Atherosclerosis is perceived as a chronic disease arising from endothelial dysfunction, inflammation, and accumulation of foam cells in the media of the arterial wall. In more advanced lesions, smooth muscle cells (SMCs) undergo a transformation from a contractile to a synthetic phenotype characterized by proliferation, migration to the intima, and production of extracellular matrix to form a fibrous cap. \(^1\)–\(^4\) The development of atherosclerosis is influenced by environmental and genetic factors. Over the past few years, genome-wide association studies have successfully identified loci associated with coronary artery disease, including the \(GUCY1A3\) locus. \(^5\)–\(^11\) Moreover, a digenic mutation in \(GUCY1A3\) and \(CC7\) was identified as the cause of premature myocardial infarction in a family study. \(^12\)

\(GUCY1A3\) encodes the \(\alpha1\)-subunit of the soluble guanylate cyclase (sGC), which forms a heterodimer in conjunction with the \(\beta\)-subunit. Expression studies revealed that the \(\alpha1\)-subunit is ubiquitously expressed in the cardiovascular system. The \(\beta\)-subunit also associates with the \(\alpha2\)-subunit, which is, however, expressed at lower levels. The \(\alpha1\)-subunit is known to be involved in the regulation of smooth muscle cell function, with a proatherosclerotic effect in the context of high-fat diet and high-cholesterol conditions. \(^12\)

Soluble guanylate cyclase (sGC), a key enzyme of the nitric oxide signaling pathway, is formed as a heterodimer by various isoforms of its \(\alpha\) and \(\beta\) subunit. \(GUCY1A3\), encoding the \(\alpha1\) subunit, was identified as a risk gene for coronary artery disease and myocardial infarction, but its specific contribution to atherosclerosis remains unclear. This study sought to decipher the role of \(Gucy1a3\) in atherosclerosis in mice. At age 32 weeks and after 20 weeks of standard or high-fat diet, \(Gucy1a3\) \(^{-/-}\)/\(Ldlr\) \(^{-/-}\) mice exhibited a significant reduction of the atherosclerotic plaque size at the aortic root and the aorta for high-fat diet animals as compared with \(Ldlr\) \(^{-/-}\) control mice. Collagen content in plaques in the aortic root was reduced, suggesting an alteration of smooth muscle cell function. Proliferation and migration were reduced in \(Gucy1a3\) \(^{-/-}\) primary aortic smooth muscle cells (AoSMCs), and proliferation was also reduced in human AoSMCs after inhibition of sGC by 1H-\([1,2,4]\) oxadiazolo \([4,3-a]\) quinoxalin-1-one. \(Gucy1a3\) deficiency in AoSMCs prevents their phenotypic switching, as indicated by the differential expression of marker proteins. The inherited \(Gucy1a3\) \(^{-/-}\) loss exerts an atheroprotective effect. We suggest that sGC activity promotes the phenotypic switching of smooth muscle cells from a contractile to a synthetic state, fostering the formation of atherosclerosis. Preventing this switch by sGC inhibition may provide a novel target in atherosclerotic disease. (Am J Pathol 2016, 186: 2220–2231; http://dx.doi.org/10.1016/j.ajpath.2016.04.010)

Supported by the German Federal Ministry of Education and Research in the context of the e:Med program (c:AtheroSysMed and sysINFLAME), the FP7 European Union project CVgenes@target (261123), and a Fondation Leducq grant (CADgenomics: Understanding Coronary Artery Disease Genes, 12CVD02), and the German Research Foundation cluster of excellence Inflammation at Interfaces.

C.d.W. and Z.A. contributed equally to this work as senior authors.

Disclosures: None declared.

Copyright © 2016 American Society for Investigative Pathology. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
levels.13,14 The primary activator of sGC is nitric oxide (NO), a signaling molecule specifically relevant in the pathogenesis of atherosclerosis. NO is synthesized by nitric oxide synthases (endothelial, neuronal, and inducible). Interestingly, the eNOS locus was also identified by genome-wide association studies to be associated with coronary artery disease.15 NO diffuses across membranes binding to the heme cofactor of sGC, which activates this enzyme. The resulting increase in cGMP modulates the activity of phosphodiesterase, ion-gated channels, or protein kinases such as cGKI/PKG1 (cGMP-dependent protein kinase type I) to exert important physiological functions.16,17

Interestingly, the nitric oxide synthases (endothelial, neuronal, and inducible).

Interestingly, the nitric oxide synthases (endothelial, neuronal, and inducible).

Interestingly, the nitric oxide synthases (endothelial, neuronal, and inducible).

Interestingly, the nitric oxide synthases (endothelial, neuronal, and inducible).

Interestingly, the nitric oxide synthases (endothelial, neuronal, and inducible).

Interestingly, the nitric oxide synthases (endothelial, neuronal, and inducible). The primary activator of sGC is nitric oxide (NO), a signaling molecule specifically relevant in the pathogenesis of atherosclerosis. NO is synthesized by nitric oxide synthases (endothelial, neuronal, and inducible). Interestingly, the eNOS locus was also identified by genome-wide association studies to be associated with coronary artery disease.15 NO diffuses across membranes binding to the heme cofactor of sGC, which activates this enzyme. The resulting increase in cGMP modulates the activity of phosphodiesterase, ion-gated channels, or protein kinases such as cGKI/PKG1 (cGMP-dependent protein kinase type I) to exert important physiological functions.16,17

Previous studies have sought to decipher the role of the NO-sGC-cGMP pathway in atherosclerosis.18–25 NO is generally considered to prevent atherosclerosis by inhibiting SMC proliferation and migration, platelet aggregation, and leukocyte adhesion.26 However, multiple studies have reported contradictory results, due in part to the differing contributions of specific cell types and the gene(s) of interest in the pathway. nNOS and eNOS are thought to be atheroprotective,18–21 whereas iNOS and cGKI are reportedly proatherosclerotic.22,23 Specifically, studies using tissue-specific knockout mice revealed that SMCs play a decisive role in the proatherosclerotic role of cGKI.24,25 Analyses of cell proliferation and migration also highlight the complexity of NO signaling, which can exert opposing effects depending on its concentration and site of action.27–29 In general, low NO concentrations stimulate cell proliferation and survival, whereas higher concentrations inhibit proliferation.30,31

