Chapter 6

Genetic variation at the Quaking locus associates with clinical restenosis after PCI and induces vascular smooth muscle cell dysfunction
ABSTRACT

**Introduction:** The mRNA binding protein Quaking (Qk) is a key regulator of neuron myelination, while more recent data point to a pivotal role in embryonic arteriogenesis. Therefore, we hypothesized that Qk might exert regulatory functions in vascular patency responses such as restenosis, a frequent complication of percutaneous coronary intervention (PCI).

**Methods and results:** We addressed the role of Qk in in-stent restenosis by analysis for association of single nucleotide polymorphisms (SNPs) throughout the Qk gene and the risk of restenosis in the prospective GENetic DEterminants of Restenosis (GENDER) study (N=3,104 patients). Seven out of twelve tested SNPs associated with risk for or protection against restenosis. The strongest associations were found for SNPs located close to the Qk transcriptional start site (2786 T/C: HR: 2.14, 95%CI: 1.24-3.68, P=0.006) and in intron 3 (57896 A/G: HR: 0.74, 95%CI: 0.6-0.92, P=0.005 and 65752 A/G: HR: 1.5, 95%CI: 1.14-1.98, P=0.003). Importantly, patients carrying the 65752G risk allele but lacking the protective 57896G allele were at a high risk to develop restenosis (HR: 1.8, 95%CI: 1.3-2.5, P<0.001). In vitro characterization of Qk deficient vascular smooth muscle cells (VSMC) revealed severely compromised cell viability, as evidenced by a decreased resistance to stress, decreased cellular proliferation, migration and extracellular matrix production. In keeping with this notion, Qk deficient Qk<sup>−/−</sup> mice showed markedly decreased neointima formation in a mouse model of in-stent restenosis.

**Conclusions:** We are the first to identify a link between Qk and the risk for in-stent restenosis after PCI, which may be attributable to its regulatory role in VSMC function. Therefore, Qk could serve as a marker for TVR risk prediction as well as a novel target to reduce restenotic risk.
INTRODUCTION

The mRNA-binding protein Quaking (Qk) is a member of the signal transduction and activation of RNA (STAR) family of transcription factors. It is located on chromosome 6 in humans and chromosome 17 in mice and is highly conserved during mammalian evolution. As a result of alternative splicing, Qk has three main isoforms derived from mRNA transcripts of 5, 6 and 7 kb, notably Qk-5, Qk-6 and Qk-7. Qk-5 is the most abundantly expressed isoform and is essential during embryogenesis, whereas Qk-6 and Qk-7 are primarily expressed post-natally. Qk has been found to play a critical role in driving oligodendrocyte differentiation, positioning Qk as a central regulator of myelination of both the central and peripheral neural network. Illustratively, the Qk viable mouse mutant (Qk\(^v\)), which carries a 1 MB deletion in the promoter region resulting in Qk-knockdown, displays severe developmental defects, characterized by a phenotype of seizures and movement-dependent tremors. However, its high expression levels in heart, brain, lung and testis as well as its broad range of putative Qk response genes points to a more general regulatory function of Qk, including vascular function. Support for this notion came from a study showing a pivotal role of Qk in embryonic vascular development, as N-ethyl-N-nitrosourea (ENU) induced Qk as well as Qk-null mouse mutants were seen to die during development because of a lack of embryonic vascular remodelling. This lethality was found to be due to a lack of local pericyte differentiation into vascular smooth muscle cells (VSMC), preventing the maturation of the yolk sac vasculature. Furthermore, Qk expression has been detected in murine VSMC of the aorta and coronary arteries, suggesting that Qk may also influence VSMC function after gestation.

In-stent restenosis after percutaneous coronary intervention (PCI) is largely dependent on VSMC proliferation. This process, harbouring potentially serious clinical consequences, is characterized by proliferation of cellular components of the vascular wall, in particular VSMCs in response to vascular injury, leading to re-occlusion of the stented arterial segment. Previous studies have established the significant contribution of various genetic factors to the occurrence of restenosis. Given the proposed regulatory role of Qk in arteriogenesis and VSMC function, we hypothesized that single nucleotide polymorphisms (SNPs) in both the promoter and coding region of the Qk gene could influence Qk expression and function, and could therefore potentially influence the occurrence of restenosis in patients who underwent PCI. Furthermore, we assessed the effects of reduced Qk expression on neo-intimal hyperplasia in vivo and studied the specific effects of Qk abrogation in aortic-derived VSMCs of Qk\(^v\) mice in vitro. We demonstrate strong two-directional associations of various Qk SNPs with the risk of restenosis after PCI and also, based on direct in vivo and in vitro data, propose a mechanistic explanation for this intriguing observation.
METHODS

GENDER project study design
The present study population has been described previously. In brief, the GENDER project was designed to study the association between various gene polymorphisms and clinical restenosis, defined by target vessel revascularization (TVR). Patients eligible for inclusion were treated successfully for stable angina, non-ST-elevation acute coronary syndromes or silent ischemia by PCI in 4 out of 13 referral centers for interventional cardiology in the Netherlands. Patients treated for acute ST elevation myocardial infarction were excluded. Patients suffering from events occurring within 1 month after PCI were also excluded from analysis, as these events were attributable predominantly to sub-acute stent thrombosis or occluding dissections, rather than to restenosis.

