CARDIOVASCULAR

Genetic interactions in the β-adrenergic receptor/G-protein signal transduction pathway and survival after coronary artery bypass grafting: a pilot study†

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Editor’s key points

• β-Adrenergic antagonists improve survival after coronary artery bypass grafting (CABG), but responses are variable.
• Genetic interactions between the β-adrenergic receptor and a related protein GαS were investigated.
• Mortality was associated with haplotype in patients undergoing CABG.
• Gene–gene interactions between the genes for β-adrenergic receptor and GαS may determine mortality after CABG.

Background. In heart failure, β-adrenergic receptor (βAR) stimulation desensitizes the receptor, uncouples the downstream GαS protein, and diminishes signal transduction. We tested the hypotheses that haplotype-tagging single-nucleotide polymorphisms (htSNPs) within the GαS gene (GNAS) (i) are functionally active and alter GαS expression, (ii) influence survival after coronary artery bypass grafting (CABG), and (iii) interact with βAR SNPs.

Methods. Amplification of GNAS intron 1 was followed by cloning, reporter assays, electrophoretic mobility shift assays, and western blots. In a pilot study, 185 patients on βAR blockade undergoing CABG were studied prospectively. The primary endpoint was cardiac-related mortality at 1 yr.

Results. Two htSNPs defined three common haplotypes with altered reporter activity, allele-specific transcription factor binding, and GαS protein expression (highest in *3 carriers followed by *2 and *1 haplotypes, P=0.013). After CABG, mortality was GNAS diplotype-dependent: *3/*3: 0%; *3/*2: 2.4%; *3/*1: 2.9%; *2/*2: 4.5%; *2/*1: 9.1%; and *1/*1: 20.0% (P=0.004). While β2AR SNPs were not associated with mortality, β2AR Arg16 allele carriers were at higher risk than Gly16 allele carriers (P=0.008). Gene–gene interaction using gene-related risk alleles demonstrated the number of risk alleles to be independently associated with death (hazard ratio 2.3; 95% confidence interval: 1.5–3.5; P=0.003). Carriers of the no-risk allele had higher maximum isoproterenol-stimulated adenylyl cyclase activities than risk allele carriers (P=0.003).

Conclusions. Interactions in the βAR/GαS pathway may be associated with altered mortality after CABG. This could reconcile previously inconclusive data regarding the effects of βAR SNPs on cardiovascular prognosis.

Keywords: adrenergic signalling; mortality; polymorphism; β-receptor; signal transduction; surgery

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Coronary artery disease (CAD) is a multifactorial disorder, a major cause of morbidity and mortality, and accounts for roughly half of all cardiovascular deaths. In CAD-associated ischaemic heart failure, β-adrenergic receptor (βAR) antagonists improve left ventricular function, mitigate heart failure symptoms, and increase survival in patients after coronary artery bypass grafting (CABG).†

However, despite their success as therapeutic agents, clinical studies have also shown that the responses to βAR antagonists are variable in patients with heart failure and that variations in the βAR genes may account not only for the development and progression of heart failure but also for the variable responses to βAR blockers. In this context, single-nucleotide polymorphisms (SNPs) in the βAR have been studied extensively. However, effects on the outcome of heart failure patients of these variants were rather inconsistent and studies regarding survival after CABG are missing. Overall, the available clinical genotype–phenotype association data have been rather disappointing and most associations originally proposed were not consistently replicated in later studies.3–7

It is possible that divergent data may result from unrecognized gene–gene interactions between βAR polymorphisms
and the downstream effector GoS, which is essential for βAR signalling. Gene–gene interactions occur when the phenotypic effects of a variation in one gene is affected by a variation in a second gene. In this respect, we have recently identified three common haplotypes in the GNAS gene encoding GoS; haplotype *3 was associated with increased GoS mRNA expression and altered intraoperative haemodynamics.8 9 This may influence the prognosis of CABG patients since experiments in transgenic mice have demonstrated that GoS overexpression increases heart rate and cardiac contractility.10 However, the functional relevance of all haplotype-tagging SNPs (htSNPs) within the GNAS regulatory region which differentiate between haplotypes *1 and *2 has not been resolved.