To date, no studies have characterized the role of sGC subunits on atherosclerosis. Consequently, the specific role of GUCY1A3 in atherosclerosis remains incompletely understood. In this study, we assessed the effect of complete GUCY1A3 deficiency on atherosclerosis. To this end, we crossed Gucy1a3+/− mice into the Ldlr−/− atherogenic background and examined the development of atherosclerotic lesions. Furthermore, we performed in vitro studies of migration and proliferation to obtain mechanistic insight into the role of Gucy1a3 in primary aortic smooth muscle cells (AoSMCs). In addition, we studied the expression of genes of the NO-cGMP-cGKI signaling pathway and marker genes characterizing smooth muscle phenotype.

Materials and Methods

Experimental Animals

All animal experiments were performed in accordance with the German animal studies committee of Schleswig-Holstein. C57BL/6J mice [wild type (WT)], and Ldlr−/− mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Gucy1a3+/− mice were kindly provided by Dr. Koesling (Ruhr-University Bochum, Bochum, Germany).32 Gucy1a3+/− mice were bred into the Ldlr−/− atherogenic background to generate Gucy1a3+/−Ldlr−/− mice.

The genotyping of the Gucy1a3 gene was performed as previously described.33 For Ldlr−/− mice, three primers were used and the PCR was performed according to the standard protocol from the Jackson Laboratory.

Atherosclerosis Study

Atherosclerotic plaque analysis was performed in females. Four different genotypes were used for the study: WT, Gucy1a3+/−, Ldlr−/−, and Gucy1a3+/−Ldlr−/−. At 12 weeks of age, and after plasma collection, each genotype was divided in two groups (n = 8 to 12 per group) and fed standard chow diet (SD) or Western type high-fat diet (HFD) containing 0.2% cholesterol and 21.2% cocoa butter (Harlan, Paderborn, Germany). During the study, animals were weighted every 2 weeks. At the end of the experiment, animals were euthanized after 20 weeks of diet by an overdose of isoflurane and cervical dislocation. Animals were perfused with phosphate-buffered saline (PBS), pH 7.4 (Lonza, Cologne, Germany), and the whole heart-aorta until the iliac bifurcation was collected and fixed in 4% paraformaldehyde for histological analysis. Plasma and several organs were obtained for posterior analysis. Tail and liver were used for regenotyping of the animals.

Lipid Metabolism Analysis

Lipid metabolism was assessed at the start and the end of the experiment for all four genotypes. Total cholesterol, high-density lipoprotein (HDL), direct low-density lipoprotein, and triglycerides from the end of experiment samples were analyzed using a Roche—Cobas 702 machine at the Institute for Clinical Chemistry of the University of Lübeck. Total cholesterol, HDL, non-HDL, and triglycerides levels in plasma from the start of the experiment samples were analyzed by high-performance liquid chromatography at the Department of Endocrinology and Metabolic Diseases at University of Leiden because of reduced sample amount.

Oxidized low-density lipoprotein was also analyzed at the end of the experiment in plasma samples from all groups under high-fat diet and for the Ldlr−/− and Gucy1a3+/−Ldlr−/− animals under standard diet. The Mouse Oxidized Lowdensity Lipoprotein (OxLDL) enzyme-linked immunosorbent assay kit (CUSABIO, Baltimore, MD) was used following the manufacturer’s instructions.

Blood Pressure Measurements

Systolic, mean, and diastolic blood pressure were measured at the end of the atherosclerosis study in high-fat diet female animals by using tail cuff plethysmography (BP-98A; Softron Co., Tokyo, Japan). Measurements were taken from a total of 10 Ldlr−/−, five Gucy1a3+/−Ldlr−/−, five WT, and five Gucy1a3+/− animals. For habituation, mice were trained.
daily (7 days). After the training period, 10 measurements per mouse were recorded daily for 5 days.

**Cytokine Analysis**

The amount of cytokines present in the plasma of the four genotypes after high-fat diet was assayed after pooling 40 μL each from five animals per group. The Mouse Cytokine Array Panel A kit (R&D Systems, Minneapolis, MN) was performed according to the manufacturer’s instructions. The same procedure was used to analyze the cytokines present in the 96-hour proliferation medium of WT and 5cylα3−/− mouse aortic smooth muscle cells.

**Atherosclerotic Plaque Analysis**

To evaluate atherosclerotic lesions, two methods were used: at the aortic root serial cryosections (10 μm) were obtained starting below the aortic roots until the proximal aorta below the aortic arch and, in addition, the so-called en face analysis. The atherosclerotic lesion area was determined at the aortic root by using oil red-O staining. Mean lesion area was calculated from 10 sections at 40-μm intervals, starting at the appearance of at least two aortic valves. For the en face analysis, the thoraco-abdominal aortas were analyzed. The adventitial tissue was removed and the aorta was opened longitudinally. Lipid-rich intraluminal lesions were stained with oil red O. Aortas were pinned into a 2.5% agarose gel and color pictures were captured using a Leica M-80 microscope and Leica IC80 HD camera using the Leica Application Suite (LAS) version 4.2 software (Leica Microsystems, Wetzlar, Germany). Images were analyzed with the GIMP software version 2.6 (The GIMP Development Team) and the amount of aortic lesion formation in each animal was measured as percentage lesion area per total area of the aorta.

