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Functional characterization of the *GUCY1A3* coronary artery disease risk locus

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Abstract

Background—A chromosomal locus at 4q32.1 has been genome-wide significantly associated with coronary artery disease risk. The locus encompasses *GUCY1A3*, which encodes the α_1-subunit of the soluble guanylyl cyclase (sGC), a key enzyme in the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) signaling pathway. The mechanism linking common variants in this region with coronary risk is not known.

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Disclosures

The authors declare no conflict of interest.

Methods—Gene and protein expression were analyzed using quantitative polymerase chain reaction (qPCR) and immunoblotting, respectively. Putative allele-specific transcription factors were identified using *in silico* analyses and validated via allele-specific quantification of antibody-precipitated chromatin fractions. Regulatory properties of the lead risk variant region were analyzed using reporter gene assays. To assess the effect of ZEB1, siRNA-mediated knockdown as well as overexpression experiments were performed. Association of *GUCY1A3* genotype and cellular phenotypes were analyzed using vascular smooth muscle cell (VSMC) migration assays and platelet aggregation analyses.

Results—Whole blood *GUCY1A3* mRNA levels were significantly lower in individuals homozygous for the lead (rs7692387) risk variant. Likewise, reporter gene assays demonstrated significantly lower *GUCY1A3* promoter activity for constructs carrying this allele. *In silico* analyses located a DNase I hypersensitivity site to rs7692387 and predicted binding of the transcription factor ZEB1 rather to the non-risk allele, which was confirmed experimentally. Knockdown of *ZEB1* resulted in more profound reduction of non-risk allele promoter activity, as well as a significant reduction of endogenous *GUCY1A3* expression. *Ex vivo* studied platelets from homozygous non-risk allele carriers displayed enhanced inhibition of adenosine diphosphate-induced platelet aggregation by the NO donor sodium nitroprusside and the phosphodiesterase 5 inhibitor sildenafil as compared to homozygous risk allele carriers. Moreover, pharmacologic stimulation of sGC led to reduced migration only in VSMC homozygous for the non-risk allele. In the Hybrid Mouse Diversity Panel higher levels of *GUCY1A3* expression correlated with less atherosclerosis in the aorta.

Conclusions—Rs7692387 is located in an intronic site that modulates *GUCY1A3* promoter activity. The transcription factor ZEB1 binds preferentially to the non-risk allele leading to an increase in *GUCY1A3* expression, higher sGC levels, and higher sGC activity after stimulation. Finally, human and mouse data link augmented sGC expression to lower risk of atherosclerosis.

Keywords

coronary artery disease; genetics; human; nitric oxide; myocardial infarction; genotype; soluble guanylyl cyclase; GWAS

Introduction

An inherited component of the risk for developing atherosclerosis has been known for a long time. However, the underlying genetic mechanisms and causative genes are largely unknown. Genome-wide association studies (GWAS) identified 56 genomic loci associated with coronary artery disease (CAD) and myocardial infarction (MI) risk[1–5]. One of these is the *GUCY1A3* locus[3]. Interestingly, only a few CAD risk loci, like those harboring *LDLR* and *PCSK9*[6–9], contain mutations which were also found to co-segregate with CAD in a Mendelian pattern of inheritance. We have recently observed such allelic series at the *GUCY1A3* CAD risk locus, for which a heterozygous loss-of-function allele was identified by linkage and a common variant by GWAS analysis to affect CAD risk, respectively[3,10].

The *GUCY1A3* gene encodes the α_1-subunit of the soluble guanylyl cyclase (sGC). The sGC complex, a heterodimer of the α_1- and a β_1-subunit, acts as the receptor for nitric oxide

(NO) and catalyzes the formation of the second messenger cyclic guanosine monophosphate (cGMP). cGMP has several cellular functions including the inhibition of platelet aggregation and smooth muscle cell relaxation[11]. Further genetic evidence pointing to critical involvement of the NO–sGC–cGMP pathway in mediating CAD and MI risk has been shown by a recent GWAS meta-analysis which identified the *NOS3* gene, encoding the endothelial NO synthase, as a further CAD risk gene[4]. Whereas the variant at this locus has already been implicated to influence *NOS3* expression[12], the molecular mechanisms affected by common variants at the *GUCY1A3* locus remain elusive.

Here we provide functional evidence that the lead single nucleotide polymorphism (SNP) at the chromosome 4q32.1 risk locus affects *GUCY1A3* gene expression and give insights into the molecular disease mechanisms at a genetic and cellular level.

Methods

In silico analyses

SNPs in high linkage disequilibrium with the *GUCY1A3* lead SNP were identified using SNP Annotation and Proxy Search[13]. The respective regions were analyzed regarding potential transcription factor binding sites using UCSC Genome Browser annotations[14]. Prediction of potential allele-specific binding of transcription factors was performed using AliBaba2.1 (http://www.gene-regulation.com/pub/databases.html)[15].

Human samples and cell lines

Human samples—Studies in samples from human individuals were performed after obtaining written informed consent. The ethics committee of the Technical University of Munich approved the protocol. All studies in human samples were in concordance with the Declaration of Helsinki. We included individuals that were treated at the German Heart Centre Munich. Whole blood RNA was available in four homozygous non-risk and risk allele carriers each. Platelet aggregation was studied in eight and 14 homozygous non-risk and risk allele carriers, respectively, not taking concurrent medication and displaying platelet counts in the normal range (150,000 – 400,000/μl).

Cell lines—Human embryonic kidney (HEK) 293 cells, megakaryoblasts (both Sigma-Aldrich, St. Louis, MO, USA), and human aortic endothelial cells (PromoCell, Heidelberg, Germany) are commercially available. Human aortic smooth muscle cells (SMC) were either commercially purchased (PromoCell, Heidelberg, Germany) or part of the Systems Genetics Resource at the University of California, Los Angeles[16] (https://systems.genetics.ucla.edu/).

Isolation of nucleic acids and genotyping

DNA from human samples was isolated from whole blood using the Gentra Puregene Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Whole blood RNA was isolated from PAXgene Blood RNA Tubes (PreAnalytiX, Hombrechtikon, Switzerland) using the PAXgene Blood RNA Kit (Qiagen, Hilden, Germany) as recommended by the suppliers. Genotyping was performed using a rs7692387 TaqMan ® Genotyping Assay

(C__29125113_10; Life Technologies, Carlsbad, CA, USA) on a ViiA7 qPCR instrument (Life Technologies, Carlsbad, CA, USA).

Murine *Gucy1a3* expression analysis and correlation with plaque formation

The Hybrid Mouse Diversity Panel has been described previously[17]. Briefly, *Gucy1a3* genotype (rs30360584) was correlated with both aortic atherosclerotic lesion area as well as expression of the gene in heart tissue and aorta.

Expression analysis of transcription factors

Expression of relevant transcription factors in distinct relevant tissues, i.e., whole blood, mammary artery, and atherosclerotic arterial wall, was analyzed in data from the Stockholm Atherosclerosis Gene Expression (STAGE) study[18]. Expression in cell lines, i.e., HEK 293 cells, human aortic SMC, human endothelial cells, and megakaryoblasts, was analyzed using reverse transcription (RT) polymerase chain reaction (PCR). Bands were visualized on agarose gels.