Accordingly, due to the direct signal transduction from βARs to GoS, it is conceivable that gene–gene interactions between these genes affect the outcome of CABG patients and predict prognosis better than the analysis of a single gene. We, therefore, tested the ‘molecular’ hypothesis that htSNPs within the GoS gene (i) are functionally active and alter GoS expression and (ii) the ‘clinical’ hypothesis that diploptypes derived from htSNPs influence survival after CABG. Furthermore, we also considered whether htSNPs within the GoS gene interact with βAR SNPs.

Methods

DNA genotyping

Primer sequences are given in Supplementary Table S1. PCR was performed using the slow-down PCR method.11 The following βAR SNPs were genotyped: β2AR (ADRB2), Ser49Gly (nucleotide 1165 C/G, rs1801253), and Arg389Gly (nucleotide 2445 G/C, rs1801252); β2AR (ADRB2), Arg16Gly (nucleotide 46 A/G, rs1042713), and Gln276Glu (nucleotide 79 C/G, rs1042714). ADRB SNPs and GNAS SNPs G(-1211)A (rs6123837) and C2445G (rs15754) were genotyped using Pyrosequencing™. The following restriction enzyme SspI (New England Biolabs, Beverly, MA, USA) was used to genotype the SNP T2291C (rs6026584): genotyping was carried out blind to clinical outcome.

Plasmid construction

Three common haplotypes in GNAS exist in Caucasians and the major haplotypes could correctly be inferred by two htSNPs, that is, G(-1211)A and T2291C.8 For all reporter constructs, we used DNA from an individual who was homozygous for the respective haplotype. For construct 1, we used primer pairs Int2_Ser/Int2_AS (+1207/2481) and, for construct 2 (+1974/2481), we used primer pairs Int3_Ser/Int2_AS. We then subcloned the resulting PCR products into pCR 2.1 vector (Invitrogen, Karlsruhe, Germany), followed by sequencing (GATC, Konstanz, Germany) and subcloning the inserts into the EcoRI site of pSEAP Basic (BD Biosciences, Palo Alto, CA, USA) in front of the secretory alkaline phosphatase gene.

Site-directed mutagenesis was used to construct the GCTG and GCCC variants of construct 2. All primers were designed using Primer Premier Software, as described.11

Transient transfections and alkaline phosphatase assay

Human embryonic kidney (HEK) 293 cells were routinely maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 U ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin (PAA, Pasching, Austria) in a 5% CO₂ atmosphere at 37°C. Transfections and alkaline phosphatase assays were done as described.8 12 Raw data for reporter gene expression were normalized to transfection efficiency. All experiments were repeated at least three times.

Preparation of nuclear extracts and electromobility shift assays

Nuclear extracts from HEK293 and HeLa cells were prepared and electromobility shift assays (EMSA) done as described with the DIG Gel Shift kit (Roche Applied Science, Mannheim, Germany) using digoxigenin-labelled double-stranded oligonucleotides which were derived from synthesized oligos (Supplementary Table S1).9

Preparation of erythrocyte membranes and western blot analysis

Ten millilitres of human blood were obtained from 31 healthy blood donors, after ethical approval and written informed consent, using a commercially available kit for blood diagnostics (Sarstedt, Nümbrecht, Germany). Erythrocyte membranes were prepared as described.13 Western blot analysis was performed with an anti-human GoS antibody (Santa Cruz Inc., Santa Cruz, CA, USA) according to the manufacturer’s instructions. Immunoreactive bands were visualized using enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Nitrocellulose blots were exposed for 15 s (non-saturation) and densitometry was used to quantify the immunoblot signal (NIH Image, Scion, Frederick, MD, USA). The GoS expression between different diplotypes was compared by multiplication of the average signal intensity by the number of pixels in that area.

βAR adenylyl cyclase activity

Right atrial appendages were obtained from 58 patients, after ethical approval and written informed consent, under chronic βAR antagonism at installation of cardiopulmonary bypass. Right atrial appendages were processed and adenylyl cyclase (AC) activity was measured as described.8 14 To calculate the net increase in AC activity after stimulation with GTP, isoproterenol, forskolin, and NaF, basal AC activity was subtracted, respectively. AC activity is given as pmol cAMP formed min⁻¹ mg protein⁻¹.