The same approach was also used to determine collagen content of the plaques, five sections at 40-μm intervals were stained using Masson’s Trichrome staining (Sigma, St. Louis, MO) following the manufacturer’s instructions. Collagen content was determined as a percentage of the total plaque, after intensity determination of collagen presence. Images were obtained using Olympus IX70 microscope coupled to the Olympus U-TV0.5XC-3 video camera and using the Leica Qwin Imaging software version 3 (Leica Microsystems).

The presence of macrophages was determined in five sections at 40-μm intervals after immunohistochemical staining using a rat monoclonal anti-monocyte and macrophage antibody (MOMA-2, 1:500; Abcam, Cambridge, UK). Polyclonal rabbit anti-rat-horseradish peroxidase (HRP) (1:100; Dako, Hamburg, Germany) was used as a secondary antibody and ImmPACT NovaRED substrate (Vector Laboratories, Burlingame, CA) was used for visualization of HRP following the manufacturer’s instructions after an exposure time of 5.5 minutes. Macrophage content was determined as a percentage of the total plaque. Images were obtained using a Keyence BZ-9000 microscope with BZII software version 1.41 (Keyence Deutschland GmbH, Neu-Isenburg, Germany). Plaque areas were delimited using GIMP software version 2.6 (The GIMP Development Team) and MOMA-2 intensity was analyzed with an in-house MATLAB script. All of the atherosclerotic plaque analyses were performed blind to the given genotype.

**Primary Aortic SMC Isolation from Mice**

Murine aortic smooth muscle cells (AoSMCs) were isolated from WT and Gucy1α3−/− mice (n = 9) and grown in 0.1% pork gelatin in PBS coated surfaces (Sigma), DMEM-Glutamax (Gibco, Life Technologies, Ober-Olm, Germany) supplemented with 20% fetal bovine serum, EU Professional (PAN Biotech, Aidenbach, Germany), and 1% penicillin streptomycin (Merck Millipore, Darmstadt, Germany). Trypsin-EDTA was used for passaging (Gibco, Life Technologies). Primary cells were used for experiments until passage 12. To ensure population purity, immunohistochemistry using the SMC marker sm22α (Abcam) at 1:100 dilution was performed. Anti-goat HRP antibody (Cell Signaling) at 1:200 dilution was used as secondary antibody and Liquid DAB + substrate Chromogen system (Dako) was used for visualization of HRP. Control sections were processed in the absence of primary antibodies.

**AoSMC Proliferation Assay**

The previously isolated primary AoSMCs were used to perform proliferation assays. Twenty-thousand cells per well in triplicate were seeded in 12-well plates per time-point (4, 24, 48, 72, and 96 hours). Two methods were used for quantification. In the first, cells were trypsinized using Trypsin-EDTA (Gibco, Life Technologies) and counted using the Newbauer chamber (Marienfeld). In the second method, cells were fixated with 4% formaldehyde (Merck) for 5 minutes, treated with Triton-X-100 0.1% in PBS for 10 minutes, and washed once with PBS (Lonza). Finally, cells were stained with a 1:10,000 DAPI (Sigma) to PBS dilution for 10 minutes. Ten representative pictures per well were taken using a Keyence BZ-9000 microscope with BZII software version 1.41 (Keyence Deutschland GmbH). Cells were counted with the ImageJ software version 1.48v (NIH, Bethesda, MD; http://imagej.nih.gov/ij) using an in-house script for DAPI signal. The total number of cells per well was extrapolated from the area of the analyzed pictures to the total area of the well. All experiments were performed in triplicate and for at least three independent times.

**AoSMC Migration Assay**

Cell migratory capability of previously isolated AoSMCs was assayed using the xCELLigence Biosensor System.
Experiments were performed in an RTCA D instrument (Roche Diagnostics, Basel, Switzerland) placed in a 5% CO2 humidified incubator maintained at 37°C. Cell migration was assessed using specifically designed 16-well plates (CIM-plate 16; Roche Diagnostics) with 8-µm pores. Forty-thousand cells per well were seeded in the upper chamber in serum-free medium conditions. FCS medium (20%) was used as chemoattractant and serum-free medium as control in the lower chamber. Data were analyzed using RTCA software version 1.2 (Acea Biosciences Inc., San Diego, CA). All experiments were performed in quadruplicate and for at least three independent times.

Effect of the NO Donor SNP and sGC Inhibitor ODQ on SMC Proliferation

Human aortic smooth muscle cells (HuAoSMCs; Invitrogen) were grown in Medium 231 (Gibco, Life Technologies) supplemented with smooth muscle growth supplement (Gibco, Life Technologies) and 1% penicillin streptomycin (Merck Millipore). Trypsin-EDTA was used for passaging (Gibco, Life Technologies) together with TN trypsin (Merck Millipore). Trypsin-EDTA was used for passaging for experiments until passage 9. 1H-[1,2,4] oxadiazolo [4,3-a] AoSMCs were harvested as described above. Cells were used for experiments until passage 9. 1H-[1,2,4] oxadiazolo [4,3-a] AoSMCs were harvested as described above. Cells were used for experiments until passage 9. 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ; Cayman Chemical) was dissolved in 30 mg/mL dimethyl formamide (VWR) following manufacturer’s instructions and used at final concentrations of 10 or 20 µmol/L applied for 24 or 48 hours. Sodium nitroprusside (SNP; Sigma-Aldrich) was used at concentrations of 50 to 200 µmol/L for 24 or 48 hours. The proliferation assay was quantified using the DAPI staining method, as explained above. All experiments were performed with six technical replicates and for at least three independent times. Sequences of the primer pairs used for amplification are denoted in Table 1.

RNA Isolation and Real-Time Quantitative PCR

Total RNA was isolated from mouse AoSMCs and organs using the RNeasy Plus Mini Kit (Qiagen, Venlo, the Netherlands), following the manufacturer’s instructions. Total RNA was then reverse transcribed into cDNA and changes in mRNA levels were determined using the ΔΔCt method, as previously described. Glyceraldehyde-3-phosphate dehydrogenase or β-actin was used as housekeeping genes. All experiments were performed in triplicate and for at least three independent times. Sequences of the primer pairs used for amplification are denoted in Table 1.

