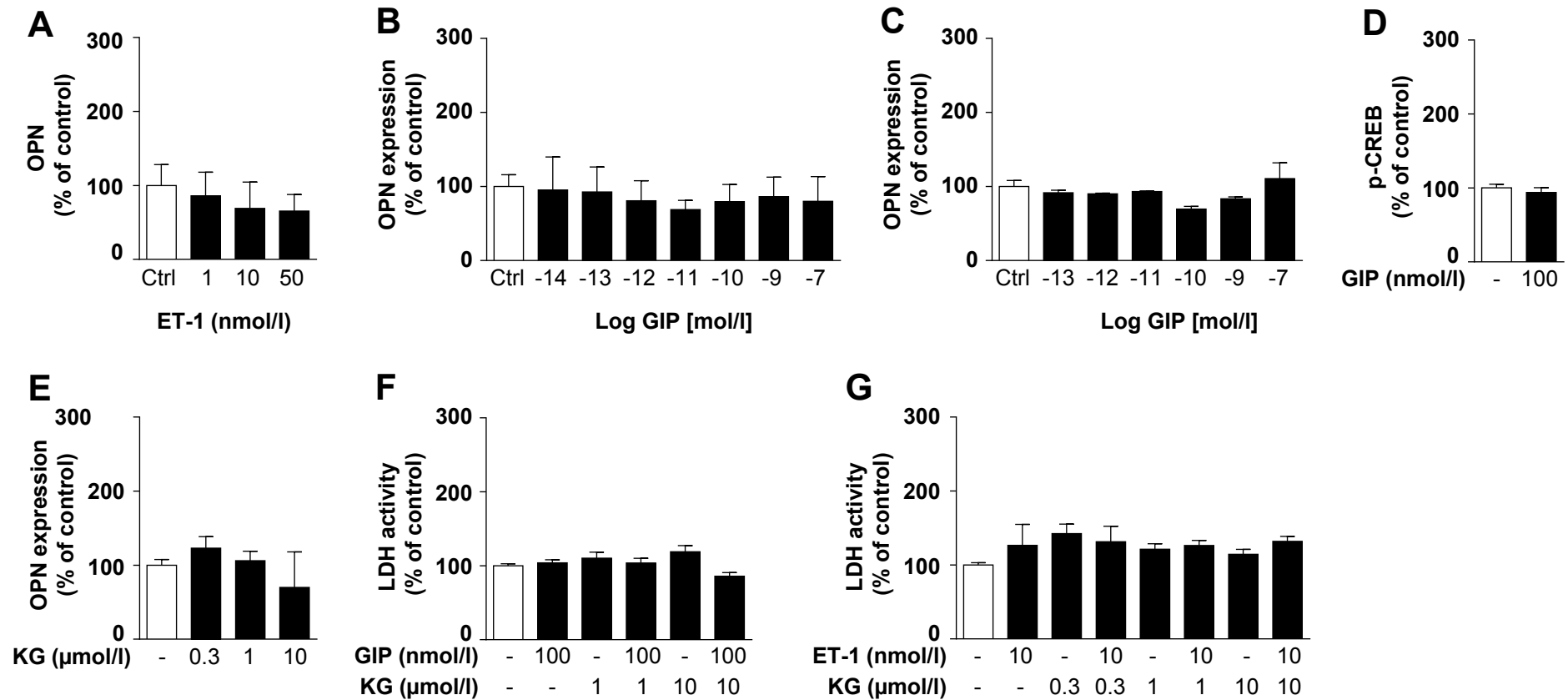


Figure S3. (A) Summarized data from western blot experiments showing OPN expression in ECs after stimulation with ET-1 for 24h. N=3 per condition. (B) and (C) Summarized data from western blot experiments showing OPN expression in ECs (B) and VSMCs (C) after stimulation with GIP for 24h. N=2-3 per condition. (D) Summarized data from western blot experiments showing p-CREB/total CREB ratio in VSMCs after stimulation with GIP (100 nmol/l, 30 min). N=4-10 per condition. (E) Summarized data from western blot experiments showing no effect of incubation with CREB inhibitor KG-501 (KG; 24h) on OPN expression in VSMCs. N=3-6 per condition. (F) and (G) LDH activity in ECs (F) and VSMCs (G) after stimulation with GIP or ET-1 in the presence or absence of KG. N=at least 3 per condition.

Figure S3

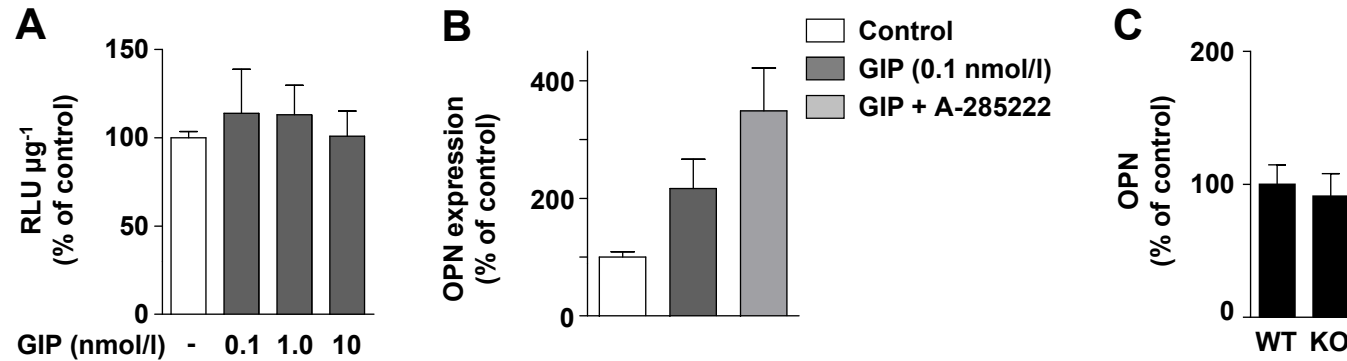


Figure S4. GIP stimulation has no effect on NFAT activation. (A) NFAT-dependent transcriptional activity in aortas from NFAT-luc transgenic mice. Each aorta was divided into four pieces and exposed to various concentrations of GIP for 12 hours. Results are expressed as relative light units (RLU) per μg protein, normalized to untreated control. The experiment was performed four times using aorta samples from at least six mice in each experiment. (B) Summarized data from western blot experiments showing OPN expression in mouse aorta upon stimulation with GIP (0.1 nmol/l) for 3 days with or without the NFAT inhibitor A-285222 (1.0 $\mu\text{mol/l}$). OPN was normalized to β -actin and is expressed as percentage of untreated control. The experiment was performed three times using aorta samples from at least five mice in each experiment. (C) GIP (0.1 nmol/l)-induced OPN expression data obtained as in B, in mouse aortas from NFATc3 competent (WT) and deficient mice (KO; N=8/group).