CABG study

After approval by the institutional review committee of the University of Duisburg-Essen and informed written consent, we extended our former study8 by establishing a follow-up programme and now include 185 consecutive patients with single- or multivessel CAD having undergone
CABG surgery to investigate cardiac-specific mortality within the follow-up period. Two hundred and sixty-eight patients were initially screened, of whom 56 were excluded because of absent βAR blockade medication, prescription of non-selective β1AR blockers, or because of recent initiation of β-blocker therapy only within 6 months before CABG. In the extended study, we changed our screening modalities, in that willingness to participate was checked before screening and scheduled combined bypass and cardiac valve repair surgery was also checked before screening. Therefore, a total of 15 patients refused to participate and 12 eventually underwent combined bypass and cardiac valve repair surgery (Supplementary Fig. S1). None of the patients had previous cardiac surgery. Clinical, laboratory, and angiographic data were obtained from the patients’ medical records and details of the study are described elsewhere.8

Information on survival status, date of death, and cause of death was obtained from medical records, phone calls with the patients’ family practitioners, or both, and, whenever necessary, the patient, his or her relatives, and/or hospitals were contacted for further information. The primary endpoint was 1 yr cardiac-specific mortality (myocardial infarction, heart failure, and cardiac arrhythmia); data for all patients with a complete 1 yr follow-up period (n=185) were analysed.

Statistical analysis

All polymorphisms were tested for conformance with the Hardy–Weinberg expectations and no evidence for deviations was detected. Reporter assay data were compared by the Wilcoxon signed-rank test. Western blot and patient characteristic clinical data were compared by linear ANOVA. Categorical variables were compared with χ² statistics or with Fisher’s exact test in the case of cells with numbers <5. The Kaplan–Meier plots were used to show the relationship between genotypes or risk factors and cardiac-specific mortality, that is, from the date of the operation to cardiac-related death up to 1 yr. Non-cardiac-related deaths within a 1 yr follow-up (n=3) were presented as censored objects. Univariate comparisons of 1 yr cardiac-related mortality and genotypes or risk factors were assessed for hazard ratio (HR), 95% confidence interval (CI), and P-values using the forward Cox proportional hazard model with diplotypes and risk factors as continuous variables.15 Non-significant variables were stepwise removed from the model. Data were analysed using the SPSS™ software package 18.0 by SPSS Inc. An α error of P<0.05 was considered to indicate statistical significance.

Results

Functional significance of htSNPs

To extend our former analysis3 and to identify functional SNPs so as to differentiate all three possible GNAS haplotypes, we engineered reporter gene constructs spanning the polymorphic regions of intron 1 comprising the htSNPs and expressed them in HEK cells, HEK293 (Fig. 1). Construct no. 1 with haplotype *3 yielded the highest reporter activity followed by haplotype *2 (n=6, P<0.05, Wilcoxon signed-rank test) and by haplotype *1 with strongly suppressed reporter activity (Fig. 1A; P=0.0001, Wilcoxon signed-rank test). Although lacking both the intron 1 SNP C1368T and the del1340 I/D polymorphism, the smaller construct no. 2 with the same haplotype configurations yielded similar results, excluding a functional effect of these latter polymorphisms. This raised the question of which of the nucleotides mediated alterations of transcriptional activity in the different haplotypes. In construct no. 2, the 2025G and 2273C alleles were present both in haplotype *2 (high activity) and in haplotype *1 (suppressed activity), while these alleles differed in haplotypes *2 and *3 (similar reporter activity), making these variants unlikely candidates. A comparison of haplotype *1 with haplotypes *2 and *3 showed allelic differences at positions 2291 and 2445. Using site-directed mutagenesis, we created constructs with all possible allelic variants at positions 2291 and 2445. As shown in Figure 1A, the 2445C allele was responsible for suppression of the promoter activity. The T2291C SNP also appeared functional since GCTG constructs showed significantly increased reporter activity compared with GCCG constructs, and GCTC constructs had greater activity compared with GCCG constructs. Subsequently, the functional SNPs 2291 and 2445 were chosen for examination of protein–DNA interactions using electrophoretic mobility shift assays (Fig. 2A and B). For T2291C, one slowly migrating complex bound to the T-allele with stronger intensity than to the C-allele (complex A), both in the presence of HEK293 and HeLa nuclear extracts, while a second one bound only to the T-allele when using HEK293 nuclear extracts (complex B). Competition experiments confirmed the specificity of allelic binding (Fig. 2A). EMSA experiments with a probe spanning the intron 1 C2445G polymorphism yielded unspecific binding (complex B) and specific binding with the C-allele in both HeLa cells and HEK293 cells (complex A, Fig. 2A). These results, therefore, are in line with the data from reporter assays, showing that the 2291T allele is associated with increased reporter activity (stronger affinity of an activator) and that the 2445C allele is associated with suppressed activity (binding of a potential repressor). Owing to the absolute linkage disequilibrium between T2291C and C2445G (D’=1 and r²=1) we, therefore, defined the T2291C as an htSNP for haplotype *2.