PCI procedure
Experienced operators, using a radial or femoral approach, performed standard angioplasty and stent placement. Before the procedure, patients received aspirin (300mg) and heparin (7500 IU). The use of intracoronary stents and additional medication, such as glycoprotein IIb/IIIa inhibitors, were carried out at discretion of the operator. In case a stent was implanted, patients received either ticlopidin or clopidogrel for at least 1 month following the procedure depending on local practice. During the study, no drug-eluting stents were used.

Follow-up and study end points
Follow-up lasted for at least 9 months, except when a coronary event occurred. TVR, either by PCI or coronary artery bypass grafting (CABG), was designated the primary end point. An independent clinical events committee evaluated the clinical events. The study protocol meets the criteria of the Declaration of Helsinki and was approved by the Medical Ethics Committees of each of the participating institutions. Written informed consent was obtained from all participating patients before the PCI procedure.

Genotyping
Blood was collected in EDTA tubes upon admission to hospital and genomic DNA was extracted following standard procedures. Validated polymorphisms in the promoter and in each linkage disequilibrium (LD)-block in the Qk gene, as defined by the algorithm of Gabriel et al., were selected using the program Haploview with genotype data from the International HapMap Consortium. The -7528 C/T (rs3857503), -4539 T/C (rs3904720), -2616 C/T (rs3857504), 2786 A/G (rs3763197), 34084 C/A (rs2759393), 57896 A/G (rs1737603), 65752 A/G (rs6937072), 71659 T/C (rs1016502), 106999 A/G (rs9458849), 120103 G/A (rs9364692), 150256 C/T (rs715020) and 161241 G/T (rs1047944) poly-
Quaking SNPs associate with restenosis and induce VSMC dysfunction

Morphisms were selected on the basis of their high minor allele frequency (>5%) and measured using the Sequenom Massarray genotyping platform (Sequenom, San Diego, CA, USA). A multiplex assay was designed using Assay designer software (Sequenom). All PCR reactions had a final volume of 5 μl and contained standard reagents and 5 ng of genomic DNA. After PCR a primer extension reaction was performed to introduce mass-differences between alleles and, after removing salts by adding resin, approximately 15 nl of the product was spotted onto a target chip with a 384 patches containing matrix. Mass differences were detected by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF; Autoflex; Bruker Daltonics, Billerica, MA, USA) and genotypes were assigned real-time using Typer 4.0 software (Sequenom). As quality controls, 5-10% of the samples were genotyped in duplo. Cluster plots were made of the signals from the low and the high mass allele. Scoring was performed by two independent researchers. Disagreements or vaguely positioned dots produced by Genotyper 4.0 (Sequenom) were left out of the results.

Femoral cuff induced vascular hyperplasia

Male Qk<sup>v</sup> mice (N=6) on a C57Bl6-J background together with wild-type age and sex-matched C57Bl6-J mice (N=6) were used (Jackson Laboratories, Bar Harbor, ME, USA). Mice were fed with water and chow diet ad libitum. At the age of 12 weeks, mice were anaesthetized with an intraperitoneal injection of 5 mg/kg Midazolam (Roche, Basel, Switzerland), 0.5 mg/kg Medetomidine (Orion, Helsinki, Finland) and 0.05 mg/kg Fentanyl (Janssen, Geel, Belgium). The femoral artery was dissected from its surroundings and sheathed with a non-constrictive cuff. Animals were sacrificed 21 days after cuff placement. Preparation for histological analyses was performed as described previously. All animal work was approved by institutional regulatory authority and carried out in compliance with guidelines issued by the Dutch government.