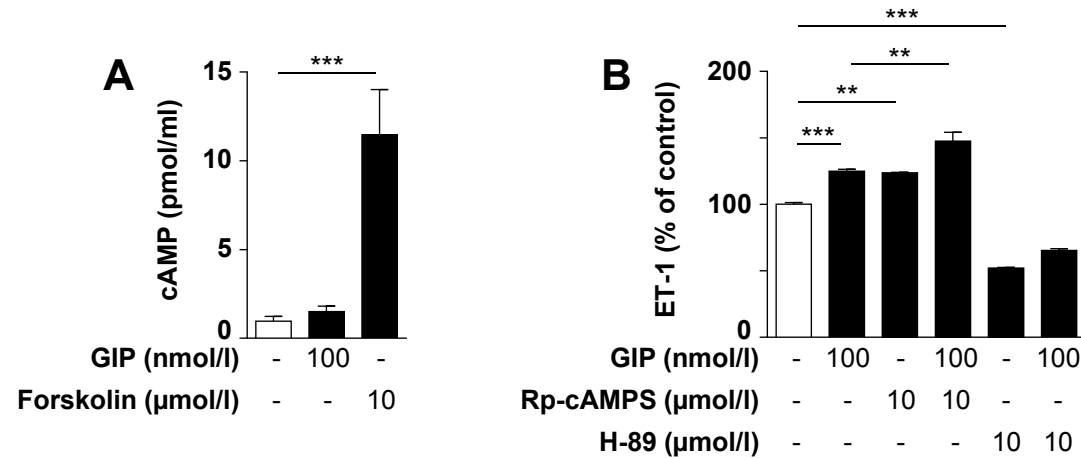


Figure S5. PKA does not mediate GIP-induced ET-1 release. (A) GIP does not stimulate production of cAMP in ECs. cAMP was measured in ECs after 30 min stimulation with GIP (100 nmol/l) or forskolin (10 μmol/l), after which cells were harvested for cAMP analysis. N=6-8 per condition. (B) ET-1 levels measured in medium from ECs after stimulation with GIP in the presence or absence of PKA inhibitors Rp-cAMPS and H-89 (both 10 μmol/l) for 24h. Rp-cAMPS was added 1h before GIP and H-89 was added 30 min before GIP, both drugs were present during the 24h GIP stimulation. N=3-6 per condition.

Figure S5

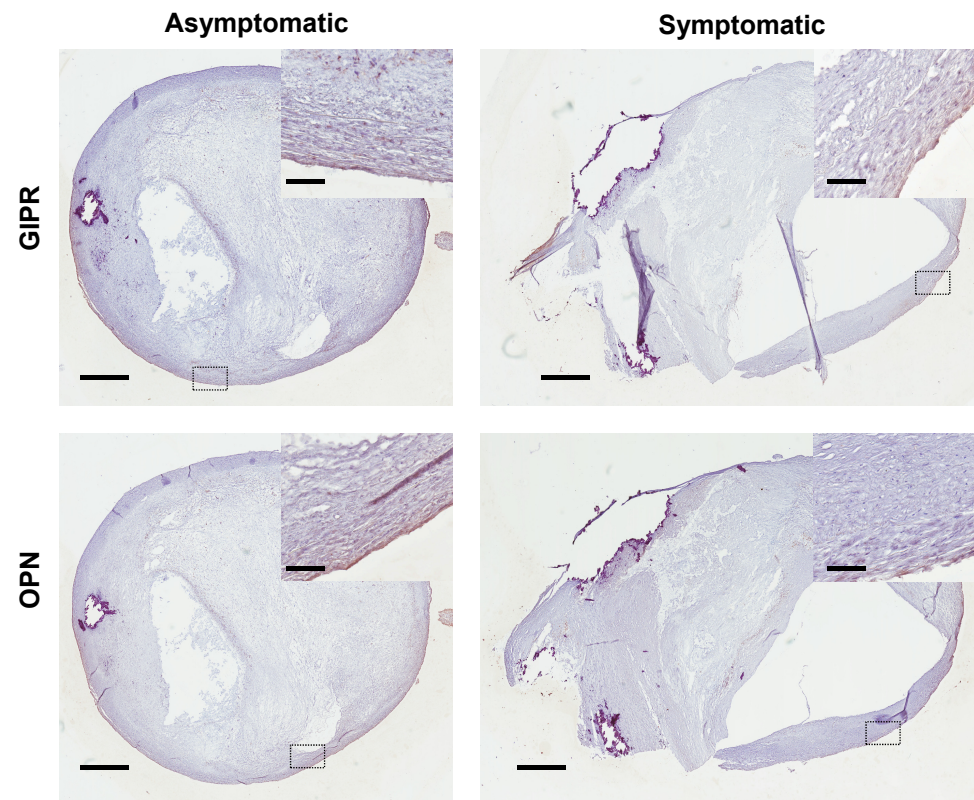


Figure S6. Immunohistochemistry staining of sections from carotid atherosclerotic plaque as described in Figure 5, with primary antibodies GIPR or OPN omitted from the protocol. Scale bars large images=1 mm, insets=100 μm.

Figure S6

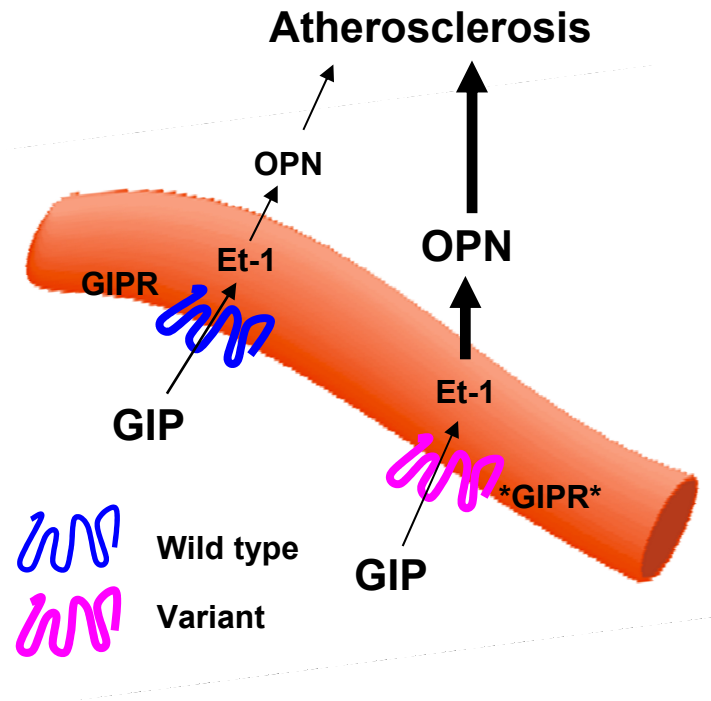


Figure S7. Schematic view of the role of GIP/GIPR and OPN in the vasculature and how this may be influenced by a common variant in the GIPR. The common risk variant in the GIPR gene (rs10423928) is shown in pink and the non-risk wild type variant in blue. GIP stimulates expression of OPN in the arterial wall through production of ET-1. In the vessels, OPN has pro-atherogenic properties and this effect seems to be enhanced in carriers of the A allele in the GIPR.

Supplemental table 1: Clinical characteristics of patients with critical limb ischemia.		
	Control	Ischemia
Number	101	85
Age, years	67.9 ± 1.5	76.0 ± 9.5***
Male sex	45 (44.6)	44 (51.8)
Body mass index	26.7 ± 4.1	24.9 ± 4.4**
Systolic blood pressure	144.3 ± 21.2	146.5 ± 22.2
Diastolic blood pressure	84.6 ± 11.1	73.9 ± 9.9***
Smoking	9 (8.9)	27 (31.8)***
Lipid-lowering medication	12 (11.9)	17 (20.0)
Values are expressed as mean ± standard deviation or N (%). **P < 0.01 and ***P < 0.001 vs. control, determined by Mann-Whitney U or Fisher's exact test.		