To assess the correspondence of these in vitro results to in vivo protein expression, we quantified Gαs protein in erythrocyte membranes by means of western blot analysis. Figure 2C shows one representative example from 31 different
individuals, with a specific 45 kDa band representing the short form of Ga\(_s\). Densitometric quantification yielded a doubled Ga\(_s\) protein expression in homozygous *3 carriers compared with homozygous *1 carriers, with homozygous *2 carriers displaying intermediate results (\(n = 31\), \(P = 0.013\) linear ANOVA; Fig. 2A). Thus, expression of Ga\(_s\) protein in non-stimulated red cells is in line with earlier results showing highest mRNA transcription in *3 carriers \(^8\) and with \textit{in vitro} reporter assays showing the highest reporter activity in haplotype *3 constructs, followed by haplotype *2 constructs, and the lowest activity in haplotype *1 constructs (Fig. 1).

**Outcome of CABG patients**

We next investigated prospectively in a pilot study whether GNAS diplotype-dependent differences in protein expression translate into an altered cardiac-specific survival. This was done by extending our former study\(^8\) to 185 patients undergoing CABG and receiving \(\beta\)AR blockade with a complete 1 yr follow-up. Table 1 shows both hSNPs G(-1211)A and T2291C which did not differ with respect to baseline characteristics such as age, sex, BMI, or clinical risk factors for adverse outcome. However, G(-1211)A was significantly associated with NYHA classification confirming results from our former study (\(n = 185\), \(P = 0.03\), \(\chi^2\)-test)\(^9\). Genotypes were tested for conformation with the Hardy–Weinberg expectations and no evidence for deviations was detected.

Overall 1 yr mortality after CABG was 7.6%. Eleven patients died from cardiac causes and three patients died from non-cardiac causes (malignant disease (\(n = 1\)), ischaemic cerebral infarction (\(n = 1\)), and pneumonia (\(n = 1\))). Univariate analysis demonstrated that cardiac-specific mortality was genotype-dependent. For G(-1211)A, 1 yr cardiac-related mortality was: AA: 0%; AG: 2.6%; and GG: 10.5% (\(n = 185\), \(P = 0.048\), \(\chi^2\)-test; Fig. 3A). For T2291C, 1 yr cardiac-related mortality was: CC: 2.3%; CT: 6.3%; and TT: 20.0% (\(n = 185\), \(P = 0.011\), \(\chi^2\)-test; Fig. 3B).

Consideration of diplotypes (Supplementary Table S2) showed a better risk stratification than the analysis of single genes. One year cardiac-related mortality was: diplotype *3/*3: 0%; *3/*2: 2.4%; *3/*1: 2.9%; *2/*2: 4.5%; *2/*1: 9.1%; and *1/*1: 20.0% (\(n = 185\), \(P = 0.004\), \(\chi^2\)-test for trend; Fig. 3C).