Reporter gene constructs and assays

To study the regulatory role of the *GUCY1A3* lead SNP (rs7692387) region, the *GUCY1A3* promoter (2,578 bp; chr4:155,666,380-155,668,957 (GRCh38/hg38)) and a 407 bp fragment flanking the lead SNP (rs7692387; chr4:155,713,878-155,714,284) were amplified and cloned into pGL4.10[luc2] reporter gene plasmids (Promega Corporation, Madison, WI, USA) upstream and downstream of the *luc2* gene, respectively. Reporter gene assays were performed in HEK 293 cells according to the manufacturer's recommendations using appropriate *Renilla* luciferase expression vectors as transfection controls. The full length open reading frame of ZEB1 was amplified by Prime star GXL proof reading polymerase (TaKaRa, Saint-Germain-en-Laye, France) and inserted into the 3xFLAG expression plasmid to obtain a ZEB1-3xFLAG fusion construct. Primers used for amplification are listed in Supplemental Table 1. HEK 293 cells were seeded at 250,000 cells per well in 24-well plates and grown to confluence for 24 h. Cells were co-transfected with 500 ng pGL4.10 constructs and 50 ng pRL-TK reporter vector using FuGENE HD Transfection Reagent (Promega Corporation, Madison, WI, USA) in a concentration of 4:1. To investigate the effect of ectopically expressed ZEB1 100 ng of the ZEB1 expression plasmid were added to each well. After 48 h incubation cells were harvested and subsequently processed with the Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) according to the manufacturer's recommendations. Signals for *Firefly* and *Renilla* luciferase were measured on the Infinite® M200 PRO reader (Tecan Group, Männedorf, Switzerland). Luciferase activities were normalized to *Renilla* signals and expressed as fold of the non-risk variant.

Allele-specific chromatin immunoprecipitation (ChIP)

ChIP assays were performed with OneDay ChIP kit (Diagenode, Liège, Belgium) according to the manufacturer´s protocol. Briefly, human aortic SMC were cross-linked by adding formaldehyde to a 1% final concentration. After nuclei extraction chromatin was sonicated using Bioruptor pico (Diagenode, Liège, Belgium). Chromatin fragments were incubated

with anti-ZEB1 antibody (Diagenode, Liège, Belgium) before agarose beads were added. Precipitated chromatin was purified by DNA-binding slurry and then treated with proteinase K. PCR-grade water was used to elute DNA from slurry. ZEB1-binding to the regulatory element including rs7692387 was verified by specific PCR-based amplification of the 407 bp regulatory element (chr4:155,713,878-155,714,284). SNaPshot multiplex PCR (Thermo Fisher Scientific, Carlsbad, CA, USA) using four different oligo probes (F1-GGCCAAGGGCAGAGACATTT; F2-CAGCTTGGCCAAGGGCAGAGACATTT; R1-GATTTGAAGATAGGTCTTTCTTTTT; R2-CAGAGAACAAGATTTGAAGATAGGTCTTT CTTTTT) was performed to quantify allele-specific binding of ZEB1.

ZEB1 knockdown by RNA interference (RNAi)

HEK 293 cells were seeded at a density of 250,000 cells per well in 24-well plates and grown to confluence; human aortic SMC were seeded in 24-well plates at a density of 100,000 cells per well. Lipofectamine RNAiMAX Transfection Reagent (Life Technologies, Carlsbad, CA, USA) was used for transfection according to the manufacturer's recommendations. Per well, 5 pmol of $siRNA_{ZEB1}$ (s229972; Life Technologies, Carlsbad, CA, USA) were incubated with Lipofectamine RNAiMAX Transfection Reagent and Opti-MEM I Reduced Serum Medium (Gibco by Life Technologies, Carlsbad, CA, USA) for 5 minutes at room temperature. Afterwards, siRNA-RNAiMAX complexes were added to the cells and incubated for 24 h. In parallel, cells were transfected with 5 pmol of $siRNA_{scramble}$ as control (Silencer® Select Negative Control No. 1 siRNA; Life Technologies, Carlsbad, CA, USA). Cells were harvested after 24/48/72 h and forwarded to quantitative real-time polymerase chain reaction (qPCR) analysis (see below). For reporter gene assays after siRNA knockdown of *ZEB1*, HEK 293 cells were seeded at 250,000 cells per well in 24-well plates and grown to confluence for 24 h. Cells were co-transfected with 500 ng pGL4.10 constructs and 50 ng pRL-TK reporter vector using FuGENE HD Transfection Reagent (Promega Corporation, Madison, WI, USA) in a concentration of 4:1. After six hours, cells were transfected with 5 pmol of $siRNA_{ZEB1}$ or $siRNA_{scramble}$. After 48 h, cells were harvested and luciferase activity was measured as described above.

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was either isolated from whole blood as described above or extracted from cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA synthesis was performed using the ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis Kit (Life Technologies, Carlsbad, CA, USA) with a mixture of oligo(dT) and random hexamer primers. Expression levels were either determined using appropriate TaqMan Gene Expression probes or detected using PerfeCTa SYBR Green FastMix Low ROX (Quanta Biosciences, Gaithersburg, MD, USA) with primers and probes listed in Supplemental Table 2. *MAPK1* or *RPLP0* served as housekeeping genes. Experiments were performed on the ViiA7 qPCR instrument (Life Technologies, Carlsbad, CA, USA). For RNAi experiments, $2^{-\Delta\Delta Ct}$ values were analyzed using one sample t-test with the reference value of 1 for control siRNA treatment and visualized. For comparison of gene expression in different individuals, ΔCt values were analyzed using Student's unpaired t-test[19] and plotted as $2^{-\Delta Ct}$ values.

Western Blot analysis

Cells were washed with Dulbecco's phosphate buffered saline (PBS; Biochrom, Berlin, Germany), resuspended in 100 µl radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, Denvers, MA, USA), and disrupted by sonication. After centrifugation for 30 min at 20,000 g and 4°C, cell lysate was separated from cell debris. Samples were mixed 1:1 with 2× Laemmli buffer (Sigma-Aldrich, St. Louis, MO, USA). Cell lysate and platelet clot samples were denaturated for 5 min at 95 °C and subjected to Western Blotting using a vertical mini gel electrophoresis (Mini-PROTEAN Tetra Cell) system and a high-performance western blotting transfer (Trans-Blot® Turbo) system (both Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's recommendations. After blotting, polyvinylidene fluoride transfer membranes (Immobilon-P, Merck Millipore, Darmstadt, Germany) were incubated with primary antibodies specifically detecting ZEB1 (D80D3, New England Biolabs, Ipswich, MA, USA), the α_1-subunit of the sGC (#NBP2-13000, Novus Biologicals, Minneapolis, MN, USA), or vasodilator-stimulated phosphoprotein (VASP) phosphorylated at $Serin_{239}$ (#3114, Cell Signalling Technology, Cambridge, UK). Detection of glyceraldehyde 3-phosphate dehydrogenase (GAPDH; D16H11; New England Biolabs, Ipswich, MA, USA) served as loading control. After incubation with appropriate secondary antibodies, detection was performed with a biomolecular imager (ImageQuant LAS 4000; GE Healthcare Life Sciences, Pittsburgh, PA, USA) using a luminol-based enhanced chemiluminescence horseradish peroxidase substrate (Thermo Fisher Scientific SuperSignal West Dura Extended Duration Substrate; Life Technologies, Carlsbad, CA, USA). Signal intensities were detected using image analysis software (ImageQuant TL; GE Healthcare Life Sciences, Pittsburgh, PA, USA) according to the manufacturer's recommendations. Signal intensities of the target proteins were normalized to signal intensities for GAPDH in the same samples.

Aortic SMC scratch wound assay

Migration was assessed using an *in vitro* scratch injury model. Human aortic SMC derived from the Systems Genetics Resource at the University of California[16] were seeded in 24-well plates (50,000 cells per well) coated with 0.1 % gelatin and grown to confluence for 24 h. The cell monolayer was scratched in the middle with a p200 pipet tip (Gilson, Middleton, WI, USA). Cells were then washed with PBS and incubated in SmBM Basal Medium supplemented with SmGM-2 SingleQuot Kit Suppl. & Growth Factors (Lonza, Basel, Switzerland). Wound area was measured directly after scratch (t0) and after 5 h (t1) using a phase-contrast microscope (EVOS FL Auto Cell Imaging System, Thermo Fisher Scientific, Waltham, MA, USA) and assessed using ImageJ[20]. Wound reclosure was measured as Δt0-t1 under vehicle (DMSO) and BAY 41–2272 (10 µM; Cayman Chemical Company, Ann Arbor, MI, USA) treatment for each genotype. Measurements were performed in triplicates in five independent experiments using passages P4-P8 of the respective cell lines.