Protein Isolation and Western Blot

Protein was isolated from mouse AoSMCs and organs, as previously described. Protein (15 µg) was used and separated in an SDS-PAGE gel and transblotted. After blocking with 5% skim milk, blots were incubated with the primary antibodies: anti-PKG1 1:200 (Cell Signaling), anti-calponin 1:200 (Sigma), anti-osteopontin 1:200 (Abcam), and anti-α-tubulin 1:1000 (Abcam), and anti-glyceraldehyde-3-phosphate dehydrogenase 1:1000 (Abcam) as control. Appropriate secondary antibodies were then used, and the protein bands were detected using the ECL Prime Western Blotting Detection Reagent (GE Health Care, Little Chalfont, UK) and quantified with ImageJ software version 1.48v (NIH, Bethesda, MD).

Statistical Analysis

Data were analyzed using the GraphPad Prism software version 6 (GraphPad Software Inc., La Jolla, CA) and presented as means ± SD. Pairwise comparisons were used to compare WT to Gucy1a3+/− and Ldlr−/− to Gucy1a3+/− Ldlr−/− using unpaired t-test. In case of analyzing more than two groups, analysis of variance with Bonferroni multiple comparison correction was performed. Statistical significance was assumed at P < 0.05.

Results

Body Mass, Lipid Metabolism, and Arterial Pressure

Body mass increased starting at an age of 12 to 14 weeks from 18 g during 20 weeks of standard diet (SD) to 23 g. This gain was enhanced in animals receiving high-fat diet (HF), and body mass reached approximately 29 g after 20 weeks. However, Gucy1a3-deficiency was without effect and all genotypes exhibited a similar increase of body mass for each diet (Supplemental Figure S1). Total cholesterol and HDL assessed at the start of the diet were similar in WT and Gucy1a3+/− animals, but a slight reduction of non-HDL and triglycerides was observed in Gucy1a3+/− mice. As expected, total cholesterol, non-HDL, and triglycerides were markedly elevated in Ldlr−/− and similarly increased in Gucy1a3+/−Ldlr−/− animals (Table 2). During 20 weeks of SD plasma lipid levels did not change markedly and only minor differences

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primers</th>
<th>Length (bp)</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gucy1a3-F</td>
<td>5’-CGACTGAACTTTGCTGACCCATTCA-3’</td>
<td>114</td>
<td>Exon 3</td>
</tr>
<tr>
<td>Gucy1a3-R</td>
<td>5’-TCTGCTTTGAGGACATGACCCATTCA-3’</td>
<td>44</td>
<td>Exon 4</td>
</tr>
<tr>
<td>Gucy1a2-F</td>
<td>5’-CGAGTACCCGTGACCCATTCA-3’</td>
<td>236</td>
<td>Exon 7</td>
</tr>
<tr>
<td>Gucy1a2-R</td>
<td>5’-TCTGCTTTGAGGACATGACCCATTCA-3’</td>
<td>77</td>
<td>Exon 9</td>
</tr>
<tr>
<td>Gucy1b3-F</td>
<td>5’-CACACTGGAAGCCCTGGGAGG-3’</td>
<td>105</td>
<td>Exon 4</td>
</tr>
<tr>
<td>Gucy1b3-R</td>
<td>5’-GGCAAAAGGAGGAGGAGGAGG-3’</td>
<td>105</td>
<td>Exon 10</td>
</tr>
<tr>
<td>C6KI-F</td>
<td>5’-GCTGCTGCCGACGACCTGACCCATTCA-3’</td>
<td>150</td>
<td>Exon 4</td>
</tr>
<tr>
<td>C6KI-R</td>
<td>5’-GCTGCTGCCGACGACCTGACCCATTCA-3’</td>
<td>150</td>
<td>Exon 5</td>
</tr>
<tr>
<td>Cnn1-F</td>
<td>5’-GCTGCTGCCGACGACCTGACCCATTCA-3’</td>
<td>97</td>
<td>Exon 2</td>
</tr>
<tr>
<td>Cnn1-R</td>
<td>5’-GCTGCTGCCGACGACCTGACCCATTCA-3’</td>
<td>97</td>
<td>Exon 3</td>
</tr>
<tr>
<td>Spp1-F</td>
<td>5’-CGATGACCCCTGACCTGACCCATTCA-3’</td>
<td>76</td>
<td>Exon 4</td>
</tr>
<tr>
<td>Spp1-R</td>
<td>5’-CGATGACCCCTGACCTGACCCATTCA-3’</td>
<td>76</td>
<td>Exon 5</td>
</tr>
<tr>
<td>Thsd1-F</td>
<td>5’-CGATGACCCCTGACCTGACCCATTCA-3’</td>
<td>98</td>
<td>Exon 2</td>
</tr>
<tr>
<td>Thsd1-R</td>
<td>5’-CGATGACCCCTGACCTGACCCATTCA-3’</td>
<td>98</td>
<td>Exon 3</td>
</tr>
</tbody>
</table>

F, forward; R, reverse.
were found between $Gucy1a3^{-/-}$ mice and their respective controls (Table 3). Twenty weeks of HFD potentiated direct low-density lipoprotein and total cholesterol levels (approximately sevenfold) in $Ldlr^{-/-}$ and to a similar extent in $Gucy1a3^{-/-}/Ldlr^{-/-}$ mice (Table 3). Thus, $Gucy1a3^{-/-}$ mice differed after 20 weeks of diet only slightly from their respective controls, namely, a small reduction of total cholesterol after SD irrespective of the presence of the LDL receptor and a minor increase in LDL in LDL receptor bearing mice after both diets. More important, differences between $Ldlr^{-/-}$ and $Gucy1a3^{-/-}/Ldlr^{-/-}$ animals were not observed on HFD and also in such animals receiving SD LDL levels were similar (Table 3). Oxidized low-density lipoprotein was comparable in all HFD groups and also similar in $Ldlr^{-/-}$ and $Gucy1a3^{-/-}/Ldlr^{-/-}$ on SD (Table 3).