Temporal gene expression cuff model

15 C57Bl6-J mice were maintained and provided with a femoral cuff as described above. At 0, 1, 2, 3, 7 and 14 days after placement of the femoral cuff, the mice were sacrificed and cuffed arteries were dissected and stored in liquid nitrogen. Trizol (Invitrogen) was used to extract total RNA from the femoral arteries, and the samples were subjected to DNAse I treatment (Promega, Madison, WS) to ensure that the samples were rid of genomic DNA. Subsequently, cDNA was generated using RevertAid M-MuLV reverse transcriptase (Fermentas, Burlington, Canada) according to manufacturer’s protocol. Semi-quantitative expression of the QKI gene was performed using the Assays-on-Demand primer and probe set for QKI (Mm 01318927_m1) and SYBR-Green (Eurogentec, Liege, Belgium) on a Bio-Rad iCycler (Bio-Rad, Hercules, CA). GAPDH was utilized as
housekeeping gene. Relative gene expression was calculated by determining the ΔΔCt value of QKI versus GAPDH.

**Histological analysis and quantification of cuffed femoral artery lesions**

All samples were routinely stained with hematoxylin-phloxine-saffron (HPS). Weigert’s elastin staining was used to visualize elastic laminae. VSMCs were visualized with α-SM actin staining (DakoCytomation, Glostrup, Denmark). Collagen content was determined using a 0.1% (w/v) solution of Sirius Red F3B dye (BDH Laboratory Supplies, Poole, UK) in aqueous picric acid for 1h. Unbound dye was removed by washing the sections with 0.01M HCl. Subsequently, the collagen-bound dye was dissolved by incubating the sections with 0.1M NaOH for 30 minutes. The amount of medial VSMC and collagen content was determined by morphometry (Leica Qwin, Wetzlar, Germany) and expressed as the percentage of total medial area consisting of α-SM actin- or Sirius red-positive area in six equally spaced serial cross-sections in all animals. Monocytes were detected with the use of an anti-CD45 antibody (Pharmingen, San Diego, CA, USA). Incorporation of 5-bromo-2′-deoxyuridine (BrdU, Sigma) into DNA as a marker of DNA synthesis was used to determine the rate of cell proliferation in cuffed vessel segments. Mice were injected intraperitoneally with 25 mg/kg BrdU three times at 72, 48, and 24 hours prior to sacrifice. Sections were incubated with a mouse monoclonal anti-BrdU antibody (DakoCytomation). Apoptotic cells were identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining according to manufacturers protocol (Roche). The number of labeled nuclei per cuffed artery were counted in six equally spaced cross-sections and expressed as a percentage of the total number of nuclei. For all immunohistochemical stainings, sections were incubated with an isotype-matched IgG at an equal titer as the primary antibody to serve as a control.

**VSMC isolation and culture**

Experiments were performed using primary mouse vascular smooth muscle cells (mVSMCs) derived by outgrowth from fragments of mouse aorta from C57Bl/6-J and Qk(v) mice seeded on fibronectin-coated plates (1 mg fibronectin dissolved in 10 mL PBS; solution left on culture dish for approximately 15 minutes). The tissue fragments and outgrowth cells were maintained in DMEM culture medium containing 20% fetal calf serum and penicillin/streptomycin (0.01 Units/ml and 0.01 μg/mL, respectively), at 37°C and 5% CO2 unless specified otherwise. The purity of VSMC outgrowth was assessed immunohistochemically using anti-mouse smooth muscle α-actin and peroxidase-conjugated goat-anti-mouse immunoglobulins, generally yielding 90-95% smooth muscle α-actin positive cells. In designated experiments, VSMCs were treated
with freshly isolated oxLDL (20 μg/mL), VLDL (50 μg/mL), and lipopolysaccharide (50 ng/mL) for the specified times. Experiments were performed with cells at passages 3-4.

**Extracellular matrix production assay**

Primary wild-type and Qk⁺ mVSMCs were seeded at a density of 1.5x10⁴ cells/cm² and grown for 24h in culture medium described above. Subsequently, the cells were cultured in DMEM containing 1% FCS and P/S and Gln for 16h. Next, the cells were extensively washed with PBS and fixed for 30 minutes using Formalfix (Thermo Scientific, Waltham, MA, USA). To assess total cellular collagen production, the cells were stained with Sirius Red F3B dye as described above. Concomitantly, a calibration curve was prepared by plating increasing concentrations of gelatin in PBS, which were air-dried and fixed in Formalfix. Total collagen content was determined spectrometrically at an optical density of 550nm on a microplate reader.

**DNA synthesis assay**

To quantify DNA synthesis, explant-derived mVSMCs from wild-type and Qk⁺ aortic explants were seeded at a density of 1x10⁴ cells/cm² and cultured in medium described above, after which the cells were cultured for 24h in serum-free DMEM. Subsequently, the cells were incubated for 12h with [³H]-thymidine (10μCi/mL) in DMEM containing 10% FCS, after which cell associated [³H]-thymidine was determined by assaying the trichloroacetic acid precipitable counts with Aquasol-2 (PerkinElmer, Waltham, MA, USA) on a Beckman LS3801 scintillation counter (Beckman, Fullerton, CA, USA).