Platelet response to nitric oxide

Preparation of platelet rich plasma—Whole blood thrombocyte count was analyzed in EDTA blood samples using an automated hematology analyzer (Sysmex Corporation, Köbe, Japan). All samples displayed thrombocyte counts in the normal range (150,000 – 400,000/

µl). Platelet rich plasma (PRP) was isolated after centrifugation of citrate blood for 10 min at 80 g and room temperature without active deceleration. The supernatant containing PRP was transferred to fresh tubes; platelet count in PRP was measured to exclude significant deviations between samples. Platelet poor plasma (PPP) was isolated after centrifugation of the precipitate for 10 min at 3000 g and room temperature. The supernatant containing PPP was transferred to fresh tubes.

Platelet aggregation analysis—Platelet aggregation analysis was performed according to the turbidimetric method of Born using a PC-controlled platelet aggregation profiler (PAP-8; Biodata Corp, Horsham, PA, USA) as described previously[21]. Sodium nitroprusside (Carl Roth GmbH, Karlsruhe, Germany) and sildenafil (Sigma-Aldrich, St. Louis, MO, USA) were solved in dimethyl sulfoxide (DMSO). PPP of the respective proband was used for blanking of the measurement channels. Prior to induction of platelet aggregation, PRP samples were incubated with sodium nitroprusside (final concentration 10 µM), or sodium nitroprusside (final concentration 10 µM) + sildenafil (final concentration 10 µM) for 2 min at 37 °C. PRP incubated with H_2O served as control and baseline measurement. All samples including the H_2O control contained similar amounts of DMSO (final concentration 1:500). Adenosine diphosphate (ADP; möLab GmbH, Langenfeld, Germany) at a final concentration of 2 µM was used to induce platelet aggregation. Platelet aggregation was recorded for 5 min after addition of ADP. Area under the curve (AUC) of platelet aggregation was the primary endpoint and displayed as arbitrary units (AU) × min.

Analysis of VASP phosphorylation—Detection of phosphorylated VASP was performed as described previously[21,22]. Briefly, after termination of platelet aggregation measurements, PRP samples were transferred to 1.5 ml tubes (Eppendorf AG, Hamburg, Germany) and centrifuged for 10 min at 20,000 g and 4 °C. Supernatant was discarded and 25 µl of 2× Laemmli buffer (Bio-Rad Laboratories, Hercules, CA, USA) as well as 25 µl RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) were added to the platelet pellet and the samples were forwarded to immunoblotting.

Statistical analysis

Distribution of data was assessed using the D'Agostino-Pearson omnibus normality test. Unless otherwise stated, normally distributed data was analyzed using Student's unpaired/paired t-test. Not normally distributed data was analyzed with Mann-Whitney-test. For comparison of more than two groups, ordinary one-way ANOVA with Tukey's multiple comparison test was used unless otherwise stated. Categorical data was analyzed using Fisher's exact test. P-values < 0.05 were regarded significant. GraphPad Prism version 7.0a for Mac OS × (GraphPad Software, La Jolla, CA, USA) was used.

Results

Decreased *GUCY1A3* expression associates with atherosclerosis

Genotype-dependent *GUCY1A3* expression was assessed in RNA extracts of whole blood samples from eight individuals. Homozygous risk allele carriers displayed significantly lower *GUCY1A3* mRNA levels compared to homozygous non-risk allele carriers (Figure

1A). To investigate whether these findings are conserved among different species, we identified a SNP affecting *Gucy1a3* mRNA expression in mice in the Hybrid Mouse Diversity Panel (rs30360584)[17]. As seen in human samples, the allele related to lower expression levels of *Gucy1a3* in mice was associated with increased atherosclerotic disease burden (Supplemental Figure 1).

In-silico predictions on the human *GUCY1A3* risk variant

The lead SNP of the human CAD-GWAS meta-analysis, rs7692387, is located within intron eight of the *GUCY1A3* gene (NG_034128.1) on chromosome 4. We assessed properties of all variants in high linkage disequilibrium (LD; $r^2>0.8$) with the lead SNP variant (Supplemental Table 3) using UCSC Genome Browser annotations[23]. None of these variants was located in the coding region. Only the lead variant was found to be located at a DNase I hypersensitivity site (chr4:156635141-156635310 (HG19), Supplemental Figure 2) indicative for regulatory relevance[24]. Next, we screened *in silico* for putative transcription factor binding sites affected by rs7692387. In addition to the rather unspecific C/EBP transcription factor family, IRF8 and ZEB1 were predicted to exhibit allele-specific binding. Whereas IRF8 was predicted to bind only to the G-allele, ZEB1 was predicted to bind to the A- (non-risk) allele (Table 1). We then investigated the expression of *IRF8* and *ZEB1* in different cell lines *in vitro* as well as relevant tissues in the Stockholm Atherosclerosis Gene Expression (STAGE) study[18]. *ZEB1* was abundantly expressed in HEK 293 cells, human vascular smooth muscle cells (SMC), human endothelial cells, and megakaryoblasts (Meg-01; Supplemental Figure 3), but also in whole blood, mammary artery, and atherosclerotic arterial wall, i.e., tissue relevant for sGC function (Table 1). By contrast, *IRF8* was not expressed in vascular SMC and megakaryoblasts. Publicly available genome-wide ChIP sequencing data generated on liver hepatocellular carcinoma (HepG) cells[25] revealed binding of the transcription factor ZEB1 within the *GUCY1A3* intronic region directly adjacent to rs7692387 (Supplemental Figure 4A). We conducted ChIP experiments using formaldehyde-crosslinked chromatin of primary fibroblasts to confirm specific binding of ZEB1 to this genomic region. Therefore, a ChIP-grade anti-ZEB1 antibody was used for precipitation followed by PCR amplification of a 407 bp fragment including the SNP rs7692387 (Supplemental Figure 4B). Thus, in consecutive experiments we focused on the influence of ZEB1 on *GUCY1A3* expression.

ZEB1 modulates *GUCY1A3* expression

To assess regulatory properties of the *GUCY1A3* lead SNP region, we performed reporter gene assays using constructs carrying the *GUCY1A3* promoter as well as the nucleotide sequence containing the non-risk or risk variant of rs7692387 (Figure 1B). Whereas the *GUCY1A3* non-risk allele construct (P+A) led to a 25% increase of luciferase activity (P=0.0001), the risk allele construct (P+G) displayed a 20% reduction of luciferase activity compared to the construct only carrying the promoter (P; P<0.0005) and a 44% reduction of luciferase activity compared to the non-risk allele construct (P<0.0001; Figure 1C). As outlined above, *in-silico* analysis predicted ZEB1-binding within the regulatory region whose binding-affinity might be directly correlated with rs7692387 genotype. To address this, we performed allele-specific quantification of ZEB1-precipitated chromatin fractions using primer extension-based SNaPshot multiplex PCR in heterozygous VSMC. While the

risk G-allele as well as the non-risk A-allele were detected in both genomic DNA and chromatin fractions used for ChIP assays, ZEB1-precipitated chromatin fraction only shows the protective A-allele indicating exclusive ZEB1-binding to the A-allele. (Figure 1D). We further investigated the effect of *ZEB1* knockdown on reporter gene expression. Using a specific RNA interference approach, we observed reduced *ZEB1* mRNA levels by 46% (P=0.01) in HEK 293 cells and 80% (P=0.01) human aortic SMC after 72 h (Figure 2A–B; Supplemental Figures 5, 6) as well as reduced protein levels. Knockdown of *ZEB1* led to a reduction in luciferase expression of rather the non-risk (P+A) construct (15%; P<0.01; Figure 2C) than the risk (P+G) construct (8%; P=0.05; Figure 2D). In line, overexpression of ZEB1 resulted in an additional increase of reporter gene expression (Figure 2E, F). ZEB1-mediated activation was stronger for the construct containing the non-risk variant (P+A, 65%, P<0.01; **E**) compared to the risk allele variant construct (P+G, 28%, P=0.0504; **F**; Supplemental Figure 7). To further confirm these observations, we investigated endogenous *GUCY1A3* mRNA levels in HEK 293 cells that were homozygous for the non-risk variant (AA-genotype). Knockdown of *ZEB1* by siRNA led to a 25% decrease in *GUCY1A3* expression after 72 h (P<0.01; Figure 2G). As *GUCY1A3* is endogenously expressed in vascular SMC, we also investigated *GUCY1A3* expression in human aortic SMC found to be heterozygous for rs7692387. Here, silencing of *ZEB1* by RNAi also led to a significant reduction in *GUCY1A3* expression (16%; P<0.01; Figure 2H).