Cytokines reflect the inflammatory process during the development of atherosclerosis. However, cytokine levels assessed in plasma from animals receiving HFD were comparable in all genotypes after normalization (Supplemental Figure S2).

Deficiency of $Gucy1a3$ is associated with an increase in arterial pressure. Systolic and diastolic pressures amounted to $119.6 \pm 3.5$ and $85.4 \pm 3.4$ mm Hg, respectively, in $Ldlr^{-/-}$ mice after HFD and were significantly increased in $Gucy1a3^{-/-}/Ldlr^{-/-}$ animals (to $127.5 \pm 3.3$ and $90.1 \pm 3.7$ mm Hg, respectively; both $P < 0.05$).

### Reduced Atherosclerosis in $Gucy1a3^{-/-}/Ldlr^{-/-}$ Mice

Plaque area was analyzed at the aortic root in all genotypes after 20 weeks of SD or HFD. As expected, lesions were not found in WT and $Gucy1a3^{-/-}$ animals after either diet (data not shown). Atherosclerotic lesions were observed in $Ldlr^{-/-}$ mice after SD and lesion area was markedly enlarged after HFD (eightfold). $Gucy1a3^{-/-}/Ldlr^{-/-}$ mice exhibited a significantly reduced lesion area after both diets (Figure 1A). The reduction was pronounced after SD (by 48%) but lesion area was also reduced significantly after HFD (by 16%).

The previous controversy on the role of the NO pathway in atherosclerosis, to confirm our results and test their reproducibility, we analyzed a second experimental group of animals receiving SD. In this independent experiment, plaque area was decreased in $Gucy1a3^{-/-}/Ldlr^{-/-}$ by 69% compared to $Ldlr^{-/-}$ mice, thus confirming the results of the initial series (Figure 1B).

### Table 2 Lipid Metabolism Levels at 12 to 14 Weeks of Age

<table>
<thead>
<tr>
<th>Variable</th>
<th>Standard diet</th>
<th>$Gucy1a3^{-/-}$</th>
<th>$Ldlr^{-/-}$</th>
<th>$Gucy1a3^{-/-}/Ldlr^{-/-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT ($n = 11$)</td>
<td>($n = 11$)</td>
<td>($n = 14$)</td>
<td>($n = 20$)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>$2.33 \pm 0.34$</td>
<td>$2.14 \pm 0.33$</td>
<td>$7.04 \pm 1.09$</td>
<td>$6.82 \pm 1.09$</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>$1.43 \pm 0.20$</td>
<td>$1.51 \pm 0.25$</td>
<td>$1.75 \pm 0.43$</td>
<td>$1.90 \pm 0.22$</td>
</tr>
<tr>
<td>Non-HDL (mmol/L)</td>
<td>$0.90 \pm 0.21$</td>
<td>$0.63 \pm 0.25^*$</td>
<td>$5.30 \pm 1.24$</td>
<td>$4.97 \pm 1.21$</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>$1.70 \pm 0.97$</td>
<td>$1.02 \pm 0.26^*$</td>
<td>$2.43 \pm 0.91$</td>
<td>$2.92 \pm 1.39$</td>
</tr>
</tbody>
</table>

Data are represented as means ± SD.

### Table 3 Lipid Metabolism Levels at 30 to 32 Weeks of Age

<table>
<thead>
<tr>
<th>Variable</th>
<th>Genotype</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>$Gucy1a3^{-/-}$</td>
<td>$Ldlr^{-/-}$</td>
<td>$Gucy1a3^{-/-}/Ldlr^{-/-}$</td>
</tr>
<tr>
<td>Standard diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$n$</td>
<td>5</td>
<td>9</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>$2.2 \pm 0.20$</td>
<td>$1.89 \pm 0.27^*$</td>
<td>$6.08 \pm 0.60$</td>
<td>$5.32 \pm 0.37^*$</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>$1.02 \pm 0.067$</td>
<td>$0.93 \pm 0.12$</td>
<td>$1.18 \pm 0.15$</td>
<td>$1.10 \pm 0.14$</td>
</tr>
<tr>
<td>dLDL (mmol/L)</td>
<td>$0.12 \pm 0.02$</td>
<td>$0.16 \pm 0.03^*$</td>
<td>$2.17 \pm 0.23$</td>
<td>$1.99 \pm 0.23$</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>$0.61 \pm 0.05$</td>
<td>$0.72 \pm 0.24$</td>
<td>$0.87 \pm 0.1$</td>
<td>$0.97 \pm 0.13$</td>
</tr>
<tr>
<td>oxLDL (mmol/L)</td>
<td>$0.45 \pm 0.16$</td>
<td>$0.45 \pm 0.16$</td>
<td>$0.36 \pm 0.07$</td>
<td>$0.36 \pm 0.07$</td>
</tr>
</tbody>
</table>

|                  |          |          |          |          |
| High-fat diet     |          |          |          |          |
| $n$               | 6        | 5        | 8        | 6        |
| Total cholesterol (mmol/L) | $3.04 \pm 0.28$ | $3.31 \pm 0.41$ | $40.95 \pm 8.38$ | $42.94 \pm 5.5$          |
| HDL (mmol/L)      | $2.92 \pm 0.35$ | $2.99 \pm 0.35$ | $1.10 \pm 0.39$ | $0.88 \pm 0.17$          |
| dLDL (mmol/L)     | $0.57 \pm 0.06$ | $0.78 \pm 0.16^*$ | $15.39 \pm 2.95$ | $16.93 \pm 2.14$          |
| Triglycerides (mmol/L) | $0.61 \pm 0.07$ | $0.69 \pm 0.18$ | $2.58 \pm 0.58$ | $2.39 \pm 0.66$          |
| oxLDL (mmol/L)    | $0.49 \pm 0.09$ | $0.65 \pm 0.32$ | $0.58 \pm 0.36$ | $0.47 \pm 0.21$          |

Data are represented as means ± SD.