Supplemental table 2: Clinical characteristics of patients undergoing carotid endarterectomy.	
Age, years	70.4 ± 8.6
Male sex	100 (66.7)
Degree of stenosis	85.0 ± 11.3
Body mass index (kg/m ²)	26.5 ± 3.5
Diabetes	55 (36.7)
Hypertension	110 (73.3)
Symptomatic	87 (58.0)
Stroke	40 (26.7)
Amaurosis fugax	17 (11.3)
Transient ischemic attacks	38 (25.3)
Values are expressed as mean ± standard deviation or N (%).	

Supplemental table 3. Results from fixed effect meta-analyses of <i>GIPR</i> rs10423928 associations.						
Phenotype	Analysis	OR (95% CI)	<i>P</i> value	<i>Q</i>	<i>P</i> heterogeneity	<i>I</i>²
Ischemic stroke	All individuals	1.057 (0.983-1.137)	0.133	4.09	0.664	0%
	T2D subjects	1.221 (1.054-1.415)	0.00799	7.52	0.276	20.2%
Myocardial infarction	All individuals	0.942 (0.882-1.006)	0.075	3.78	0.707	0%
	T2D subjects	0.929 (0.814-1.059)	0.27	5.29	0.508	0%
Phenotype	Analysis	Beta (95 % CI)	<i>P</i> value	<i>Q</i>	<i>P</i> heterogeneity	<i>I</i>²
Systolic blood pressure*	All individuals	0.001 (-0.001-0.003)	0.366	8.82	0.184	32.0%
	T2D subjects	-0.005 (-0.011-0.002)	0.159	4.74	0.577	0%
Diastolic blood pressure	All individuals	0.107 (-0.046-0.260)	0.17	4.32	0.634	0%
	T2D subjects	-0.131 (-0.637-0.376)	0.613	7.7	0.613	22.1%
Pulse pressure*	All individuals	0.001 (-0.003-0.005)	0.627	10.73	0.097	44.1%
	T2D subjects	-0.009 (-0.021-0.004)	0.171	4.87	0.561	0%

* Systolic blood pressure and pulse pressure values were log transformed
Individual analyses were adjusted for age, sex and BMI.

Supplemental table 4. <i>GIPR</i> rs10423928 and risk of ischemic stroke.						
Study ID	N (total)	N (cases)	OR	SE(OR)	95% CI	<i>P</i> value
All individuals						
PPP-Botnia	4746	57	1.100	0.230	0.730-1.658	0.648
MDC	27209	1402	1.073	0.050	0.979-1.175	0.134
METSIM	6497	169	0.959	0.130	0.735-1.250	0.755
SDR	1738	101	1.185	0.206	0.842-1.666	0.330
DGI	1690	78	0.985	0.192	0.672-1.443	0.938
Steno T2D	269	26	0.538	0.214	0.246-1.174	0.119
MPP	6447	314	1.071	0.105	0.883-1.298	0.488
T2D subjects only						
PPP-Botnia	269	10	1.360	0.664	0.523-3.538	0.528
MDC	2360	151	1.406	0.195	1.072-1.845	0.014
METSIM	919	44	1.238	0.301	0.768-1.994	0.381
SDR	1738	101	1.185	0.206	0.842-1.666	0.330
DGI	1052	112	1.018	0.167	0.739-1.402	0.914
Steno T2D	269	26	0.538	0.214	0.246-1.174	0.119
MPP	864	79	1.470	0.285	1.004-2.150	0.047
Analyses were adjusted for age, gender and BMI. In the DGI study, information from the HILMO inpatient registry was only available in diabetic subjects. This additional information was used in the analysis of only T2D subjects, hence the higher number of cases in that analysis.						

Supplemental table 5. <i>GIPR</i> rs10423928 and risk of myocardial infarction.						
Study ID	N (total)	N (cases)	OR	SE(OR)	95% CI	P value
All individuals						
PPP-Botnia	4761	133	0.898	0.134	0.670-1.203	0.470
MDC	26979	1608	0.958	0.043	0.877-1.047	0.347
METSIM	6507	347	0.849	0.085	0.698-1.032	0.101
SDR	1673	124	0.994	0.162	0.723-1.368	0.971
DGI	1698	135	0.763	0.123	0.556-1.048	0.095
Steno T2D	270	17	0.907	0.388	0.391-2.100	0.819
MPP	6660	563	1.001	0.076	0.861-1.162	0.995
T2D subjects only						
PPP-Botnia	271	31	0.998	0.312	0.541-1.840	0.994
MDC	2330	175	1.098	0.150	0.840-1.436	0.494
METSIM	921	101	0.644	0.127	0.438-0.947	0.025
SDR	1673	124	0.994	0.162	0.723-1.368	0.971
DGI	1062	219	0.908	0.118	0.704-1.173	0.460
Steno T2D	270	17	0.907	0.388	0.391-2.100	0.819
MPP	900	120	0.888	0.160	0.624-1.265	0.511
Data has been adjusted for age, gender and BMI. In the DGI study, information from the HILMO inpatient registry was only available in diabetic subjects. This additional information was used in the analysis of only T2D subjects, hence the higher number of cases in that analysis.						

Supplemental table 6. Association of <i>GIPR</i> rs10423928 with blood pressure.												
	Log (systolic blood pressure)				Diastolic blood pressure				Log (pulse pressure)			
Study ID	Beta	SE	P value	N	Beta	SE	P value	N	Beta	SE	P value	N
All individuals												
PPP-Botnia	0.0048	0.0028	0.090	3891	0.1788	0.2292	0.435	3891	0.0096	0.0059	0.104	3891
MDC	0.0011	0.0014	0.435	22731	0.1105	0.1038	0.287	22730	0.0013	0.0026	0.628	22728
METSIM	-0.0031	0.0027	0.247	5038	-0.1195	0.2156	0.579	5038	-0.0051	0.0054	0.346	5038
SDR	-0.0035	0.0063	0.581	1288	-0.4963	0.4934	0.472	1288	0.0002	0.0120	0.984	1288
DGI	-0.0038	0.0059	0.520	1210	0.4338	0.4418	0.326	1209	-0.0203	0.0118	0.087	1209
Steno T2D	-0.0201	0.0132	0.128	281	-0.2757	1.0227	0.788	282	-0.0469	0.0249	0.061	280
MPP	0.0037	0.0028	0.185	6747	0.3193	0.2201	0.147	6747	0.0039	0.0046	0.391	6747
T2D subjects only												
PPP-Botnia	0.0123	0.0186	0.660	114	2.1893	1.2620	0.086	114	-0.0082	0.0384	0.831	114
MDC	-0.0136	0.0090	0.129	637	-0.7635	0.6494	0.240	637	-0.0234	0.0171	0.173	637
METSIM	-0.0096	0.0086	0.265	508	-0.7941	0.7330	0.279	508	-0.0117	0.0167	0.485	508
SDR	-0.0035	0.0063	0.581	1288	-0.4963	0.4934	0.472	1288	0.0002	0.0120	0.984	1288
DGI	0.0004	0.0082	0.962	582	0.6947	0.6346	0.274	581	-0.0133	0.0167	0.425	581
Steno T2D	-0.0201	0.0132	0.128	281	-0.2757	1.0227	0.788	282	-0.0469	0.0249	0.061	280
MPP	0.0025	0.0081	0.756	915	0.2084	0.6239	0.738	915	0.0055	0.0134	0.682	915
Analyses were adjusted for age, gender and BMI and performed in individuals without anti-hypertensive treatment. In the SDR study, information on antihypertensive treatment was not available and all individuals were included.												