**Cellular migration assay**

Primary mMVSMCs were cultured in medium described above, and 24h prior to seeding into a transwell plate, cells were incubated in culture medium containing 1% FCS and Gln. DMEM containing 10% FCS, P/S, Gln as well as the chemotactic peptide n-formyl-methionine-leucine-phenylalanine (fMLP; 0.1nM) was added to the basolateral side of a semi-permeable membrane with a pore size of 12 μm (ChemoProbe, UK). Subsequently, the cells were detached from the culture dish using trypsin-EDTA and 2x10⁴ cells were seeded onto the membrane and allowed to migrate for 24h. After centrifugation, adherent mVSMCs were fixed, stained with Mayer’s hematoxylin, and the number of cells that had completely migrated to the basolateral side of the chamber was assessed microscopically and scored manually.

**Cellular outgrowth assay**

Aortas were harvested from both wild-type and Qk⁺ mice after sacrificing. The aortas were excised from the base of the aortic arch until the iliac bifurcation. Subsequently, the aortas were cut longitudinally to expose the luminal portion of the vessel and the
endothelial cells were removed by gently scraping. After this, the aortas were cut into approximately 0.5 cm² pieces, and placed on fibronectin-coated culture dishes. The explants were cultured in DMEM containing 20% FCS, P/S and Gln. After 7 days, phase-contrast photomicrographs of cellular outgrowth from individual pieces of aortic tissue were taken. Using Leica QWin software, the photomicrographs were used to quantitate both the total area of the designated piece of tissue and its corresponding cellular outgrowth was calculated based on: \( A_{\text{outgrowth}} = \left( \text{Total Area} \left( A_{\text{outgrowth}} + A_{\text{tissue}} \right) \right) - A_{\text{tissue}} \).

**Western blot analysis**

Protein expression was assessed by Western blot analysis with chemiluminescence detection. Qk-5 was detected using a rabbit polyclonal anti-human Qk antibody (Bethyl Laboratories, Montgomery, TX, USA). As a reference α-tubulin was detected using a monoclonal antibody (Clone B-5-1-1, Sigma). Horseradish peroxidase conjugated anti-rabbit and anti-mouse IgG F(ab')₂ (Amersham Pharmacia, Roosendaal, the Netherlands) were used as secondary antibodies. Bound Fab fragment was detected with enhanced chemiluminescent reagents (Boehringer, Ingelheim, Germany) and exposed on Kodak X-Omat blue XB-1 film.

**Statistics**

Allele frequencies were determined by gene counting. The Chi-squared test was used to test whether genotype frequencies at the SNP locus complied with Hardy-Weinberg equilibrium. Hazard ratios (HR) with 95% confidence intervals (CI) were calculated using a Cox-proportional hazards model. All analyses were adjusted for clinical and procedural risk factors for restenosis, such as diabetes, smoking, hypertension, stenting, total occlusion and residual stenosis >20%. All polymorphisms were also assessed using dominant and recessive models, and the model with the lowest Akaike information criteria was used in the multivariable regression analysis. SPSS software (version 12.0.1, SPSS Inc, Chicago, IL, USA) was used for all GENDER analyses. All *in vitro* and *in vivo* results were analysed via a 2-tailed Student’s t-test and ANOVA when appropriate using Graphpad Prism 4.0 Software (Graphpad software, La Jolla, CA, USA). P-values <0.05 were considered significant.

**RESULTS**

**Patient characteristics**

Characteristics of the GENDER cohort have previously been described in detail. In brief, a total of 3,146 patients had a complete follow-up (99.3%) with a median duration of 9.6 months (interquartile range 3.9 months). Out of 3,146 patients, 42 had an event
Quaking SNPs associate with restenosis and induce VSMC dysfunction in the first 30 days. These patients were excluded from further analysis, according to the protocol. Baseline characteristics of the population are shown in Table 1. Bare metal stents were used in 2309 patients (74.4%), whereas the rest of the population was treated with plain balloon angioplasty. Of all patients, 304 patients (9.8%) underwent TVR during follow-up, 51 patients (1.6%) died and 22 (0.7%) had a myocardial infarction.

Quaking genotyping
Due to lack of genomic DNA or inconclusive genotyping (vaguely positioned dots or genotyping errors), not all 3,104 patients could be successfully genotyped. For each SNP, genotype counts and minor allele frequencies are presented in Table 2 and figure 1A. There were no significant deviations from Hardy-Weinberg equilibrium. Out of the 12 SNPs we assessed, 4 were found to significantly increase the risk of TVR using multiple regression analysis: -2616 T/T (promoter region; HR 1.91, 95% CI 1.11-3.28), 2786 G/G (intron 1; HR 2.14, 95% CI 1.24-3.68), 65752 G/G (intron 3; HR 1.57, 95% CI 0.87-2.8) and 106999 G/G (intron 3; HR 1.51, 95% CI 1.15-1.98) (Table 2 and figure 1A).