Multivariable Cox’s regression analysis revealed GNAS diplotype (HR 3.1 for each allele; 95% CI: 1.3–7.3), older age (HR 1.1; 95% CI: 1.0–1.2), diabetes mellitus (HR 4.2; 95% CI: 1.0–13.6), and NYHA classification (HR 2.9; 95% CI: 1.2–7.2) as independent predictors for cardiac-related mortality.

Concerning ADRB SNPs, these were also tested for conformation with the Hardy–Weinberg expectations and no evidence for deviations was detected. Polymorphisms in ADRB1 were not associated with cardiac-specific mortality (\(n = 185\), \(P > 0.1\), \(\chi^2\)-test; data not shown). However, in ADRB2 polymorphisms, mortality was associated with the Arg16 allele (\(n = 185\), \(P = 0.008\), \(\chi^2\)-test; Fig. 4A), while the
Glu27Gln SNP showed only a trend \( n=185, P=0.07, \chi^2\)-test; Fig. 4B).

Considering the directly linked signal transduction from \( \beta \)-AR to \( G\alpha\)s, we considered risk alleles from the respective genotypes, which had already shown significant associations with outcome in univariate analysis (i.e. \( G\alpha\)s diplotype and \( ADRB2 \) Arg16Gly). From \textit{in vitro} results and survival studies, we therefore defined haplotype *3 as a no-risk allele, while haplotype *2 was defined as one risk allele and haplotype *1 was defined as two risk alleles. For \( ADRB2 \), every Arg16 allele was defined as one risk allele. Combination of \( ADRB2 \) genotypes and \( G\alpha\)s diplotypes finally yielded seven risk alleles (Supplementary Table S3). As shown in Figure 4C, consideration of risk alleles showed a better risk stratification than the analysis of single genes \( n=185, P=0.0003, \chi^2\)-test for trend). The cardiac-specific mortality of patients

\[ \begin{align*}
\text{A} & \quad 2291C + 2291T \\
\text{Competition} & \quad + + + + + + + + \\
\text{HeLa} & \quad + + + + + + + + \\
\text{HEK} & \quad + + + + + + + + \\
\text{B} & \quad 2445G + 2445C \\
\text{Competition} & \quad + + + + + + + + \\
\text{HeLa} & \quad + + + + + + + + \\
\text{HEK} & \quad + + + + + + + + \\
\text{C} & \quad \begin{array}{c}
\ast 3/\ast 3 \\
\ast 3/\ast 2 \\
\ast 3/\ast 1 \\
\ast 2/\ast 1 \\
\ast 1/\ast 1
\end{array} \\
\text{D} & \quad \begin{array}{c}
\ast 3/\ast 3 \\
\ast 3/\ast 2 \\
\ast 3/\ast 1 \\
\ast 2/\ast 1 \\
\ast 1/\ast 1
\end{array} \\
\text{Gas expression} & \quad \begin{array}{c}
1.5 \times 10^5 \\
1.0 \times 10^5 \\
5.0 \times 10^4 \\
0 \times 10^4 \\
0 \times 10^4
\end{array} \\
P & = 0.013
\end{align*} \]

\[ \begin{align*}
n & = 5 \\
n & = 7 \\
n & = 6 \\
n & = 3 \\
n & = 5 \\
n & = 5 \\
n & = 5
\end{align*} \]

**Fig 2** \( G\alpha\)s allele-specific transcription factor binding and western blot analysis. (a) HeLa and HEK293 nuclear extracts were incubated with equal amounts of probes with different alleles in the absence or presence of a molar excess of unlabelled probe. Representative blot from two independent experiments with similar results. 2291T and 2291C oligos were used for competition gel shift experiments. The 2291T probe evoked one stronger band (complex A) and one additional band (complex B) in contrast to the 2291C probe. Specificity was proven by competition of 100-fold molar excess of the unlabelled probe. The faster migrating complex C shows no specificity as seen from non-competition with the unlabelled probe. (a) 2445G and 2445C oligos were used for competition gel shift experiments. The 2445C probe produced one additional band (complex A) in contrast to the 2445G probe. Specificity was proven by competition of 100-fold molar excess of the unlabelled probe. Again, the faster migrating complex B shows no specificity as seen from non-competition with the unlabelled probe. (c) Erythrocyte membranes were prepared from 31 healthy blood donors with different \( G\alpha\)s diplotypes. Forty micrograms of protein were used for SDS–PAGE and equal total protein loading was verified by Ponceau S-staining of the blot. Representative blots probed with a \( G\alpha\)s antibody. The 45 kDa band represents the short form of \( G\alpha\)s. Note the density differences between different \( G\alpha\)s diplotypes. (d) Relative quantification of \( G\alpha\)s expression in erythrocyte membranes by densitometry of western blots. Comparisons were done using linear ANOVA.
Table 1. Patient characteristic variables and statistics as related to genotypes. Data are shown as n (%) for dichotomous variables and means (SD) for continuous variables. P-values refer to $\chi^2$-test or ANOVA.