GUCY1A3 genotype affects human vascular SMC migration after sGC stimulation

To test whether the *GUCY1A3* genotype influences vascular SMC migration, we performed a scratch wound assay in cells derived from a homozygous risk allele carrier and cells derived from a homozygous non-risk allele carrier from the Systems Genetics Resource at UCLA[16]. The cell line homozygous for the risk allele (GG) displayed significantly reduced *GUCY1A3* mRNA levels compared to a cell line derived from a homozygous non-risk allele carrier (AA; Figure 3A). We determined reclosure areas of the scratch wounds under vehicle treatment for the two cell lines and compared these to reclosure areas after treatment with the specific sGC stimulator BAY 41–2272. In the homozygous non-risk allele donor cell line, significant reduction in scratch wound reclosure was detected (P<0.01; Figure 3B–C) whereas BAY 41–2272 had no effect on migration in the cell line derived from the homozygous risk allele carrier (Figure 3D, E). VSMC from a heterozygous individual displayed an intermediate effect (Supplemental Figure 8).

GUCY1A3 genotype affects NO-mediated inhibition of platelet function

Homozygous risk allele carriers displayed significantly reduced platelet α_1-sGC protein levels compared to homozygous non-risk allele carriers (Figure 4). In order to evaluate the functional implications of reduced α_1-sGC protein levels we went on to analyze the enzymatic activity of sGC. Thus, we studied the production of its second messenger cGMP, which is known to inhibit platelet aggregation in platelet rich plasma samples of homozygous non-risk allele carriers (n=8) and homozygous risk allele carriers (n=14). Homozygous non-risk allele carriers displayed slightly increased platelet counts (Supplemental Table 4). Adenosine diphosphate (ADP)-induced platelet aggregation was similar in homozygous risk allele and homozygous non-risk allele carriers (Figure 5A). By contrast, addition of sodium nitroprusside as NO donor led to stronger reduction of platelet

aggregation in non-risk compared to risk allele carriers (P<0.001; Figure 5B). To confirm whether the effect was mediated via an accumulation of cGMP, we inhibited cGMP degradation by the additional supplementation of the phosphodiesterase 5 (PDE5) inhibitor sildenafil. The addition of sildenafil led to a further reduction of platelet aggregation in both genotypes with a stronger effect in homozygous non-risk allele carriers (P<0.0001; Figure 5C). NO application to platelets of individuals representing the three genotypes revealed that with increasing numbers of non-risk alleles the inhibitory effect of NO became stronger (Supplemental Figure 9). To exclude an influence of prevalent atherosclerosis on platelet aggregation, we also analyzed the subgroups of individuals in which atherosclerosis had been excluded by coronary angiography (Supplemental Table 5) revealing similar results (Supplemental Figure 10).

GUCY1A3 genotype affects cGMP levels in platelet rich plasma

The observation that addition of sildenafil enhanced the inhibition of platelet aggregation by the NO donor sodium nitroprusside points to a mechanism involving increased cGMP levels in platelets. To confirm this observation, we evaluated the cGMP-dependent phosphorylation of vasodilator-stimulated phosphoprotein (VASP) by protein kinase G after measurement of platelet aggregation in homozygous non-risk and risk allele carriers (n=8 each). The addition of sodium nitroprusside led to increased phosphorylation of VASP at $Serin_{239}$ in homozygous non-risk allele carriers compared to risk allele carriers (P<0.01). Again, this effect could be enhanced by addition of the PDE5 inhibitor sildenafil with significantly stronger cGMP-dependent phosphorylation of VASP in homozygous non-risk allele carriers compared to risk allele carriers (P=0.02; Figure 6).

Discussion

In a series of experiments using human samples and cell lines, we provide evidence that SNP rs7692387, which is genome-wide significantly associated with CAD, is involved in regulation of *GUCY1A3* gene expression. The SNP is located at a DNase I hypersensitive region that overlaps an intron of the *GUCY1A3* gene and interferes with the Zinc Finger E box-binding Homeobox 1 transcription factor (ZEB1) in a genotype-specific manner. As a consequence of impaired *GUCY1A3* expression, aortic SMC of a homozygous risk allele carriers showed decreased inhibitory effects of sGC stimulation on cell migration. We further observed that production of the second messenger cGMP and inhibition of platelet aggregation after exposure to a NO donor was impaired in platelets of homozygous risk allele carriers, an effect that was pronounced by the addition of the PDE5 inhibitor sildenafil. In agreement with these human data, lower *Gucy1a3* expression correlated with more aortic atherosclerosis in a population of genetically diverse mice[17]. The genotype-dependent effects found in this study might contribute to the understanding of the genetic and functional relevance of sGC activity for CAD and MI risk[3].

The impact of genetic variation in the *GUCY1A3* gene on human atherosclerosis has been highlighted by previous studies which showed a unique digenic loss-of-function mutation affecting *GUCY1A3* to co-segregate with CAD and MI in an extended family[10]. Besides, it has been shown that rare coding variants in the *GUCY1A3* gene are enriched in patients

suffering from premature CAD and MI[10,21]. Here we investigated a risk variant for CAD and MI which has a high allele frequency in the population[3]. In fact, ~60% of all Western Europeans are homozygous for the risk allele whereas only ~4% are homozygous for the non-risk variant. Like most of the variants identified by genome-wide association studies the *GUCY1A3* risk variant rs7692387 appears to act through regulatory mechanisms rather than alterations of protein function or activity[3]. As it has been shown for a variant at the *SORT1* locus[26], the nucleotide exchange in the risk compared to the non-risk allele at this locus seems to lead to a modulation of the binding site for a transcription factor, i.e., ZEB1, thereby directly affecting expression of the target gene.

ZEB1 is a transcription factor that may repress or activate transcription[27]. Interestingly, missense mutations in ZEB1 – also known as TCF8 – have been associated with Posterior Polymorphous Corneal Dystrophy[28], and Late-Onset Fuchs Corneal Dystrophy[29] in humans. An increased prevalence of cardiovascular diseases has been reported in the latter disease[30]. ZEB1 also seems to play a role in vascular remodeling as mice with reduced levels of the murine counterpart displayed retarded differentiation of aortic SMC, although SMC differentiation marker genes, including *Acta2* and *Myh11*, were still expressed. Whereas the total knockout was lethal, mice lacking one allele were vital and showed enhanced neointima formation after wire insertion-mediated injury compared to wildtype mice[31], which is in agreement with our human data that suggest a higher risk for coronary atherosclerosis with less ZEB1-mediated *GUCY1A3* expression.

Recently, the *NOS3* gene encoding the endothelial nitric oxide synthase (eNOS) has been identified to be also genome-wide significantly associated with CAD[4]. These results as well as our genetic and functional data presented here accentuate the impact of cGMP signaling in atherosclerosis. Targeting this pathway thus might represent a promising therapeutic strategy for CAD/MI patients. Clinical studies investigating organic or inorganic nitrates for therapeutic sGC stimulation in cardiovascular diseases, e.g., the NITRITE-AMI and the NIAMI studies, revealed contradictory results[32,33], potentially due to enhanced oxidative stress by the study medication. Rather than administration of NO donors direct targeting of sGC might thus be a promising therapeutic strategy. Indeed, a number of sGC activators and stimulators[34] are available and approved for the treatment of, e.g., pulmonary hypertension[35]. Additionally, the nitric oxide-independent sGC activator cinaciguat has already been shown to display beneficial effects in an ischemia-reperfusion-injury model[36].