Table 1. Demographic, clinical and lesion characteristics of patients with TVR and without TVR during follow-up.

<table>
<thead>
<tr>
<th></th>
<th>TVR (n=304)</th>
<th>No TVR (n=2800)</th>
<th>Total (n=3104)</th>
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</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>61.7 ± 10.1</td>
<td>62.2 ± 10.8</td>
<td>62.1 ± 10.7</td>
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<tr>
<td>BMI (kg.m⁻²)</td>
<td>26.9 ± 3.7</td>
<td>27.0 ± 3.9</td>
<td>27.0 ± 3.9</td>
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<tr>
<td>Male sex</td>
<td>220 (72.4%)</td>
<td>1996 (71.3%)</td>
<td>2216 (71.4%)</td>
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<tr>
<td>Diabetes</td>
<td>63 (20.7%)</td>
<td>390 (13.9%)</td>
<td>453 (14.6%)</td>
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<td>Hypercholesterolemia</td>
<td>188 (61.8%)</td>
<td>1702 (60.8%)</td>
<td>1890 (60.9%)</td>
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<td>Hypertension</td>
<td>138 (45.4%)</td>
<td>1121 (40.0%)</td>
<td>1259 (40.6%)</td>
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<td>Current smoker</td>
<td>62 (20.4%)</td>
<td>700 (25.0%)</td>
<td>762 (24.5%)</td>
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<tr>
<td>Family history of MI</td>
<td>121 (39.8%)</td>
<td>977 (34.9%)</td>
<td>1098 (35.4%)</td>
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<tr>
<td>Previous MI</td>
<td>109 (35.9%)</td>
<td>1130 (40.4%)</td>
<td>1239 (39.9%)</td>
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<td>Previous PCI</td>
<td>64 (21.1%)</td>
<td>493 (17.6%)</td>
<td>557 (17.9%)</td>
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<td>Previous CABG</td>
<td>36 (11.8%)</td>
<td>340 (12.1%)</td>
<td>376 (12.1%)</td>
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<td>Stable angina</td>
<td>198 (65.1%)</td>
<td>1881 (67.2%)</td>
<td>2079 (67.0%)</td>
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<td>Multivessel disease</td>
<td>148 (48.7%)</td>
<td>1284 (45.9%)</td>
<td>1432 (46.1%)</td>
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<td>Peripheral vessel disease</td>
<td>12 (3.9%)</td>
<td>92 (3.3%)</td>
<td>104 (3.4%)</td>
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<td>Lipid lowering medication</td>
<td>171 (56.3%)</td>
<td>1516 (54.1%)</td>
<td>1687 (54.3%)</td>
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<td>Restenotic lesions</td>
<td>27 (8.9%)</td>
<td>181 (6.5%)</td>
<td>208 (6.7%)</td>
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<td>Total occlusions</td>
<td>56 (18.4%)</td>
<td>372 (13.3%)</td>
<td>428 (13.8%)</td>
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<td>Type C lesion</td>
<td>94 (30.9%)</td>
<td>708 (25.3%)</td>
<td>802 (25.8%)</td>
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<td>Proximal LAD</td>
<td>70 (23.0%)</td>
<td>619 (22.1%)</td>
<td>689 (22.2%)</td>
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<td>RCX</td>
<td>75 (24.7%)</td>
<td>764 (27.3%)</td>
<td>839 (27.0%)</td>
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BMI: body mass index, MI: myocardial infarction, LAD: left anterior descending branch of the left coronary artery, RCX: circumflex branch of the left coronary artery
Table 2. Distribution and multivariate analysis of 12 Qk polymorphisms in association with TVR.

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Minor allele frequency</th>
<th>Genotype Counts</th>
<th>TVR risk (%)</th>
<th>Genetic model</th>
<th>HR (95%CI)</th>
<th>P-value</th>
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<td>CC</td>
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<td>0.94 (0.79-1.11)</td>
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<td>1393</td>
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<td>2090</td>
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<td>9.7</td>
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Values are corrected for diabetes, smoking, hypertension, stenting, total occlusion and residual stenosis >20%. TVR = target vessel revascularization; HR = hazard ratio; CI = confidence interval
Quaking SNPs associate with restenosis and induce VSMC dysfunction

In contrast, three SNPs were seen to significantly protect against TVR: 34084 A/A (intron 1; HR 0.68, 95% CI 0.52-0.88), 57896 G/G (intron 2; HR 0.74, 95% CI 0.6-0.92) and 71659 C/C (intron 3; HR 0.76, 95% CI 0.61-0.94). Only 5 SNPs were not significantly associated with TVR in the multiple regression analysis model. Importantly, analysis of the sub-cohort of patients who received one or more coronary stents did not reveal any major differences compared to results the total population (table 3 and figure 1B). Therefore, our findings can be extrapolated to the current PCI era in which plain balloon angioplasty is hardly being used anymore.