$P = 0.05$.

dLDL, direct low-density lipoprotein; HDL, high-density lipoprotein; oxLDL, oxidized low-density lipoprotein; WT, wild type.
In addition, atherosclerosis was quantified by the en face method in the thoracic and abdominal aorta. Lesions constituted 5.26% ± 2.66% of the total aortic area in Ldlr<sup>-/-</sup> animals after HFD and this proportion was reduced to 2.67% ± 1.75% (P < 0.05) in Gucy1a3<sup>-/-</sup>/Ldlr<sup>-/-</sup> mice receiving the same diet (Figure 1C). Ldlr<sup>-/-</sup> and Gucy1a3<sup>-/-</sup>/Ldlr<sup>-/-</sup> animals receiving SD exhibited a low incidence of lesions in the aorta and differences were not observed between genotypes (Figure 1D).

Next, we assessed collagen content by trichrome staining and macrophage numbers by MOMA-2 staining at the aortic root in animals fed HFD. After normalizing for lesion size, we observed a reduced collagen content in Gucy1a3<sup>-/-</sup>/Ldlr<sup>-/-</sup> compared to Ldlr<sup>-/-</sup> (Figure 2A). By contrast, macrophage content was not different between genotypes (Figure 2B), suggesting a role for SMCs rather than macrophages in the protective effect of Gucy1a3 deficiency.

Reduced Proliferation and Migration in Gucy1a3<sup>-/-</sup> AoSMCs

We next studied migration and proliferation using isolated SMCs from thoracic aorta (AoSMCs) of WT and Gucy1a3<sup>-/-</sup> animals. AoSMCs were cultured and stained with Sm22α to verify a pure SMC population (Figure 3A). Migration was analyzed in the absence (control) or presence of 20% fetal bovine serum as a stimulus. Migration was reduced in AoSMCs obtained from Gucy1a3<sup>-/-</sup> animals compared to WT in the presence of fetal bovine serum. Without stimulation (control), migration of cells was limited and no difference was evident between genotypes (Figure 3B). Cell proliferation was quantified either manually (Neubauer chamber) or using an automated method after DAPI staining in independent experiment series. Cells started to proliferate after 24 hours in culture, but in both experiments Gucy1a3<sup>-/-</sup> AoSMCs proliferated significantly less that WT AoSMCs (Figure 3, C and D). Cytokine array analysis performed on medium harvested after 96 hours of growth in culture from WT and Gucy1a3<sup>-/-</sup> cell cultures revealed no differences between genotypes (Supplemental Figure S3).

Effect of Modulation of the NO Pathway on Proliferation in Mouse and Human AoSMCs

To further define the role of the NO pathway in SMC proliferation, we treated WT mouse AoSMCs and human aortic smooth muscle cells (huAoSMCs) with the NO donor SNP<sup>34,35</sup> or the sGC inhibitor ODQ<sup>32,36</sup>. Exposure to SNP significantly reduced huAoSMC proliferation after 48 hours of culture with 24 hours of treatment at the highest concentration used (200 μmol/L). After prolongation of the culture time (72 hours, with 48 hours of

---

_Figure 1_ Atherosclerotic lesions in Gucy1a3<sup>-/-</sup> mice. A: Representative images of oil red O<sup>0</sup>-stained aortic roots and lesion area quantification in the aortic root of animals receiving standard diet (SD) or high-fat diet (HFD). B: Representative images of the oil red O<sup>0</sup>-stained aortic roots and quantification of the lesion area at the aortic root of an independent group of animals receiving SD. C: Representative images of the oil red O<sup>0</sup>-stained aortas and quantification of the area covered by plaques in the aorta examined en face in HFD animals. D: Representative images of the oil red O<sup>0</sup>-stained aortas and quantification of the area covered by plaques (%) in the aorta examined en face in SD animals. Values of individual animals are additionally depicted (dot or square). Parenthesis indicates number of animals per group. All mice are in a genetic Ldlr-deficient background (Ldlr<sup>-/-</sup>). Data are presented as means ± SD (A–D). *P < 0.05, **P < 0.01. Scale bar = 100 μm (A and B).
modulation of SMC in the previously isolated WT and Gucy1a3−/− AoSMCs. The deletion of Gucy1a3 was confirmed in Gucy1a3−/− AoSMCs (Figure 5A). The transcriptional expression of a marker of the contractile phenotype (calponin, Cnn1) was strongly enhanced in Gucy1a3−/− AoSMCs (40-fold) compared to WT, whereas genes of markers of the synthetic phenotype (osteopontin, Spp1; thrombospondin, Tspd1) were only modestly induced at the mRNA level (Figure 5A). The enhanced expression of Cnn1 was confirmed at the protein level, because it was not detected in WT but abundantly found in Gucy1a3−/− AoSMCs (Figure 5B). α-Actin, an additional marker of the contractile phenotype, was also significantly more abundant in Gucy1a3−/− cells at the protein level (Figure 5B). In contrast, the level of the synthetic phenotype marker osteopontin was not differentially expressed at the protein level between genotypes (Figure 5B). This suggests that in these AoSMCs the deletion of Gucy1a3 preserves a contractile phenotype (ie, the corresponding marker proteins are reduced in WT and proteins are accordingly more abundant in Gucy1a3−/− SMCs).