<table>
<thead>
<tr>
<th></th>
<th>G(-1211)A (rs6123837)</th>
<th>T2291C (rs6026584)</th>
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<tbody>
<tr>
<td></td>
<td>AA (n=22)</td>
<td>AG (n=77)</td>
</tr>
<tr>
<td>Frequency (%)</td>
<td>11.9</td>
<td>41.6</td>
</tr>
<tr>
<td>Age, yr (range)</td>
<td>66 (41–85)</td>
<td>68 (45–83)</td>
</tr>
<tr>
<td>Female sex (n, %)</td>
<td>5 (23)</td>
<td>11 (14)</td>
</tr>
<tr>
<td>Diabetes mellitus (n, %)</td>
<td>7 (32)</td>
<td>28 (36)</td>
</tr>
<tr>
<td>BMI (kg m$^{-2}$)</td>
<td>28.4 (3.7)</td>
<td>28.3 (3.7)</td>
</tr>
<tr>
<td>Smoking/former smoking (n, %)</td>
<td>1 (5)/12 (55)</td>
<td>12 (16)/41 (53)</td>
</tr>
<tr>
<td>Preoperative creatinine (mg dl$^{-1}$)</td>
<td>1.2 (0.3)</td>
<td>1.2 (0.3)</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>54 (14)</td>
<td>54 (14)</td>
</tr>
<tr>
<td>NYHA classification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I–II</td>
<td>17 (77)</td>
<td>52 (68)</td>
</tr>
<tr>
<td>III</td>
<td>5 (23)</td>
<td>19 (25)</td>
</tr>
<tr>
<td>IV</td>
<td>0 (0)</td>
<td>6 (8)</td>
</tr>
<tr>
<td>Left main coronary artery stenosis &gt;50% (n, %)</td>
<td>8 (36)</td>
<td>23 (30)</td>
</tr>
<tr>
<td>Preoperative cTnI &gt;0.1 μg litre$^{-1}$ (n, %)</td>
<td>3 (14)</td>
<td>9 (12)</td>
</tr>
<tr>
<td>No. of grafts</td>
<td>3.0 (1.1)</td>
<td>3.4 (1.0)</td>
</tr>
<tr>
<td>Use of internal mammary graft (n, %)</td>
<td>30 (91)</td>
<td>74 (96)</td>
</tr>
<tr>
<td>Mitral insufficiency (moderate or severe) (n, %)</td>
<td>5 (14)</td>
<td>10 (13)</td>
</tr>
<tr>
<td>Cardiopulmonary bypass time (min)</td>
<td>130 (36)</td>
<td>128 (46)</td>
</tr>
<tr>
<td>Aortic cross-clamp time (min)</td>
<td>87 (30)</td>
<td>88 (33)</td>
</tr>
</tbody>
</table>
with all risk alleles (n=6) was 50% compared with zero in patients without risk alleles (n=10), and multivariable analysis revealed an HR of 2.3 for cardiac-specific death for each risk allele (95% CI: 1.5–3.5).