Several limitations of this study have to be taken into account. First, it was not possible to show an increase of *GUCY1A3* expression by overexpressing ZEB1 which was due to the strong expression of ZEB1 in every cell line tested. Second, VSMC migration and platelet function experiments presented in this study do not fully resemble the *in vivo* human situation. Particularly, it was not possible to study a large set of VSMC carrying both genotypes. Nevertheless, the data uniformly point towards a stronger effect of NO on inhibition of migration and platelet aggregation in *GUCY1A3* non-risk allele carriers. Third, a recently published paper suggests that complete knockout of *Gucy1a3* in mice may reduce atherosclerotic plaque formation on a proatherogenic background ($Ldlr^{-/-}$)[37]. In contrast to this study, we and others investigated a partial loss of sGC activity and showed experimentally in mice and functionally in human materials that this leads to increased

disease risk[10,38–40]. Thus, in the total *Gucy1a3* knockout model compensatory mechanisms affecting NO/cGMP signaling, including a marked compensatory increase of *Gucy1a2* expression in *Gucy1a3*$^{-/-}$ VSMC[37], may not have been adequately addressed. Indeed, the total knockout of genes might be more useful to investigate molecular mechanisms rather than complex diseases such as atherosclerosis. As an example, a recent analysis of murine knockout models associated with an atherosclerotic phenotype only revealed limited overlap when compared with human genetic data from CAD-GWAS[41]. The mouse population approach used by the Hybrid Mouse Diversity Panel[17] might be more informative in this regard as it is able to discriminate gene expression in mice quantitatively rather than qualitatively. Importantly, data from this resource are concordant with our findings on the human risk allele at the *GUCY1A3* locus.

Taken together, the extensive genetic and functional data illustrated here document for the first time that a common variant in the *GUCY1A3* gene affects sGC expression, SMC migration, as well as platelet function. In conjunction with human genetic data[3,10] it can be concluded that dysregulation of sGC activity increases coronary risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Clinical perspective

What is new?

- The mechanism underlying the association of common variants at the *GUCY1A3* locus with coronary artery disease was elucidated.
- We show that the locus has regulatory properties with the risk allele leading to reduced expression of *GUCY1A3*.
- The *GUCY1A3* lead SNP (rs7692387) itself modulates binding of the transcription factor ZEB1, resulting in reduced *GUCY1A3* expression in carriers of the risk allele.
- As a consequence, risk allele carriers display impaired inhibition of vascular smooth muscle cell migration and platelet aggregation after stimulation of the soluble guanylyl cyclase (sGC).

What are the clinical implications?

- The genotype-dependent effects found in this study contribute to the understanding of the genetic and functional relevance of sGC activity for CAD and MI risk.
- Modulating sGC activity or inhibiting the resulting cellular effects caused by reduced expression of *GUCY1A3* might be a promising therapeutic strategy for individuals at risk carrying *GUCY1A3* risk alleles.

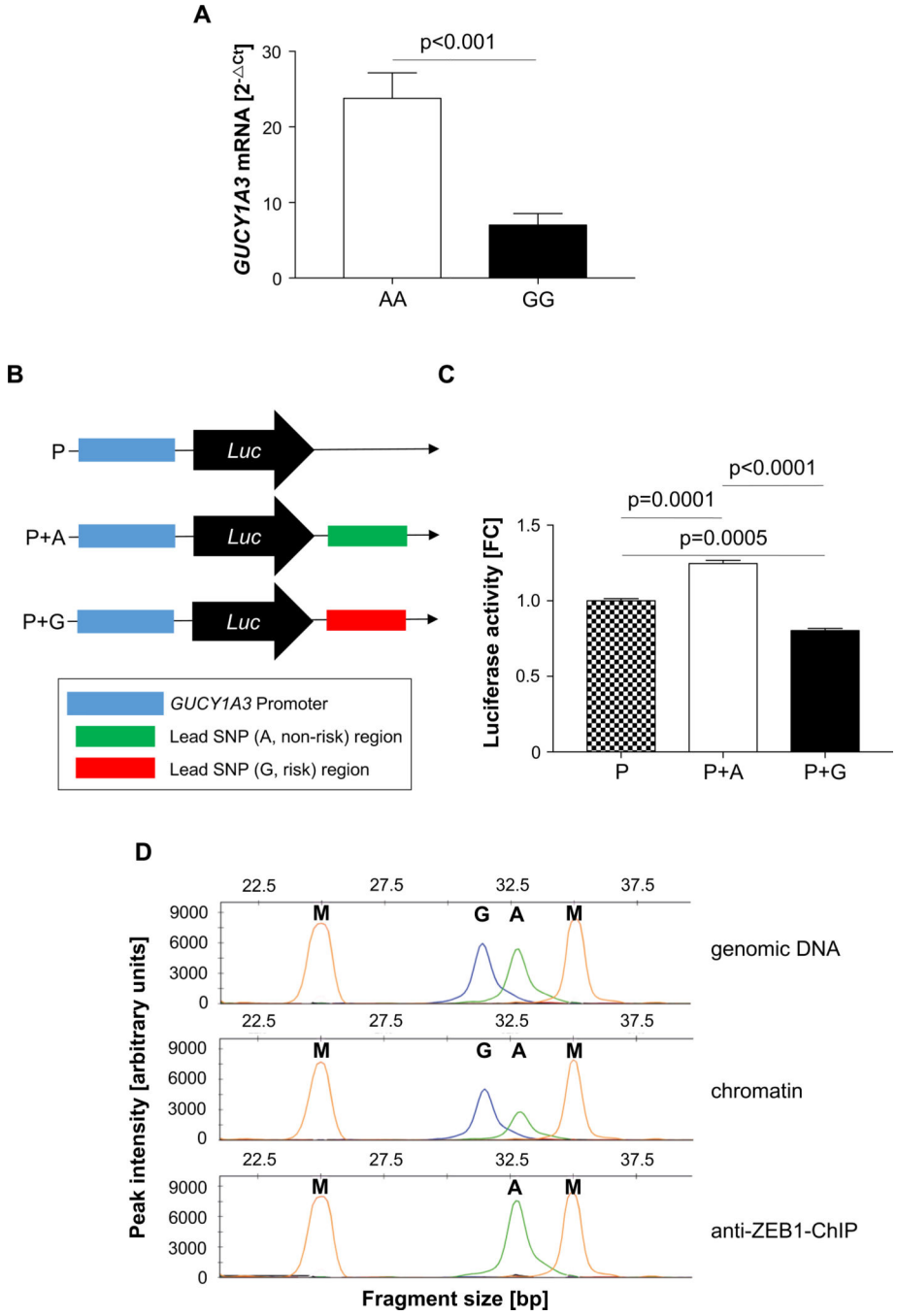

Figure 1. Coronary artery disease risk variant rs7692387 at the *GUCY1A3* locus is associated with reduced *GUCY1A3* expression in whole blood and has regulatory properties

A. *GUCY1A3* transcript levels in whole blood were significantly reduced in homozygous rs7692387 risk allele carriers (n=4) compared to homozygous non-risk allele carriers (n=4). Student's t-test. Data are mean and s.e.m. **B**. Reporter gene construct for the analysis of regulatory activity of the lead SNP region. **C**. The risk allele (G) variant led to a 44% decrease in luciferase activity compared to the non-risk allele (A) variant. 3 experiments, ordinary one-way ANOVA with Tukey's multiple comparisons test. Data are mean and

s.e.m. **D**. Single base extension analysis of genomic DNA (top lane) and enriched chromatin (middle lane) from rs7692387 heterozygous VSMC revealed signals for the risk (G, blue) and non-risk allele (A, green). After precipitation of ZEB1 (bottom lane), only the non-risk allele (A, green) signal was detectable. DNA fragments used as size markers (M) are indicated in orange.