To determine whether the preserved expression of markers of the contractile phenotype was specific for cultured AoSMCs, which mimic the intimal SMCs rather than medial AoSMCs present in basal conditions in the aorta, we analyzed their expression levels in the thoracic aorta of Gucy1a3−/−/Ldlr−/− and Ldlr−/− receiving SD, where plaque area was minimal in both genotypes (Figure 1D). In SMCs harvested from this lesion-free aorta, transcriptional expression of calponin was not different between genotypes (Figure 5C) and accordingly expression of osteopontin and thrombospondin was not detected in this tissue (data not shown), indicating that the contractile phenotype remained preserved also in the presence of Gucy1a3 under these conditions.

Expression of Genes of the NO-sGC-cGKI Pathway in Mouse AoSMCs

Finally, we studied the expression of genes of the NO-cGMP pathway in cells and atherosclerotic tissues. Genes of the NO-sGC-cGKI pathway were up-regulated at the mRNA level in Gucy1a3−/− AoSMCs, especially cGKI (Figure 6A). cGKI, alias protein kinase G (PKG1), overexpression was also observed at the protein level because it was not detected in WT but was present in Gucy1a3−/− AoSMCs (Figure 6A).

To determine whether the overexpression was specific for cultured AoSMCs mimicking the intimal state of the atherosclerosis lesion, the near lesion-free thoracic aorta from Gucy1a3−/−/Ldlr−/− and Ldlr−/− animals receiving SD was examined as outlined before. Gucy1a3 deficiency was confirmed and, in contrast to cultured AoSMCs, the transcriptional expression of cGKI was not enhanced in this tissue derived from Gucy1a3−/− but rather slightly reduced compared to WT tissue (Figure 6B). No differential
regulation was observed for Gucy1b3 or Gucy1a2 (Figure 6B). In nonvascular tissue (heart) deficiency of Gucy1a3 was confirmed and differential regulation of the genes of the NO-sGC-cGKI pathway was not observed (Figure 6C). Likewise, cGKI was similarly expressed at the protein level in heart tissue obtained from Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup>Gucy1a3<sup>−/−</sup> (Figure 6D).

**Discussion**

This study demonstrates that deletion of the α1-subunit of the sGC, which is encoded by Gucy1a3, attenuates atherosclerotic lesion formation in a murine model in vivo. This protective effect was most pronounced at moderate proatherosclerotic conditions (Ldlr<sup>−/−</sup> genetic background with normal chow) but still present during severe atherosclerosis (Ldlr<sup>−/−</sup> fed hypercholesteremic chow). This surprising observation suggests that Gucy1a3 promotes the formation of atherosclerotic lesions in mice. We suggest that this deleterious effect is related to a function of sGC in SMCs, which promotes their migration and proliferation because SMCs deficient for Gucy1a3 proliferated less and exhibited a reduced migratory activity in vitro. A similar anti-proliferative effect was also observed in human SMCs by pharmacological inhibition of the sGC. On the contrary, high concentrations of exogenously applied NO exhibited a similar inhibitory potency, suggesting that NO exerts opposite effects depending on its concentration. Gene expression profiling indicates that the reduced proliferative activity of Gucy1a3 deficient SMCs is related to a preservation of the contractile phenotype of SMCs. Accordingly, our results indicate that sGC activity is required to promote the switch from the contractile to the synthetic phenotype, which aggravates under proatherosclerotic condition lesion development.

We assessed the role of sGC in atherosclerosis because GUCY1A3 is among the genes identified in human genome-wide association studies to contribute to the risk of coronary artery disease and myocardial infarction (ie, the major manifestations of atherosclerosis).<sup>5,12,38</sup> Furthermore, a rare loss-of-function mutation in GUCY1A3 was identified in an extended family presenting with premature myocardial infarction. Thus, we expected an aggravated atherosclerosis in Gucy1a3 deficient mice. However, we observed surprisingly the opposite in our initial experimental series (ie, a markedly reduced atherosclerotic lesion size at the aortic root in Gucy1a3<sup>−/−</sup> mice; consequently, platelet aggregation accelerated and thrombus formation enhanced, which is perfectly consistent with an increased risk of the acute event.
myocardial infarction. In contrast, the current study points to a proatherosclerotic role of Gucy1a3 in SMC as also demonstrated for the cGKI by Wolfsgruber et al.25

In aortic lesions, collagen content was reduced in Gucy1a3−/− mice, whereas macrophage content remained unaltered. This further underlines the hypothesis that SMCs are accountable for the observed effect, although pharmacological activation of sGC exerted antiatherosclerotic effects in ApoE-deficient mice reportedly because of a modulation of macrophage functions.36 Recruitment, adhesion, and, finally, migration of monocytes and lymphocytes into the vessel wall and atherosclerotic plaques are crucial steps initiating and fostering the disease by local inflammatory processes.37,40 These processes may be modulated through Gucy1a3, and reportedly activation of sGC limits inflammatory processes through cGMP and cGKI signaling.41,42 However, we did not find significant differences in macrophage content in lesions and also cytokine levels in murine plasma, reflecting the inflammatory processes were not altered. In contrast, phenotypically switched SMCs are responsible for matrix and collagen synthesis in response to injury. This transition from a quiescent, highly specialized contractile state (medial SMC) to a proliferative, migratory, and synthetic phenotype is accompanied by an altered localization of SMC toward the intima (intimal SMC).37,43 As a consequence, intimal SMCs differ functionally from medial SMCs.3,37,44 SMCs in culture also undergo this phenotypical switch and mimic effects produced during the atherosclerosis process.45,46

Our in vitro data demonstrate that primary AoSMCs derived from Gucy1a3-deficient mice migrate and proliferate to a lesser extent than AoSMCs from WT animals. The pharmacological inhibition of sGC in human AoSMCs mimicked the antiproliferative effect of Gucy1a3 deficiency. Exogenously applied NO at high concentrations exhibited likewise an antiproliferative effect in human AoSMCs as well as in mouse WT AoSMCs. This is consistent with previous results using NO donors25 and corroborates the classic protective role of NO.47 The antiproliferative effect of NO is most likely independent of sGC/cGKI, as demonstrated in murine cells in this study and also previously suggested,25,27,29,48 whereas sGC (current study) and