Interestingly, patients who carry the 65752 A/G and G/G alleles together with the 57896 A/A allele were at high risk of restenosis compared to the rest of the population (HR 1.77, 95% CI 1.29-2.44; P<0.001) during 15 months of follow-up (figure 1C). This particular genotype was observed in 10% of the total population. This demonstrates that
### Table 3. Distribution and multivariate analysis of 12 Qk polymorphisms in association with TVR in the stented subgroup.

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<th>Polymorphisms</th>
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Values are corrected for diabetes, smoking, hypertension, stenting, total occlusion and residual stenosis >20%. TVR = target vessel revascularization; HR = hazard ratio; CI = confidence interval.
specific combinations of protective and hazardous genotypes can have additive effects on outcome in this high risk patient group.

**Effect of Qk abrogation on VSMC function**

Since post-operative restenosis is a fibrocellular hyperplastic response to vascular injury mediated by VSMC dedifferentiation, migration and proliferation, we sought to determine whether altered Qk expression could affect VSMC function. For this, we utilized Qk$^v$ mice, which contain a 1 Mb deletion in the Qk promoter region resulting in ablated transcription. Primary mouse VSMC cultures were established by aortic outgrowth and shown to be SM-a-actin positive by immunohistochemistry. Western blot analysis revealed a marked decrease in Qk-5 expression, the most prominent isoform (figure 2A). Next, we assessed the effect of serum starvation as well as proatherogenic stimuli on Qk-5 expression. We observed a striking down-regulation of Qk-5 in 1% FBS, suggesting that a shift to a non-proliferative phenotype is accompanied by reduced Qk-5 expression (figure 2B). Treatment of VSMC for 24h with either oxLDL, VLDL or LPS did not influence Qk-5 expression, whereas remarkably that of Qk-6 was markedly decreased upon stimulation with oxLDL and VLDL, suggesting differential responses of VSMC Qk isoforms to pro-inflammatory stimuli (figure 2C).

Primary WT and Qk$^v$ VSMCs displayed pronounced phenotypic differences even under non-stimulated conditions. Qk$^v$ cells were characterized by a flattened, enlarged morphology with prominent stress-fibers as opposed to WT cells which displayed a healthy phenotype (figure 3A,B). To gain further insight into the consequences of decreased Qk-5 expression for VSMC phenotype, we assessed cellular proliferation, migration and extracellular matrix production in primary WT and Qk$^v$ VSMC. Qk$^v$ cells displayed a 45% decrease in outgrowth from aortic explants 7d after plating (WT

![Figure 2.](image)

**Figure 2.**

Qk protein expression analysis in primary mVSMCs. Decreased Qk-5 expression is confirmed in Qk$^v$ cells (A). In primary WT mVSMCs, Qk-5 protein expression is markedly reduced upon serum starvation, suggesting a decreased Qk-5 functionality in non-proliferative mVSMCs (B). No differences are observed for Qk-5 expression upon incubation with pro-atherogenic stressors such as oxLDL, VLDL and LPS, but incubation with oxLDL and to a lesser degree VLDL caused a decrease in the Qk-6 isoform protein expression (C). α-tubulin is used as housekeeping control.
Chapter 6

1.8x10^6 ± 5.6x10^5 μm² vs. Qk^v 1.0x10^6 ± 6.0x10^5 μm² VSMC area; N=34; P<0.001; figure 3D). The proliferation rate of primary Qk^v VSMC was 3.5 fold lower than that observed in WT mice as assessed by [3H]-thymidine incorporation (WT 2,149 ± 806 vs. Qk^v 607 ± 292 DPM/well; N=25;P<0.001;figure 3E). Moreover, transwell cellular migration was significantly decreased with Qk^v cells (WT 116 ± 32 vs. Qk^v 40 ± 7 migrated cells; N=5; P<0.001; figure 3C). Finally, Qk^v cells showed a 50% reduction in extracellular matrix production than their WT counterparts (36 ± 1 vs. 18 ± 5 μm/ml; N=6; P<0.05; figure 3F). Altogether, these data point to a significant role of Qk-5 in VSMC homeostasis, underscoring a causal relationship of Qk SNPs and the occurrence of clinical restenosis.