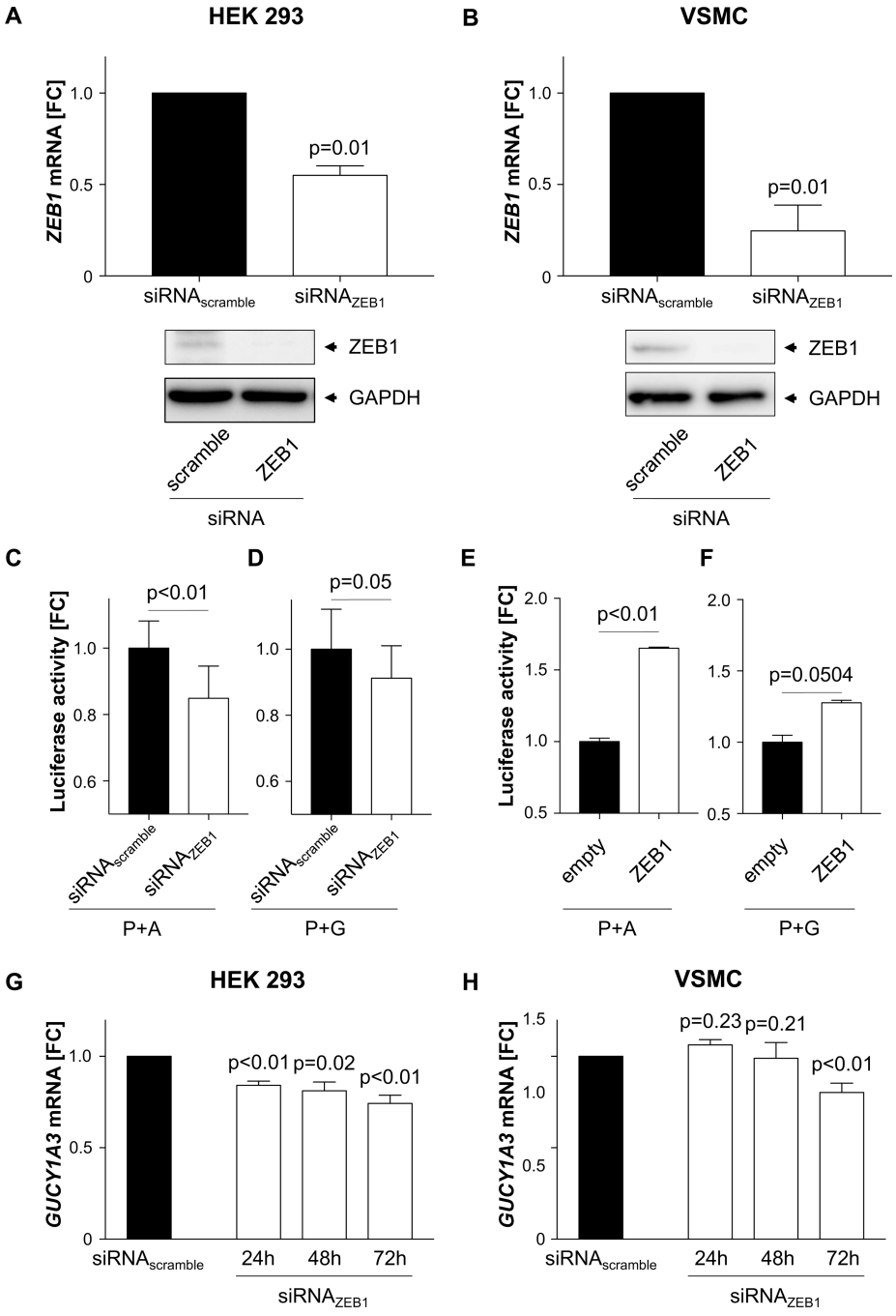

Figure 2. Knockdown of *ZEB1* influences expression of *GUCY1A3*

A, B. Application of $siRNA_{ZEB1}$ led to a significant reduction in *ZEB1* mRNA levels in HEK 293 cells (**A**) and human aortic SMC (VSMC, **B**) compared to control ($siRNA_{scramble}$). 3–4 experiments, one-sample t-test. **C, D**. siRNA-mediated knockdown of *ZEB1* significantly decreased luciferase activity rather of the non-risk allele (P+A) construct (**C**) whereas the risk allele (P+G) construct was only weakly influenced by silencing of *ZEB1* (**D**). 9 experiments, paired t-test. **E, F**. Overexpression of ZEB1 resulted in an increase of *GUCY1A3* promoter activity for both reporter plasmids. This gain in reporter gene

expression was more prominent for the construct containing the regulatory element with the non-risk allele (P+A, **E**) as compared to the risk allele construct (P+G, **F**). 3 experiments, paired t-test. **G, H**. Endogenous *GUCY1A3* expression in HEK 293 cells (**G**) and VSMC (**H**) was significantly reduced after 72 h secondary to knockdown of *ZEB1*. 4–5 experiments, one-sample t-test. Data are mean and s.e.m.