**Intracellular biochemical consequences of risk alleles**

The genetic constellations associated with an increased risk of death after CABG independently showed biochemical alterations at the endpoint of the βAR/G-protein-signalling cascade, that is, AC activity. When we re-analysed cAMP data derived from our former study and stratified the data to GNAS/ADRB2 risk alleles, we found that carriers with no-risk allele displayed the greatest AC activity when compared with risk allele carriers (Table 2). We compared risk allele-dependent AC activities and showed that basal AC activity was increased by 40% in no-risk allele carriers compared with carriers of all risk alleles (n=58, P=0.005 linear ANOVA). Furthermore, direct Gαs stimulation using GTP and direct Gαs stimulation using NaF resulted in a 52% (n=58, P=0.001, linear ANOVA) and 68% (n=58, P=0.002, linear ANOVA) increase in AC activity in no-risk allele carriers compared with carriers of all risk alleles, respectively. Interestingly, stimulation of the complete β-adrenergic signal pathway via βAR using isoprenaline also revealed large differences in AC activity, with a doubled activity in no-risk allele carriers compared with carriers with all risk alleles (n=58, P=0.003, linear ANOVA; Table 2). In contrast, direct (not adrenoceptor/G-protein mediated) AC stimulation with forskolin was risk allele independent, indicating a specific effect of ADRB2 Arg16Gly genotypes and GNAS diplotypes on AC-mediated cAMP generation.

**Discussion**

Our data demonstrate important genetic interactions of functional polymorphisms in the βAR/Gαs-signalling pathway that evoke major alterations of biochemical
events after βAR/G-protein signalling. Based on biochemical, functional, and expression measurements and a candidate-gene approach, our data are in line with the predicted roles of the gain-of-function mutations and we recommend taking into account a direct interaction of GNAS haplotypes with βAR SNPs.

Several authors have proposed that genetic variants may be associated with an altered outcome after CABG, but no data exist regarding SNPs in the βAR/G-protein-signalling pathway. Our study provides clinical, molecular, and functional biochemical evidence for risk alleles as independent predictors of cardiac mortality after CABG, as derived from genetic interaction of ADRB2 and GNAS. Furthermore, predicted mortality based on combinations of variants of the genes in the βAR/Gαs-signalling pathway is more reliable when compared with the analysis of the single genes. In turn, this approach also makes sense from a biochemical perspective since risk genes were not only associated with altered cardiac-related mortality but also with clear-cut alterations of signal transduction, as shown by cellular AC activity and the response to β-agonists in vitro, that is, the physiological endpoint of the βAR/G-protein-signalling pathway.

While experiments in transgenic mice have addressed the potential importance of the Gαs protein in the maintenance and augmentation of left ventricular function and hypertrophy, the impact on survival of altered Gαs expression in humans is unknown. We therefore investigated in detail the haplotype structure of GNAS and could demonstrate the functional relevance of both SNPs, T2291C and C2445G, which represent hSNPs for haplotype *2. Owing to their absolute linkage disequilibrium, that is, inheritance of the alleles without any recombination, the T2291C SNP was associated with zero mortality, while diminished expression (homozygous haplotype *1) is associated with a 20% cardiac-related 1 yr mortality, with an independent HR of 3.1. Quantitatively, the HR of 3.1 associated with GNAS haplotypes is substantial and, for example, in a range similar to that evoked by the presence of diabetes mellitus. As haplotype *3 carriers show the highest Gαs protein expression, we hypothetized that enhanced Gαs-mediated signal transduction under βAR antagonism is beneficial for the cardiac-related outcome after CABG. This hypothesis is supported by experiments in transgenic mice under chronic βAR blockade demonstrating less affection by evoked heart failure and augmented left ventricular function upon overexpression of Gαs.

To extend our comprehensive analysis of the GNAS with regard to β-adrenergic signal transduction, we also analysed the effect of ADRB SNPs on cardiac-related mortality. While two functionally important SNPs have been described for the ADRB1 gene, Ser49Gly and Arg389Gly, three functionally important SNPs have been described for the ADRB2 gene, Arg16Gly, Gln27Glu, and the rare Thr164Ile. We found no association of the ADRB1 SNPs with mortality. This is consistent with non-replicable results on the impact of ADRB1 SNPs on prognosis and survival of heart failure.