**Restenosis in vivo model analyses**

As a next step we have studied the implication of Qk dependent impairment of VSMC function on restenosis in vivo. Firstly, total vessel area of cuffed femoral arteries in Qk^v and WT mice did not differ at 2 weeks post surgery (WT 19,458 ± 4,942 vs. Qk^v 17,593 ± 4,433 μm²; P=0.42; figure 4A). However, in keeping with our in vitro finding that both the proliferative capacity and production of ECM is reduced in Qk^v VSMC, both medial area (WT 9,870 ± 1,798 vs. Qk^v 7,670 ± 931 μm²; P<0.01) and intimal hyperplasia (WT...
Quaking SNPs associate with restenosis and induce VSMC dysfunction

3,537 1,147 ± vs. Qk⁻/⁻ 1,779 ± 758 µm²; P<0.05; figure 4B,D) were significantly lower in cuff induced femoral artery lesions of Qk⁻/⁻ mice. Of note, luminal stenosis of the cuffed femoral artery segment was considerably lower in Qk⁻/⁻ than in WT mice (20% and 34%, respectively P<0.05; figure 4C,E). These findings suggest that attenuation of Qk gene and protein expression not only affects VSMC function but also results in a clinically relevant decreased fibroproliferative response upon vascular injury. Immunohistochemical analysis of the VSMC content of the neointimal lesions revealed that lesions in Qk⁻/⁻ mice contained a significantly lower percentage of SM-α-actin staining (WT 62.5 ± 4.7% vs. Qk⁻/⁻ 46.5 ± 4.1%; P<0.05; figure 4F). Although not significant, Qk⁻/⁻ mice also tended to display a decreased neo-intimal proliferative response, as assessed by cellular BrdU incorporation (WT 1.30 ± 0.5% vs. Qk⁻/⁻ 0.45 ± 0.15%; P=0.14). No significant differences were seen in apoptosis (TUNEL staining), nor in collagen and CD45⁺ leukocyte content in Qk⁻/⁻ vs. WT lesions (data not shown).

To assess normal Qk gene expression development following cuff placement in WT mice, we performed a temporal Qk gene profile analysis as shown in figure 5. Following
cuff placement, Qk gene expression is unchanged the first 2 days. However, starting at day three until day seven, a significant increase in Qk expression was observed in femoral arteries, coinciding with neointimal hyperplasia. Two weeks later, Qk expression falls back to basal levels. These data indicate that Qk has a regulatory role in VSMC differentiation in response to neointimal proliferative triggers.

**DISCUSSION**

In the present study we demonstrate the association of Qk SNPs with clinical restenosis and suggest that this association is the result of Qk regulated VSMC dysfunction. To date, the majority of studies pertaining to Qk have assessed its role in central nervous system development, studies that have primarily been performed in a murine setting. More recently, human studies and clinical data have started to emerge, and provide links between Qk and human diseases such as Canavan disease and schizophrenia.\textsuperscript{16, 17} In the latter case, SNPs in the Qk gene were seen to associate with the occurrence of schizophrenia in a Swedish cohort, possibly caused by aberrant Qk splicing.\textsuperscript{17, 18} Furthermore, previous work has identified that alterations in Qk isoform expression are linked to the development of glioblastoma multiforme and ataxia.\textsuperscript{19, 20} Our study is the first to assess the impact of Qk SNPs on clinical restenosis and cardiovascular disease. We have identified 7 SNPs that conferred enhanced risk of or protection against TVR, with the most significant associations for SNPs located in the promoter region and introns 1, 2 and 3 of Qk. Importantly, these analyses included a correction for possible confounding factors, thereby increasing the strength of the current analysis. Furthermore, separate analysis of the stented population revealed similar significant outcomes, which
Quaking SNPs associate with restenosis and induce VSMC dysfunction

is particularly relevant for current clinical practice in which plain balloon angioplasty is only rarely employed because of the higher risk of restenosis of this procedure. Of note, only bare metal stents were applied, thereby excluding bias due to interference of Qk with the activity of stent-coated drugs. Remarkably, subjects with both the 57896AA protective allele and 65752AG and GG risk alleles, a genotype prevalent in 10% of the total population, exhibited a 2 fold enhanced risk for restenosis, the strongest of all associations observed before in previous analyses of the GENDER study. Cloning and functional characterization of individual SNPs in vitro will likely clarify the effects of each SNP. Further studies are needed to examine wether protective SNPs confer reduced Qk expression or activity and if deleterious SNPs enhance Qk expression or vice versa.