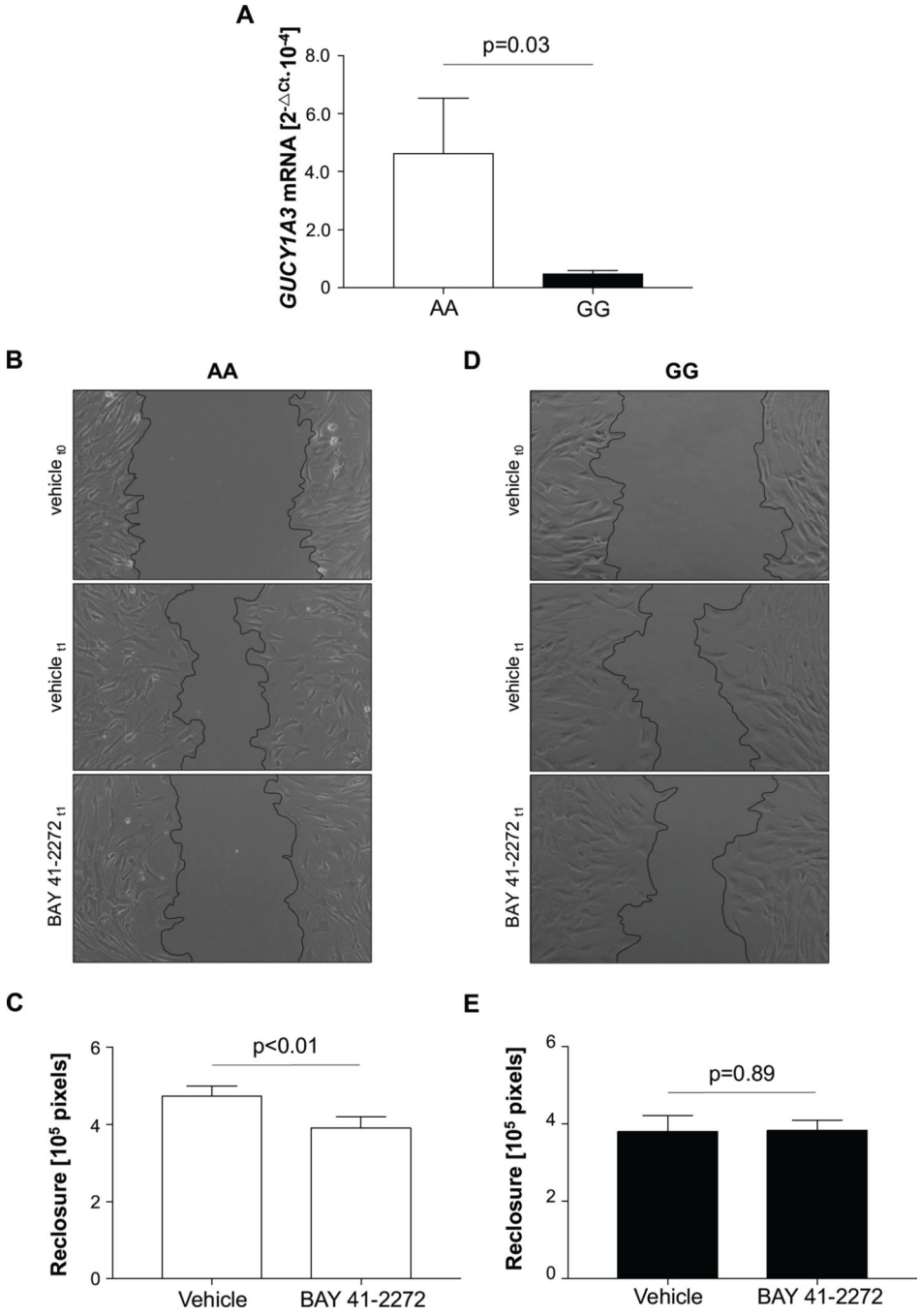

Figure 3. *GUCY1A3* genotype influences effect of sGC on migration in human aortic SMC

A. Risk allele aortic SMC displayed significantly reduced *GUCY1A3* mRNA levels compared to non-risk allele aortic SMC. 4 experiments. **B**. At timepoint t0, a scratch wound was set. Reclosure of the wound via migration of aortic SMC was determined at timepoint t1 (5 h). Differences in reclosure areas were assessed after application of vehicle (DMSO) or the sGC stimulator BAY 41–2272 in homozygous non-risk allele aortic SMC (**B, C**) and homozygous risk allele aortic SMC (**D, E**). Application of BAY 41–2272 led to a reduction

in migration of the non-risk allele aortic SMC ($P<0.01$, **C**) but not of the risk allele aortic SMC (**D**). 5 experiments, paired t-test. Data are mean and s.e.m.

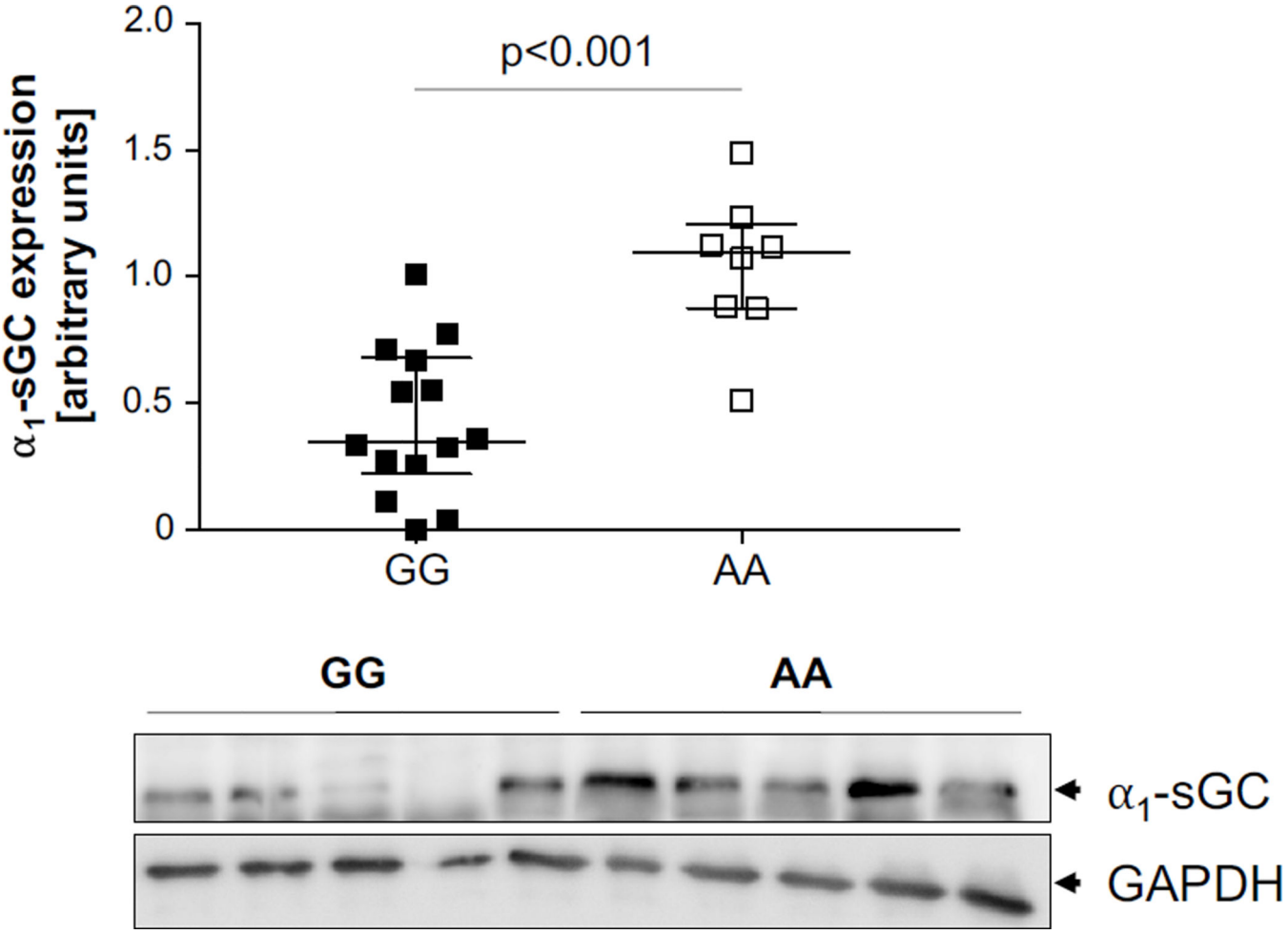

Figure 4. *GUCY1A3* genotype influences expression of α_1-sGC in platelets

Expression of α_1-sGC was significantly higher in homozygous non-risk allele carriers (n=8) compared to homozygous risk allele carriers (n=14; P<0.001). Mann-Whitney test. Data are median and interquartile range.