Supplemental table 7. Association of <i>GIPR</i> rs10423928 with risk of ischemic stroke, myocardial infarction and retinopathy in T1D subjects.						
Study ID	N (total)	N (cases)	OR	SE(OR)	95% CI	P value
Ischemic stroke						
FinnDiane	3005	85	1.0778	0.1894	0.7638-1.5209	0.670
Steno T1D	828	35	0.6525	0.2091	0.3482-1.2229	0.183
Myocardial infarction						
FinnDiane	3003	109	0.9038	0.1478	0.6560-1.2451	0.536
Steno T1D	828	26	0.9791	0.3241	0.5118-1.8733	0.949
Retinopathy						
FinnDiane	2993	1188	0.9778	0.0687	0.8521-1.1221	0.749
Steno T1D	862	417	1.0355	0.1244	0.8183-1.3105	0.771
Analyses were adjusted for age at onset of diabetes, disease duration and gender.						

Supplemental table 8. Association of <i>GIPR</i> rs10423928 with blood pressure in T1D subjects.												
	Log (systolic blood pressure)				Diastolic blood pressure				Log (pulse pressure)			
Study ID	Beta	SE	<i>P</i> value	N	Beta	SE	<i>P</i> value	N	Beta	SE	<i>P</i> value	N
FinnDiane	0.0056	0.0045	0.216	1662	0.0699	0.3532	0.843	1660	0.0128	0.0097	0.187	1660
Steno	0.0004	0.0102	0.966	453	0.3574	0.7696	0.643	453	-0.0096	0.0213	0.652	453
Univariate analyses were performed in individuals free from hypertensive treatment.												

Supplemental table 9. Correlations between mRNA levels and clinical, biochemical and histological parameters in relation to *GIPR* genotype, in human carotid atherosclerotic plaques.

	TT				TA/AA			
	GIPR		OPN		GIPR		OPN	
	r	N	r	N	r	N	r	N
mRNA								
<i>GIPR</i>	-	-	0.580***	94	-	-	0.537***	53
<i>OPN</i>	0.580***	94	-	-	0.537***	53	-	-
Clinical data								
Degree of stenosis (%)	-0.012	94	-0.019	94	0.093	53	-0.100	53
Number of events	0.091	94	0.096	94	0.306*	53	0.343*	53
Age (years)	-0.080	94	0.144	94	0.105	53	0.063	53
Body mass index (kg/m ²)	-0.063	94	-0.197	94	0.215	53	0.308*	53
Histology (% area)								
Oil-Red-O	0.337**	91	0.449***	91	0.270	49	0.534***	49
CD68	0.158	72	0.168	72	0.346*	36	0.555***	36
Elastin	0.249	57	0.592***	57	0.255	32	0.127	32
α-actin	-0.330**	82	-0.228*	82	-0.289	37	-0.537**	37
Masson	-0.123	93	-0.026	93	0.042	50	0.114	50
Calcium	-0.088	78	0.070	78	0.261	42	0.056	42
Extracellular matrix (mg/g)								
GAG	-0.229	38	-0.194	38	0.065	22	0.128	22
Collagen	-0.297	38	-0.532**	38	0.226	22	0.487*	22
Elastin	-0.116	38	-0.400*	38	0.327	22	0.423*	22
Hydroxyapatite	0.099	38	-0.031	38	-0.014	22	-0.179	22
Plaque cytokines (pg/g)								
Eotaxin	0.051	71	-0.075	71	-0.269	38	-0.455**	38
Fractalkine	-0.004	76	-0.265*	76	-0.179	45	-0.223	45
IFN-γ	-0.042	76	-0.245*	76	-0.096	43	-0.073	43
IL-10	0.189	76	0.171	76	0.287	45	0.471**	45
IL-12p40	0.063	68	0.127	68	0.057	34	0.032	34
IL-12p70	-0.066	76	-0.182	76	-0.219	45	-0.192	45

Supplemental table 9. Continued								
	TT				TA/AA			
	GIPR		OPN		GIPR		OPN	
	r	N	r	N	r	N	r	N
IL-1 β	0.140	76	0.280*	76	0.426**	45	0.288	45
IL-6	0.254*	76	0.283*	76	0.173	45	0.203	45
MCP-1	0.318**	76	0.414***	76	0.221	45	0.218	45
MIP-1 β	0.185	76	0.292*	76	0.213	45	0.384**	45
PDGF-AB/BB	0.251*	76	0.065	76	0.103	45	0.099	45
RANTES	0.325**	76	0.208	76	0.224	45	0.294*	45
sCD40L	0.231	76	-0.002	76	0.023	43	-0.158	43
TNF- α	0.000	76	0.019	76	-0.100	45	0.065	45
VEGF	-0.007	72	-0.134	72	-0.271	42	-0.391*	42
<i>Plasma cytokines (pg/mL)</i>								
Eotaxin	0.156	75	0.017	75	-0.050	45	0.157	45
Fractalkine	0.209	76	-0.056	76	0.065	42	0.246	42
IFN- γ	0.240*	76	0.021	76	-0.147	45	0.195	45
IL-10	-0.161	76	-0.179	76	-0.066	45	0.105	45
IL-12p40	0.091	75	-0.093	75	0.039	42	0.206	42
IL-12p70	0.085	76	-0.097	76	-0.210	45	0.105	45
IL-1 β	0.069	76	-0.147	76	-0.295*	45	0.038	45
IL-6	0.078	76	0.008	76	-0.114	45	0.004	45
MCP-1	-0.116	76	-0.061	76	0.089	45	-0.011	45
MIP-1 β	0.024	76	-0.098	76	-0.110	45	0.152	45
PDGF-AB/BB	-0.175	54	-0.185	54	-0.268	37	-0.107	37
RANTES	0.060	76	0.112	76	0.131	45	-0.046	45
sCD40L	-0.177	61	-0.132	61	0.045	38	-0.081	38
TNF- α	0.175	76	-0.053	76	-0.037	45	0.160	45
VEGF	0.233*	76	0.105	76	-0.204	42	0.065	42
<i>Blood samples</i>								
Hemoglobin (g/L)	0.128	94	0.020	94	-0.017	53	0.185	53
White blood cell counts (*10 ⁹ /L)	-0.132	94	-0.151	94	-0.057	53	-0.080	53
Platelets count (*10 ⁹ /L)	-0.176	94	-0.078	94	-0.127	53	-0.084	53
International Normalized Ratio	0.091	93	0.041	93	-0.204	52	-0.072	52