The in vitro analyses point to a causal involvement of Qk in restenosis. In fact reduced Qk expression results in VSMC dysfunction. Qk-5 protein expression was significantly reduced in Qk- derived VSMCs, whereas expression of Qk-6 and Qk-7 did not appear to be affected in Qk+ mice. It has previously been shown that Qk isoforms show differential patterns of temporal expression during differentiation in various cell types. Interestingly, Qk-/ mice have previously been found to be embryonically lethal, an effect that was attributed to a loss of Qk-5 expression during vascular development and maturation. Our findings suggest that as a result of decreased Qk-5 expression, Qk- VSMCs lose their capacity to function properly, displaying many of the hallmarks of stressed cells, highlighted by a senescent morphology. This was substantiated in four functional assays, all pointing to decreased VSMC function as indicated by altered phenotype and decreased migration, proliferation, outgrowth and extracellular matrix production, features of the cobble-stone shaped synthetic phenotype. Therefore, Qk is likely to have a critical regulatory role in VSMC function and/or differentiation. Li et al. demonstrated that vitelline arteries of Qk null mice, which die at day 10 after gestation, are essentially devoid of a-actin+ VSMCs, a feature that was attributed to an inability of endothelial cells to differentiate into VMSCs pointing to a vital role of Qk in VSMC differentiation. The defective VSMC phenotype could be rescued in vitro by adding Qk and PECAM-1 positive cells to a culture of para-aortic splanchnopleural cells derived from Qk-/- mice, suggesting the presence of a paracrine factor mediating the differentiation of endothelial cells into VSMCs. In addition, Bohnsack et al. demonstrated the paracrine effects of Qk-mediated release of retinoic acid regulating visceral endoderm survival and endothelial cell function. We extend these observations by showing that even decreased, not completely abrogated Qk expression has serious implications on VSMC function in vitro, without requiring paracrine effects of neighboring endothelial cells.

Although we observed both protective and enhanced risk ratio’s of restenosis within our Qk SNP analysis, Qk+ mice displayed a decreased neo-intimal response after femoral cuff placement which was in line with our in vitro observations. This is likely to be caused by decreased VSMC migration and proliferation, as SM-α-actin and, to a lesser extent,
BrdU staining was decreased in neo-intimal lesions of Qk\(^+\) mice. The temporal Qk gene expression analysis strengthens this hypothesis, while Qk expression was significantly raised seven days following femoral cuff placement in wild type mice. Therefore, Qk is imperative for the physiological neo-intimal proliferative response. It should be noted that the 1-MB deletion in the Qk gene in Qk\(^+\) mice not only affects Qk expression, but also that of Parkin as well as of the Parkin co-regulate gene (Pacrg).\(^{23}\) Although we have solid evidence that underscores a direct relationship of Qk with VSMC function, and while others have previously shown that expression of Qk-6 rescues the dysmyelination phenotype of Qk\(^+\) mice,\(^{22}\) it cannot be excluded that parkin or Pacrg are partly responsible for VSMC dysfunction and/or restenosis as well.

The actual downstream effectors in this regulatory role of Qk on VSMC function are hitherto unknown. Recently, Galarneau and Richard, based on a computational analysis for Qk response elements, composed a list of 1,433 putative downstream targets of Qk.\(^3\) These targets included genes involved in development, cell adhesion, morphogenesis, organogenesis, transport, cell differentiation, cell growth and/or maintenance, and cell communication, implicating Qk proteins as regulators of cell fate and proliferation. Many of these putative targets are implicated in cancer, a disease process heavily dependent on (neo)vascularization. Extending these observations, we hypothesize based on these putative targets that Qk has additional functions in cardiovascular disease next to restenosis, such as atherogenesis, vascular and cardiac remodeling.

Finally, our observations strengthen the hypothesis that proteins which are originally associated with central nervous system development and maintenance also play a crucial role in vascular homeostasis.\(^{24}\) In this regard, Nogo-B, a protein involved in the inhibition of axonal growth and repair, was shown to regulate VSMC as well as endothelial cell migration \textit{in vitro} and vascular remodeling \textit{in vivo}.\(^{25}\) Furthermore, Nogo-B overexpression was recently shown to reduce neo-intima formation following vascular injury.\(^{26}\) Although these effects are opposite to the effects of Qk, it shows that genes involved in neural cell maintenance may have substantial influence on vascular cells as well.

In conclusion, Qk SNPs are associated with the occurrence of restenosis in a large prospective cohort. As in this study decreased Qk activity could be linked to VSMC dysfunction, we propose that Qk may serve as a future genetic marker for restenosis risk, as well as a target for anti-restenosis therapy. The development of compounds which directly target the Qk transcriptional route responsible for regulation of VSMC function would be a feasible next step towards direct \textit{in vivo} therapeutic targeting.
REFERENCES