Figure 4 Proliferation of mouse and human aortic smooth muscle cells (SMCs) on pharmacological treatment. Human aortic smooth muscle cells (huAoSMCs; A–C) and primary mouse aortic SMCs (D and E) harvested from wild-type animals were used. A: HuAoSMCs were treated for 24 or 48 hours with the NO-donor sodium nitroprusside (SNP; 50, 100, 200 μmol/L) starting 24 hours after initial seeding, and cell number was assessed after the treatment interval. B: Effect of 10 μmol/L 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ) treatment for 20 or 44 hours starting 4 hours after cell seeding on cell proliferation in huAoSMCs. Controls were treated using solvent (dimethylformamide). C: HuAoSMCs were treated for 24 hours with ODQ started 24 hours after seeding. Controls were treated with solvent and proliferation was assessed after the treatment interval. D: Proliferation of mouse AoSMCs treated for 24 or 48 hours with 200 μmol/L SNP started 24 hours after seeding. E: Mouse AoSMCs were treated for 24 or 48 hours with 20 μmol/L ODQ alone or combined with 200 μmol/L SNP, controls received the solvent of ODQ (dimethylformamide). *P < 0.05, **P < 0.01, and ****P < 0.0001.
cGKI activation at low NO concentrations exert a proliferative effect onto SMCs.

Further support for the idea that SMCs deficient for Gucy1a3 retain their highly specialized contractile phenotype comes from gene expression studies performed on cultured AoSMCs. Marker proteins like calponin and α-actin, indicative of a contractile phenotype, are expressed at the mRNA and protein level at higher amounts in SMCs with Gucy1a3 deficiency. Western blot analysis and quantification of cGKI (D). Staining for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) indicates equal loading of gels. *P < 0.05, **P < 0.01, and ****P < 0.0001.

**Figure 5** Expression of contractile and synthetic marker proteins in mouse aortic smooth muscle cells, aorta, and heart. A: Relative mRNA levels of Gucy1a3, as well as contractile (calponin, Cnn1) and synthetic marker proteins (osteopontin, Spp1; thrombospondin, Thsd1) in cultured primary mouse aortic smooth muscle cells from wild-type (WT) and Gucy1a3−/− animals. B: Western blot analysis of calponin, α-actin, and osteopontin in cultured primary mouse aortic smooth muscle cells. Staining for α-tubulin indicated equal loading of gels. For α-actin and osteopontin, Western blot quantification is available on right panels. C: Relative mRNA levels of calponin in lesion-free aorta from Ldlr−/− and Gucy1a3−/−/Ldlr−/− animals under SD. n = 3 to 4 per group (A and C). *P < 0.05, **P < 0.01, and ****P < 0.0001.
isolated from Gucy1a3-deficient mice than in cultured SMCs harvested from WT. In contrast, protein levels for osteopontin, which indicates a synthetic phenotype, were not altered. Interestingly, expression of calponin in lesion-free aortic tissue was not different between genotypes. This suggests that culture of SMCs initiates the down-regulation of calponin, α-actin, and possibly other contractile proteins. Gucy1a3 and thus sGC activity is required to promote this down-regulation. However, if SMCs are in their physiological environment in the absence of atherosclerotic environmental stimuli (lesion-free aorta) the phenotypic switching is also prevented in WT aorta. Currently, the molecular mechanisms that are involved in sGC/cGKI dependent promotion of phenotypic switching on injury are unknown. These may include the modulation of transcription factors being responsible for the preservation of the contractile state (eg, serum response factor, myocardin) or epigenetic mechanisms preventing access to key sites on the genome.

Several possible causes for the atheroprotective effect of Gucy1a3 deficiency were excluded. First, other genes of the NO-cGMP-cGKI signaling pathway were expressed at higher levels in cultured primary AoSMCs on Gucy1a3 deletion. This included Gucy1a3β, Gucy1a2, and specifically cGKI. For the latter, an enhancement was also demonstrated at the protein level. However, in light of the fact that a distinct up-regulation was neither observed in lesion-free aortic tissue (where SMCs retain their contractile state) nor in heart tissue, we conclude that the differential expression level is related to the phenotypic switch occurring in WT but not in Gucy1a3−/− AoSMCs. Consequently, cGKI is not up-regulated in Gucy1a3−/− AoSMCs but rather down-regulated in WT and the differences observed are more likely a consequence rather than being causative. Second, altered arterial pressure is unlikely having contributed to the protective effect of Gucy1a3 deficiency. Arterial pressure in Gucy1a3−/−/Ldlr−/− mice receiving HFD was slightly increased, which is in accordance with previous studies.32,49 If anything, enhanced pressure is known to exacerbate atherosclerotic lesions. Third, body mass was unaffected and plasma lipid levels only minorly altered by Gucy1a3 deficiency. Specifically, LDL levels were unchanged by the lack of Gucy1a3 in proatherosclerotic Ldlr−/− mice, excluding a metabolically driven protective effect.

In summary, our data demonstrate that loss of Gucy1a3 exerts an atheroprotective effect in prone mice. The in vivo and in vitro data suggest that Gucy1a3 and sGC activity promotes phenotypic switching of SMC from a quiescent contractile to a synthetic proliferative state. The preservation of the contractile phenotype may be fostered by inhibition of sGC in SMC and it remains to be clarified if this mechanism can be exploited therapeutically. However, a potential negative impact of sGC inhibition on platelet function that promotes platelet aggregation has to be considered.

Acknowledgments

We thank Maren Behrensen, Annett Liebers, Sandra Wrobel, Sabine Stark, and Petra Bruse for excellent technical assistance.

Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2016.04.010.

References