Supplemental table 9. Continued								
	TT				TA/AA			
	GIPR		OPN		GIPR		OPN	
	r	N	r	N	r	N	r	N
Creatinin (mmol/L)	-0.021	94	-0.053	94	0.075	53	0.097	53
CRP (mg/L)	0.226*	88	0.050	88	-0.025	47	-0.260	47
Cholesterol (mmol/L)	0.243*	85	0.144	85	-0.013	48	0.029	48
Triglycerides (mmol/L)	0.224*	84	0.073	84	0.097	46	0.098	46
LDL (mmol/L)	0.205	82	0.132	82	-0.050	48	0.009	48
HDL (mmol/L)	-0.076	85	0.032	85	-0.019	47	-0.052	47
HbA1c (mmol/mol)	0.038	38	-0.019	38	0.644*	11	0.106	11

ONLINE SUPPLEMENTAL DATA

Investigated cohorts

We explored association of the *GIPR* rs10423928 SNP with cardiovascular events in individuals from the following studies: Prevalence, Prediction and Prevention of Diabetes-Botnia (PPP-Botnia (1)), The Malmö Diet and Cancer Study (MDC (2)), METabolic Syndrome In Men (METSIM (3)), Scania Diabetes Registry (SDR (4)), Diabetes Genetic Initiative (DGI (1)), Steno type 1 and type 2 diabetes studies (Steno T1D and T2D (5; 6)), Malmö Preventive Project (MPP (7; 8)) and Finnish Diabetic Nephropathy study (FinnDiane (9)). All studies comply with the Declaration of Helsinki and all participants gave their written informed consent. A brief description of the studies and approval numbers for the ethical protocols are found below.

Prevalence, Prediction and Prevention of diabetes (PPP-Botnia)

The PPP-Botnia, ethical approval #574/.E5/03, is a population-based study from the Botnia region, which includes approximately 7% of the population aged 18-75 years (mean age 51 ± 17 years).(1) Diagnosis of diabetes was confirmed from subject records or on the basis of fasting plasma glucose concentration ≥ 7.0 mmol/l and/or 2h glucose ≥ 11.1 mmol/l. Systolic and diastolic blood pressure were measured twice during the screening visit. Participants were asked whether they were diagnosed with diabetes, had suffered a MI, stroke and several other diseases, as well as about any medication taken.(1)

The Malmö Diet and Cancer study (MDC)

The MDC study, ethical approval #LU 51-90, is a prospective cohort study that includes men born between 1923 and 1945 and women born between 1923 and 1950 from Malmö, Sweden. (2) Individuals underwent measurement of anthropometric dimensions (BMI, waist and hip circumference) and blood pressure and filled out a questionnaire on health, lifestyle,

socioeconomic factors and medications.(10) Blood pressure was measured once after 10 min rest in supine position. Baseline diabetes was defined as self-reported history of a physician's diagnosis of diabetes or use of anti-diabetic medications drugs or fasting whole blood glucose ≥ 6.1 mmol/l. Information on stroke and myocardial infarction was obtained from the Swedish Hospital Discharge Registry, the Stroke register of Malmö, and the National Cause of Death Registry, defined as non-fatal myocardial infarction or death due to ischemic heart disease (ICD codes 410-414) or ischemic stroke (ICD code 434). The latter was verified by computer tomography, magnetic resonance imaging or autopsy as previously described.(11) By definition, transient ischemic attacks (ICD-9 code 435) were not counted as CVD events. Incident cases of stroke or myocardial infarction were included in the analyses and prevalent cases were excluded. In the analyses of T2D subjects, incident cases of stroke or myocardial infarction and prevalent and incident diabetics were included. Incident diabetes was added as an additional covariate.

The METabolic Syndrome In Men (METSIM)

The METSIM study, ethical approval #171/2004, includes men aged 45-70 years, randomly selected from the population register of Kuopio in eastern Finland (population 95,000).(3) Every participant had a 1-day outpatient visit to the Clinical Research Unit at the University of Kuopio, including an interview on the history of previous diseases and current drug treatment and an evaluation of glucose tolerance and cardiovascular risk factors. Diagnosis of diabetes was defined by a fasting plasma glucose concentration ≥ 7.0 mmol/l and/or 2h glucose ≥ 11.1 mmol/l or drug treatment for diabetes. Blood pressure was measured in the sitting position after a 5-min rest with. (12) Stroke events were ascertained based on medical records. WHO criteria for stroke were used in the ascertainment of a new stroke as well as a previous stroke: a clinical syndrome consisting of a neurological deficit observed by a neurologist and persisting >24 hours (nonfatal stroke), without other diseases explaining the symptoms. Thromboembolic and hemorrhagic strokes, but not subarachnoid hemorrhagic stroke, were included in the diagnosis of stroke.

Scania Diabetes Registry (SDR)

A Diabetes Registry in Southern Sweden (Scania Diabetes Registry), ethical approval #LU 35-99, was initiated in 1996 and hitherto 7,365 patients with different kinds of diabetes have been registered at outpatient clinics in the region.(4) At annual follow-ups, signs of retinopathy, nephropathy, neuropathy and cardiovascular disease are recorded. Stroke or myocardial infarction events were obtained from the Swedish Hospital Discharge Register and the Stroke Registry of Malmö.(13) Incident cases of stroke or myocardial infarction were included in the analyses.

Diabetes Genetic Initiative (DGI)

Patients with T2D, geographically matched controls and discordant sib-ships were selected from Finland and Sweden.(14) The Finnish cohort, ethical approval #39/08/95, consisted largely of patients from the Botnia study on the west coast of Finland with a smaller sample from other regions of Finland. The Swedish cohort consisted of patients from Southern Sweden and Skara; ethical approvals #LU 305-94 and #LU 161-92. Patients with T2D were classified according to WHO criteria with fasting plasma glucose ≥ 7.0 mmol/l or a 2h glucose ≥ 11.1 mmol/l during an OGTT. Control subjects were defined as normal glucose tolerant, with fasting plasma glucose < 6.1 mmol/l and 2h glucose < 7.8 mmol/l and age within 5 years of the age at onset of the patients with T2D. Blood pressure was measured as previously described.(14) Self-reported stroke and MI were used for all but the Skara cohort. Stroke and MI in the Skara cohort was defined based upon hospital records and central registries in Sweden. Inpatient registry information (HILMO) on incident cardiovascular events was available in Finnish diabetics only. Accordingly, this information was only included in the analysis of T2D subjects only. Individuals already included in the PPP-Botnia study were removed from the analyses of DGI.