Concerning ADRB2 SNPs, the Arg16Gly genotype patients were at higher risk for death and there also was a trend for Gln27Glu genotypes, which are in close linkage disequilibrium. Owing to the sample size restrictions, the very rare (minor allele frequency in Caucasians 0.02–0.04) Thr164Ile SNP was not analysed. Our results confirm earlier studies showing a protective role for the Glu27 and Gly16 alleles regarding the incidence of cardiac events, myocardial infarction, and outcome after acute coronary syndrome. Finally, studies in humans have shown enhanced agonist-mediated desensitization associated with the Arg16 or Gly27 alleles.

Based on our data of increased Gαs expression in haplotype *3 carriers and those showing diminished βAR desensitization in ADRB2 Gly16 carriers, we aimed to substantiate our molecular and protein expression data with biochemical data at the endpoint of the β-adrenergic/G-protein signal cascade. We therefore re-analysed AC assays from our former study using membrane preparations from atrial cardiac muscle that also were derived from CABG patients under βAR blockade. Using this approach, we could show that Gαs-stimulated AC activity was markedly increased in patients not carrying risk alleles (GNAS

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Risk allele-dependent AC activity. Values are given in pmol min⁻¹ mg⁻¹. P-values refer to linear ANOVA</th>
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<tbody>
<tr>
<td></td>
<td>No-risk allele (n=4)</td>
</tr>
<tr>
<td>Unstimulated (baseline)</td>
<td>35.4 (16.0)</td>
</tr>
<tr>
<td>GTP (10 μM)</td>
<td>47.3 (21.9)</td>
</tr>
<tr>
<td>NaF (10 mM)</td>
<td>101.0 (33.0)</td>
</tr>
<tr>
<td>Isoproterenol (100 μM)</td>
<td>87.3 (21.0)</td>
</tr>
<tr>
<td>Forskolin (100 μM)</td>
<td>349.3 (101.2)</td>
</tr>
</tbody>
</table>
homozygous *3 plus ADRB2 Gly16Gly) when compared with carriers of all risk alleles (GNAS homozygous *1 plus ADRB2 Arg16Arg). Most importantly, stimulation of the complete β-adrenergic/G-protein signal pathway via βAR stimulation by isoprenaline also resulted in large AC activity differences, with a doubled activity in carriers of no-risk allele compared with carriers of all risk alleles. In contrast, responses to forskolin, a direct (not receptor mediated) stimulator of the catalytic unit of the AC and hence independent of Gαs, were unaltered. These results, therefore, clearly demonstrate that the combined effect of diminished β2AR response (Gly16) together with increased Gαs expression (haplotype *3) is responsible for the observed profound changes in βAR/G-protein-mediated signal transduction, whereas the concentration, activity, or both of the AC catalytic unit itself remains independent of these genetic factors.

It is generally accepted that the profound activation of the sympathetic system in patients with ischaemic heart failure is inversely correlated with survival. A general feature in human heart failure is a decrease in cardiac βARs, notably in β1AR, leading to a shift in the β1:β2AR ratio towards β2AR. Moreover, data have demonstrated changes in the compartmentation of the β2AR–cAMP signalling in heart failure leading to β1-like signalling properties and implying a shift in signalling from the β1AR–Gαs–cAMP axis towards the β2AR–Gαs–cAMP axis. These recent data may support our results which show an effect of ADRB2 SNPs on the outcome of patients, a proportion of which had heart failure, while no effect was detected for ADRB1 SNPs.

Examination not only of single genes but hypothesis-driven investigation of genetic interactions in a signal transduction pathway using a candidate-gene approach also sheds new light on the general question why many genetic association studies addressing only single genes often cannot be replicated.

We used a candidate-gene approach driven by a valid hypothesis based on molecular experiments, an approach considered of ‘high value’. While we were able to support our outcome data by related biochemical findings, we are aware that the number of lethal cardiac events in the CABG study is quite small and may not yield enough power to draw a definitive clinical conclusion. Moreover, no correction for multiple testing was done for different genotypes. Therefore, this study should be regarded a hypothesis-generating pilot study rather than definitive. Accordingly, further and larger studies are needed to substantiate our results.

**Supplementary material**

Supplementary material is available at *British Journal of Anaesthesia* online.

**Conflict of interest**

None declared.

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