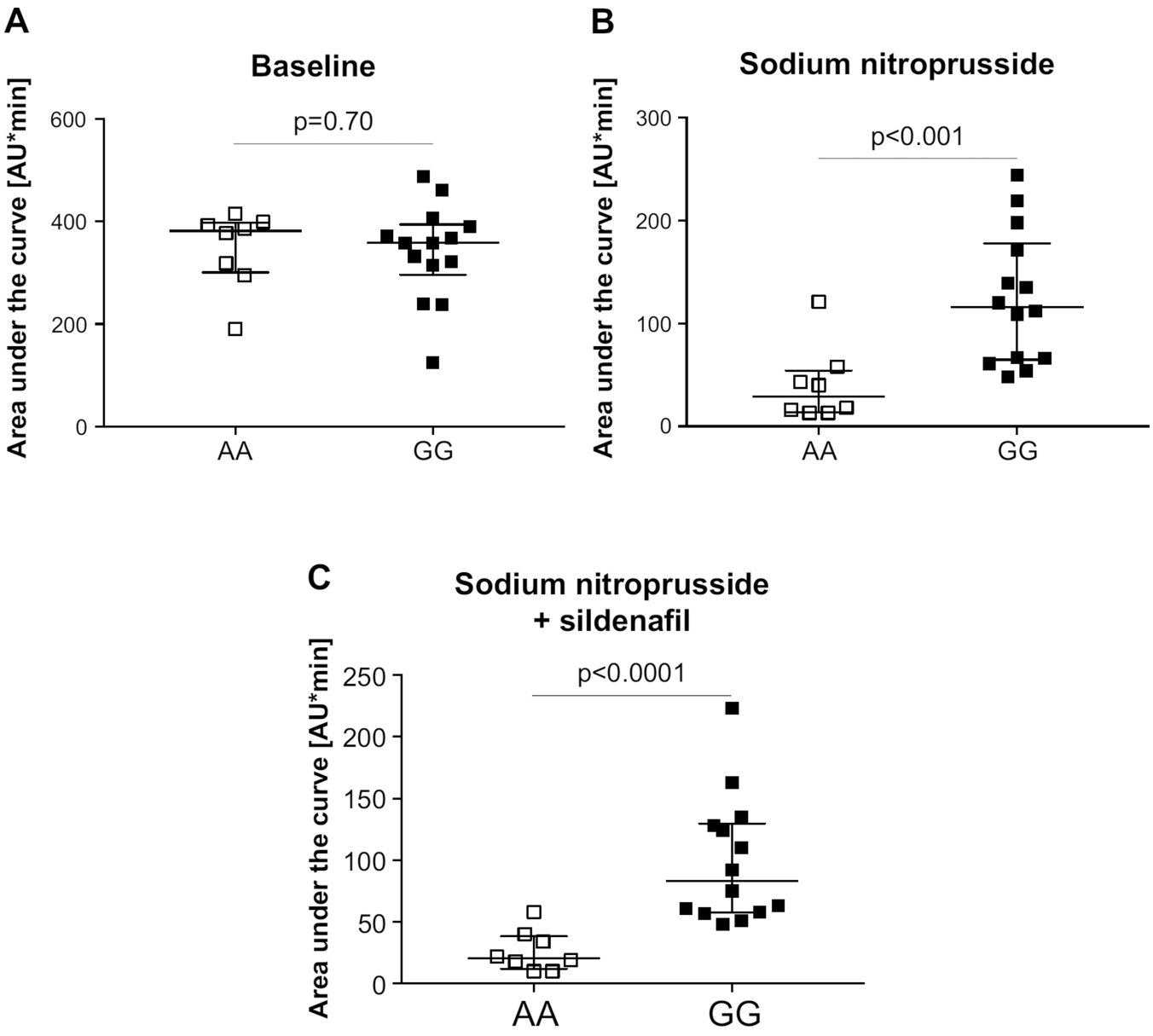

Figure 5. Genotype-dependent platelet response to nitric oxide *in vitro*

A. Baseline ADP-induced platelet aggregation did not differ between the genotypes (n=8 and n=14). **B, C**. Supplementation of the nitric oxide donor sodium nitroprusside (10 μM) led to significantly stronger inhibition of ADP-induced platelet aggregation in homozygous non-risk allele carriers (n=8) compared to homozygous risk allele carriers (n=14; P<0.001; **B**) with stronger effect after additional inhibition of cGMP degradation by sildenafil (10 μM; P<0.0001; **C**). Mann-Whitney test. Data are median and interquartile range.

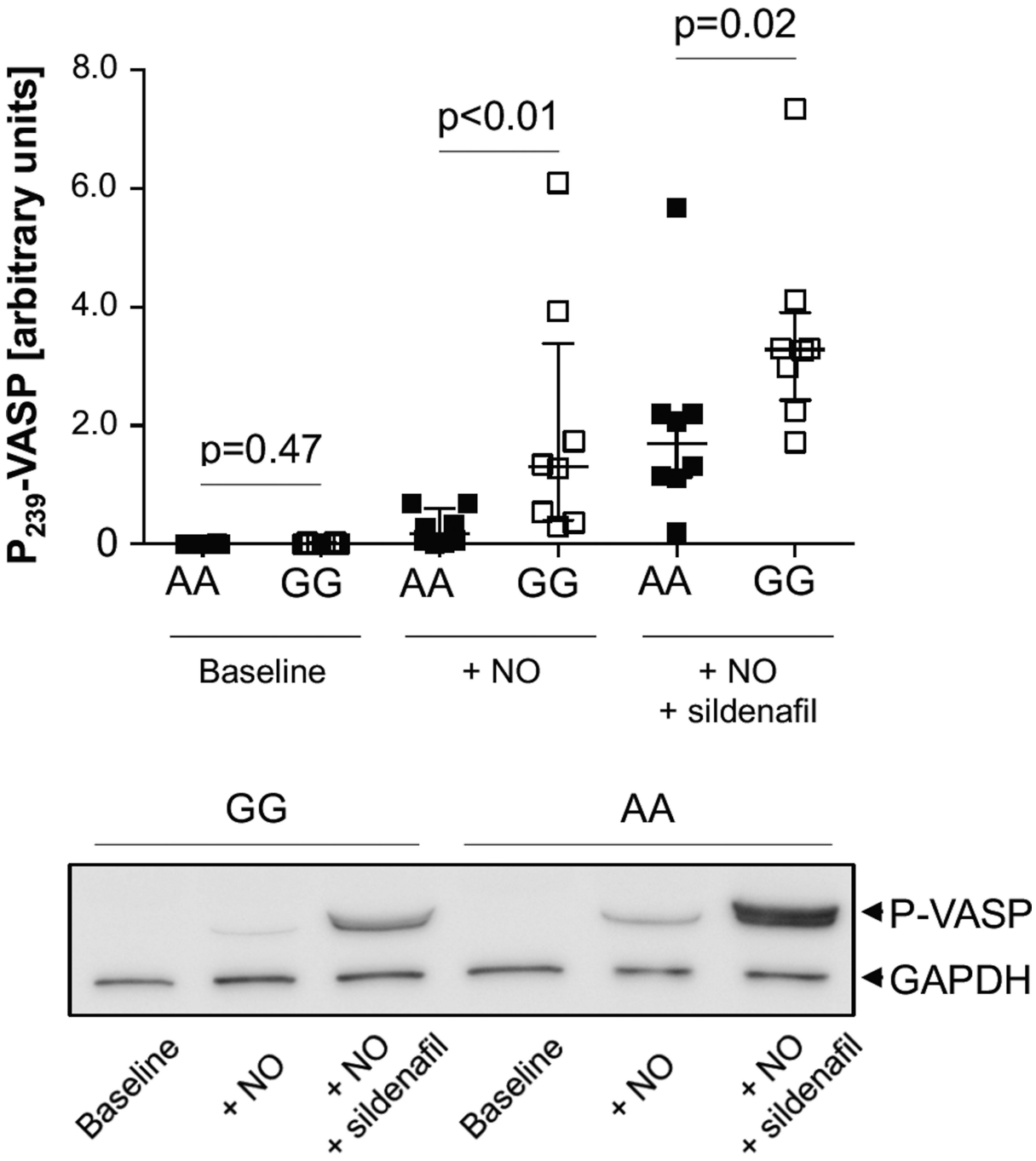

Figure 6. Nitric oxide leads to stronger cGMP-dependent phosphorylation of VASP in homozygous non-risk allele carriers

Phosphorylation of VASP by the cGMP-dependent protein kinase G (PKG) was assessed via Western Blot. PKG-dependent phosphorylation of VASP was stronger in homozygous non-risk allele carriers (n=8) after addition of the NO donor sodium nitroprusside (P<0.01). After addition of sildenafil to sodium nitroprusside, homozygous non-risk allele carriers (n=8) still displayed significantly stronger phosphorylation of VASP compared to homozygous risk allele carriers (n=8; P=0.02). Mann-Whitney test. Data are median and interquartile range.

Table 1

Characteristics of putative allele-specific transcription factors

Transcription factor	Predicted allele		Expression in relevant tissue*	Expression in cell types†
	A-allele	G-allele		
IRF8	−	+	WB/IMA/AAW	HEK/EC
ZEB1	+	−	WB/IMA/AAW	HEK/SMC/Meg01/EC

*expression according to STAGE study[18] data: *WB*, whole blood; *IMA*, internal mammary artery; *AAW*, atherosclerotic arterial wall;

†expression in cell types for further investigation: *HEK*, HEK 293 cells; *SMC*, human vascular SMC; *Meg-01*, human megakaryoblastic leukaemia cells; *EC*, human endothelial cells.