Steno T1D and T2D studies

T1D study (ethical approval #KA93141): From 1993 to 2001, all adult Danish Caucasian patients with T1D and diabetic nephropathy attending the outpatient clinic at Steno Diabetes Center were invited to participate in a study of genetic risk factors for the development of diabetic micro- and macrovascular complications. T1D was considered present if the age at onset of diabetes was ≤ 35 years and the subsequent time to definite insulin therapy ≤ 1 year. Of these, 73% accepted, resulting in a total of 489 cases with established diabetic nephropathy defined by persistent albuminuria (>300 mg/24h) in two out of three consecutive measurements in sterile urines, presence of retinopathy, and absence of other kidney or urinary tract disease. In four cases without retinopathy, the diagnosis of diabetic glomerulopathy was verified by a kidney biopsy. Absence of diabetic nephropathy (controls) was defined as persistent normoalbuminuria (<30 mg/24h) after more than 15 years of T1D in patients not treated with angiotensin converting enzyme inhibitors or angiotensin II receptor blockers. In total, 463 patients were included as controls (76% of patients fulfilling the criteria). All patients had blood samples and phenotypic characteristics collected as part of an European case-control study collaboration (EURAGEDIC).⁽⁶⁾ Office blood pressure was measured twice with a sphygmomanometer after at least 10 min rest in the sitting position. Diabetic retinopathy was assessed by fundus photography after pupillary dilatation and graded as nil, simplex, and proliferative retinopathy. Major cardiovascular events were myocardial infarction or stroke.

In a prospective, observational study design, ~~the~~ patients were followed until an endpoint was reached, to the last visit at Steno Diabetes Center or until the 1st of September 2006. The endpoints were all-cause mortality, cardiovascular mortality, major cardiovascular events, decline in GFR and development of end-stage renal disease (ESRD). Deaths were classified as cardiovascular deaths unless an unequivocal non-cardiovascular cause was established.⁽¹⁵⁾

T2D study (ethical approval #KA95038g): This is a case-control study of T2D patients with cases having diabetic nephropathy and controls without nephropathy, including patients collected as

part of an European case-control study collaboration (PREDICTIONS) and additional patients, according to the previously described criteria.(5) T2D patients aged 35-75 with a documented duration of diabetes of ≥ 5 years attending the outpatient clinic at the Steno Diabetes Center were eligible. Diagnosis of diabetes was established in accordance with WHO criteria: fasting plasma glucose ≥ 7.0 mmol/l, a two-hour value in an oral glucose tolerance test ≥ 11.1 mmol/l, or random plasma glucose ≥ 11.1 mmol/l in the presence of symptoms. T2D was diagnosed by lack of fulfilling the criteria for T1D. For cases, inclusion criteria were albuminuria >300 mg/dl and known overt diabetic retinopathy. In cases without retinopathy, the diagnosis of diabetic glomerulopathy was verified by a kidney biopsy. Exclusion criteria were end stage renal failure, known causes of renal failure other than diabetes and non-Caucasian ethnic origin. Regarding controls, these were matched pair-wise to cases in term of gender and diabetes duration. Exclusion criteria for controls were micro-albuminuria, non-Caucasian ethnic origin, and in case of use of RAAS-blocking medication, unknown albuminuria status prior to start of treatment. Office blood pressure was measured twice after at least 10 min rest in the sitting position. Prior major cardiovascular events were defined as a history of stroke and/or myocardial infarction obtained from the patient's medical records. In a prospective, observational study design, the patients were followed until they died, to the last visit at Steno Diabetes Center or until the 1st of August 2009. If a patient had died before 1 of August 2009, the date of death was recorded.

Malmö Preventive Project (MPP)

In the MPP study, ethical approval #85/2004 and #154/2004, 33,346 Swedish subjects in Malmö in southern Sweden participated in a health screening during 1974-1992. (7; 8) Men were included from 1974-1990 and women, from 1980-1992. Of those persons participating in the initial screening, 4,931 have died, and 551 are lost to follow-up. Twenty-five thousand eligible participants were invited to a re-screening visit during 2002-2006; this included a physical examination and fasting blood samples. Of those invited, 17,284 persons participated in the re-

screening; of these 1,223 were excluded because of incomplete medical information or lack of a DNA sample (or type 2 diabetes at baseline). Thereby, 16,061 non-diabetic subjects, 2,063 of whom developed T2D during follow-up, were available for the current analyses.(8) Diagnosis of diabetes was confirmed from patient records or based upon a fasting plasma glucose concentration ≥ 7.0 mmol/l (126 mg/dl). Blood pressure (mm Hg) was measured twice after 10 min rest in the supine position.(7) Stroke and myocardial infarction events were ascertained from the Swedish Hospital Discharge Register, and the Stroke Register of Malmö,(13) in which original medical records (including imaging studies, when available) were examined. Individuals that also participated in the MDC study were removed from the MPP analyses.

FinnDiane Study

The FinnDiane (Finnish Diabetic Nephropathy) study, ethical approval #491/E5/2006, is an ongoing nationwide prospective multicenter study with the aim to identify risk factors for T1D and its complications.(9) All adult patients with T1D from 21 university and central hospitals, 33 district hospitals, and 26 primary health care centers across Finland were invited to participate and the response rate exceeds 78%. T1D was defined as disease onset before the age of 35 years, insulin treatment initiated within 1 year of diagnosis, and serum C-peptide level below 0.3 nmol/l. Anthropometric measures as well as blood pressure were recorded, and blood and urine samples were collected. Information about concomitant disorders and medication was obtained using a standardized questionnaire, including cardiovascular disease as myocardial infarction and stroke (defined as a verified hemorrhagic or ischemic event. The presence of proliferative retinopathy was defined as a history of laser treatment.

Genotyping

Genotyping of rs10423928 was performed as previously described.(16) Briefly, matrix-assisted laser desorption ionization-time of flight mass spectrometry on the Sequenom MassARRAY

platform (San Diego, CA) was used for PPP-Botnia, METSIM and SDR studies, and an allelic discrimination method with a TaqMan assay on the ABI 7900 platform (Applied Biosystems, Foster City, CA, USA) was used for MPP, MDC, DGI, FinnDiane, Steno and incretin clamp studies. We obtained an average genotyping success rate of >95.5%, and the average concordance rate in all studies >99.9%. Hardy-Weinberg equilibrium was fulfilled in all studied populations ($p > 0.50$).

Cells, tissue and animals

Experiments involving animals were approved by the Animal Ethical Committee in Lund and Malmö (#M 29-12 and #M 915).

Cells: Primary human coronary artery smooth muscle cells (HCASMCs, Cascade Biologics), were cultured in M231 supplemented with either Smooth Muscle Growth Supplement (SMGS) or Smooth Muscle Differentiation Supplement (SMDS). Human microvascular endothelial cells (HMEC-1, CDC/Emory University) were cultured in M200 supplemented with endothelial cell growth supplement (ECGS). Vascular smooth muscle cells (VSMCs) from mouse aortic explants were isolated and cultured as previously described (17) and used between passages 3 and 12. Before stimulation with GIP or ET-1, cells were serum starved for 24h.

Arteries and mice: We used human myometrial resistance arteries from premenopausal women undergoing hysterectomy for non-oncologic reasons as previously described; ethical approval #LU 39-02.(17) Also, carotid arteries from male Wistar Kyoto rats (N= 2; Charles Rivers, France), coronary arteries from healthy domestic pigs (N=2) and mouse aortas and carotid arteries. Mice of the following strains were used: Naval Medical Research Institute (NMRI; Taconic, Europe), FVBN NFAT luciferase transgenic mice (NFAT-luc (17)) and diabetic Akita^{+/-} LDLr^{-/-} (B6.Cg-*Ins2*^{Akita}LDLr^{tm1Her}/J) and control LDLr^{-/-} littermates (Jackson Laboratories).

Critical limb ischemia: This clinical material is described in previous publications, ethical approval #LU 751-00.(18-20) Plasma from 85 (of 259) patients with confirmed diagnosis of critical limb ischemia and 101 (of 219) controls was available for analysis. A brief description of the material included is shown in Supplementary Table 1. Diagnosis was done in accordance with TASC scientific criteria (21) One-year mortality after admission was assessed from Swedish population registries.

Human carotid atherosclerotic plaques: One hundred and fifty human carotid plaques and corresponding plasma were collected at carotid endarterectomy and analyzed (ethical approval #472/2005 LUND). A brief description of the material is shown in Supplementary Table 2. The indications for surgery were plaques associated with ipsilateral symptoms (transient ischemic attacks - TIA, stroke or amaurosis fugax) and stenosis greater than 70%, or plaques not associated with symptoms and stenosis > 80%. Patients with atrial fibrillation, aortic valve disease, mechanical heart valves, chronic inflammatory disease, ipsilateral carotid artery occlusion or restenosis after previous carotid endarterectomy were excluded from this study. Cardiovascular risk factors, such as hypertension (systolic blood pressure > 140 mm Hg), diabetes, coronary artery disease, smoking (in the past or currently), fasting lipoproteins (total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides) were recorded, as well as, the use of medications (anti-hypertensive drugs, diabetes treatment, and statins). All patients were preoperatively assessed by a neurologist. After surgical removal, plaques were snap-frozen in liquid nitrogen at the operating room. Plaques were weighed; cross-sectional fragments of one-mm from the most stenotic region were taken for histology and adjacent fragments for RNA isolation. The remaining of the plaques were homogenized as previously described (22) for protein and cytokine analyses.

Incretin Clamp Study: Forty-seven healthy subjects were examined with hyperglycemic clamps with infusion of GIP; ethical approval #KF 01-016/0.(23) Hyperglycemic clamps (7 mmol/l; 2-hr) in conjunction with a primed continuous infusion of GIP were performed as previously described.(23) Glucose infusion was initiated at t:-30 min and terminated at t: 120 min. At t:-2

min, a bolus of GIP was infused to increase the plasma concentration to approximately 1000 pmol/l. At t=0 min, a continuous infusion of GIP (240 pmol/kg*h) was initiated and terminated at t=120. Plasma levels of OPN were analyzed in samples drawn at t=0 and t=105 min.

Confocal immunofluorescence

GIPR: For detection of GIPR protein in native arteries we used 10 µm sections of human myometrial resistance arteries, rat carotid arteries, pig coronary arteries and aortas from NMRI mice. For detection of GIPR protein in cells we used VSMCs, HCASMCs and HMEC-1. Arteries and cells were stained as previously described.(17; 24) Two different primary antibodies were used, rabbit polyclonal anti-GIPR (1:1600; extensively described in (25)) detecting the N-terminal part of GIPR, and rabbit polyclonal anti-GIPR (1:300, H-70, Santa Cruz Biotechnology) detecting an internal region of the protein. Both primary antibodies were used with secondary Cy5-anti-rabbit IgG (1:500, Jackson ImmunoResearch).

OPN: Aortas from NMRI (N=11) mice were dissected free of surrounding tissue in Ca²⁺-free ice-cold physiological saline solution and divided into 7-8 mm long pieces before placed in organ culture in DMEM medium (Biochrom AG, Berlin, Germany) supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin and 5 mmol/L glucose. Vessels were cultured for 2 or 3 days and under various stimulatory conditions, as specified in the text. GIP was added fresh every 24h. After culture, arteries were fixed in 4% formaldehyde, sectioned and stained with a primary rabbit anti-mouse OPN antibody (1:500, IBL Hamburg, Germany) and a secondary Cy5-anti-rabbit IgG (1:400 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA) as previously described.

vWF and α-SMA: Sections from mouse aorta were stained with von Willebrand factor (vWF; 1:400, Dako) and α-smooth muscle actin (α-SMA; 1:400, Sigma Aldrich) to detect endothelial and smooth muscle cells, respectively.

The fluorescent nucleic acid dye SYTOX Green (1:3000, Molecular Probes, Invitrogen, Paisley, UK) was used for nuclear identification. Sections and cells were examined by monitoring Cy5 and green fluorescence in a Zeiss LSM 5 Pascal laser scanning confocal microscope. Multiple fields for each vessel or cover slip were imaged and analyzed under blind conditions. For quantification of OPN expression, three to five boxes were randomly positioned within the vessel media layer and mean pixel intensity (range 0 to 255 grayscale values) after background subtraction was calculated using the Zeiss LSM 5 Pascal Analysis software. Specificity of immune staining was confirmed by the absence of staining when primary or secondary antibodies were omitted from the protocol.

Western blotting

OPN: Western blotting for OPN in arteries was performed as previously described (26). Briefly, intact aortas (ascending and descending parts) from NMRI mice were divided into four pieces and cultured DMEM medium (Biochrom AG, Berlin, Germany) supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin and 5 mmol/l glucose for up to 3 days with various treatments as specified in the text. GIP was added fresh every 24h. Serum starved cells (ECs and VSMCs) were stimulated as indicated in the text before protein was harvested. Extracted protein was loaded onto 12.5% Tris-HCl gels (Bio-Rad Laboratories, Sundbyberg, Sweden), separated by gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories). Primary antibodies against mouse (1:500 dilution in 3% BSA, IBL, Hamburg, Germany) and human OPN (1:1000, MP11B10 monoclonal antibody developed by Solursh and Franzén, obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242) and HRP-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA), were used and bands detected with chemiluminescence (Supersignal West Dura, Pierce Biotechnology, Rockford, IL). Western blotting of β -actin (1:3000 dilution, GenScript

Corporation, Piscataway, NJ) was used as a loading control and the intensity of bands was quantified using the Quantity One Analysis software version 4.6.5 (Bio-Rad Laboratories).

GIPR: Expression of GIPR was analyzed in cells and in pancreas homogenate from GIPR competent and GIPR KO mice, kindly provided by Drs. D. Drucker and Y. Seino, using a rabbit polyclonal anti-GIPR as described (1:500 (9)).

Phosphorylated and total CREB: Serum starved cells were stimulated with GIP or ET-1 as indicated in the text and protein extracted, separated by gel electrophoresis as described above, and transferred to nitrocellulose membrane. Primary antibodies detecting active, phosphorylated CREB (p-CREB) and total CREB (#9198 and #9197, Cell Signaling Technology) were used at 1:1000 dilution with secondary antibodies and development as described above.

Luciferase reporter assay

Phenotypically normal NFAT-luc transgenic mice were used. These mice express nine copies of an NFAT binding site from the interleukin-4 promoter, positioned 5' to a minimal promoter from the α -myosin heavy chain gene (-164 to +16) and inserted upstream of a luciferase reporter gene. Aortas were stimulated *ex vivo* with various concentrations of GIP before measuring luciferase activity as previously described.(27) Optical density was measured using a Tecan Infinite M200 instrument (Tecan Nordic AB, Mölndal, Sweden) and normalized to protein content measured with the EZQ protein quantitation kit (Molecular Probes, Invitrogen, Paisley, UK).

LDH activity

Cell death was measured by quantification of lactate dehydrogenase (LDH) activity in the culture medium after treatment of cells as indicated in the text, using a Cytotoxicity Detection Kit (Roche Applied Science) according to the manufacturer's instructions.

ELISA assays

The levels of OPN in plasma were assayed using Quantikine human OPN ELISA kit (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. Absorbance was measured at 450 nm and the lower limit of detection for the assay is 11 pg/ml. The level of ET-1 in plasma and culture medium was determined using Human Endothelin-1 Immunoassay (R&D Systems, Abingdon, UK) according to the manufacturer's instructions, with a lower limit of detection of 0.25 pg/ml. GIP levels were measured in human serum from patients with critical limb ischemia and from individuals in PPP-Botnia (see below for a description of this cohort) using Human GIP (Total) ELISA Kit (Millipore, St. Charles, Missouri) according to the manufacturer's instructions. The lower limit of detection for the assay is 10.1 pg/ml. Direct cAMP ELISA (Enzo Life Sciences) was used to measure cAMP levels in cells, lysed in 0.1 N HCl containing 0.1% Triton X-100 and 100 μ mol/l 3-Isobutyl-1-methylxanthine (IBMX).

Proliferation

For proliferation studies, cells were incubated for 48h with or without growth supplement and DNA synthesis was measured by thymidine incorporation. Cells were pulsed with 1 mCi [methyl-³H]thymidine (Amersham Biosciences, Uppsala, Sweden); during the last 24 hours of stimulation, macromolecular material was harvested on glass fiber filters using a Filter Mate Harvester (Perkin Elmer, Buckinghamshire, UK) and analyzed using a liquid scintillation counter (Wallac 1450 MicroBeta, Ramsey, MN).

Cytokine assessment

Cytokine levels were measured in aliquots of 50 μ l human carotid plaque homogenates and plasma. Plaque homogenates were centrifuged at 14,000 rpm for 10 min and 25 μ l of the supernatant was used for measuring the following cytokines: fractalkine, interferon- γ (IFN- γ), interleukin (IL)-10, IL-12(p70), IL-12(p40), IL-1 β , IL-6, monocyte chemoattractant protein-1 (MCP-1), macrophage

inflammatory protein-1beta (MIP-1 β), platelet derived growth factor-AB/BB (PDGF-AB/BB), regulated on activation normal T cell expressed and secreted (RANTES), vascular endothelial growth factor (VEGF), sCD40L and tumor necrosis factor-alpha (TNF- α). The procedure was performed according to the manufacturer's instructions (Milliplex Kit - Human Cytokine/Chemokine Immunoassay - Cat. No.: MPXHCYTO-60K-15 Kit Lots #1691735 and 1702424, Millipore, Electrabox Diagnostica AB, USA) and analyzed with Luminex 100 IS 2.3 (Austin, Texas).

Plaque immunohistochemistry

Sections of the one-mm-thick fragment of carotid atherosclerotic plaques were thawed, fixed with ice-cold acetone and permeabilized in 0.5% Triton-X100 before blocking in 10% serum. Primary antibodies for GIPR (1:800 dilution (25)), OPN (1:100 dilution, MPIIIB10, CD68 (1:100 dilution, DakoCytomation, Glostrup, Denmark) and α -actin (1:50 dilution, M0851, DakoCytomation, Glostrup, Denmark) were used together with biotinylated secondary goat anti-rabbit (1:200 dilution, Vector Laboratories, Burlingame, CA) for GIPR and rabbit anti-mouse (1:200 dilution, DakoCytomation, Glostrup, Denmark) for OPN, CD68 and α -actin. The DAB detection kit was used for color development (Vector Laboratories). For detection of lipids, sections were fixed with Histochoice (Amresco, Ohio, USA), dipped in 60% isopropanol and then in 0.4% Oil Red O in 60% isopropanol (for 20 min). Masson's trichrome using Ponceau-acid fuchsin (Chroma-Gesellschaft, Schmidt GmbH, Germany) and aniline blue (BDH, Dorset, England) was used to assess plaque collagen content. To assess the calcified areas, the area of the holes where calcium had been present was measured. Mounted slides were scanned with ScanScope Console Version 8.2 (LRI imaging AB, Vista California, USA) and photographed with Aperio image scope v.8.0 (Aperio, Vista, California, USA). The area of the plaque (% area) constituted by the different components was quantified blindly using Biopix iQ 2.1.8 (Gothenburg, Sweden).

RNA isolation, cDNA synthesis and quantitative PCR

Total RNA was isolated from cultured cells using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions, and from mouse arteries and human plaque biopsies using TriReagent as previously described.(28; 29) Concentration, purity and degradation of RNA samples was evaluated using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and Experion DNA 1K gel chips (Bio-Rad, Hercules, CA, USA). Reverse transcription was performed with RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences). GIPR and OPN mRNA levels were quantified by real-time PCR using TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer's protocols. For human tissue, assays ID were Hs00164732_m1 for *GIPR* and Hs00959010_m1 for *OPN* (*SPPI*); for mouse tissue: Mm01316351_g1 and Mm01316349_g1 for *GIPR* and Mm00436767_m1 for *OPN*. Target gene expression was normalized to the expression of the following housekeeping genes: human *HPRT1* (HPRT, article # 4326321E), human *PPIA* (cyclophilin A, article # 4326316E) and human *POLR2A* (polymerase (RNA) II (DNA directed) polypeptide A, 220kDa, assay ID Hs00172187_m1); mouse *PPIb* (cyclophilin B, assay ID Mm00478295_m1), mouse *GAPDH* (article # 4352339E) and eukaryotic 18S rRNA (assay ID Hs99999901_s1). The PCR reactions were run on an ABI 7900 HT instrument (Applied Biosystems), all assays in triplicates. Relative quantity of *GIPR* and *OPN* mRNA was calculated using the comparative threshold method (Ct-method) and normalizing was done using Genorm v.3.5 software.

Chemicals

A-285222(17; 30; 31) was provided by Abbott Laboratories. GIP was obtained from Bachem, Germany; all other chemicals were from Sigma Aldrich.

Statistics

For in vitro studies, results are expressed as means \pm SEM, where applicable and GraphPad (Prism 5.0) was used for the statistical analyses. Statistical significance was determined using two-tailed Student's t-test, or one-way analysis of variance followed by Bonferroni test for normally distributed variables. Mann-Whitney or Kruskal-Wallis tests were used to determine statistical difference between two or more groups, respectively, for variables that were not normally distributed. SPSS version 17.0 was used to analyze non-parametric, bivariate correlations. Associations between genotype and cardiovascular disease outcomes were investigated using logistic regressions. In the T1D studies, age at onset, diabetes duration and gender were used as covariates while the T2D analyses were adjusted for age, gender and BMI. Age was not available in the SDR and instead age at onset and diabetes duration was used. Only individuals without anti-hypertensive treatment were included in linear regression analyses of associations between genotype and blood pressure variables. Treatment information was not available for the SDR, so all individuals were included from that cohort. Fixed effect meta-analyses were performed with the metan command in STATA. Non-normally distributed variables were logarithmically transformed before analysis. Analyses were performed using SPSS version 17.0 and STATA/SE version 12.1. **P < 0.05, **P < 0.01 and ***P < 0.001.*

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