Genetic dysregulation of endothelin-1 is implicated in coronary microvascular dysfunction

Short title: Genetic dysregulation of ET-1 and microvascular dysfunction

Authors: Thomas J. Ford[1,2,5], David Corcoran[1,2], Sandosh Padmanabhan[1], Alisha Aman[1], Paul Rocchiccioli[1,2], Richard Good[1,2], Margaret McEntegart[1,2], Janet J. Maguire[3], Stuart Watkins[2], Hany Eteiba[2], Aadil Shaukat[2], Mitchell Lindsay[2], Keith Robertson[2], Stuart Hood[2],Ross McGeoch[4], Robert McDade[2], Eric Yii[1], Naveed Sattar[1], Li-Yueh Hsu[4], Andrew E. Arai[4], Keith G. Oldroyd[1,2], Rhian M. Touyz[1], Anthony P. Davenport[3], Colin Berry[1,2*]

Affiliations:

1 - British Heart Foundation Glasgow Cardiovascular Research Centre, Institute of Cardiovascular and Medical Sciences, University of Glasgow, UK

2 - West of Scotland Heart and Lung Centre, Golden Jubilee National Hospital, UK

3 - Experimental Medicine and Immunotherapeutics, University of Cambridge, UK

4 - Laboratory for Advanced Cardiovascular Imaging, National Heart, Lung, and Blood Institute, National Institutes of Health, Department of Health, Bethesda, MD, USA.

5 – Department of Cardiology, Gosford Hospital, New South Wales, Australia

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Competing interests

C.B. is employed by the University of Glasgow which holds consultancy and research agreements with companies that have commercial interests in the diagnosis and treatment of angina. The companies include Abbott Vascular, AstraZeneca, Boehringer Ingelheim, Coroventis, GSK, HeartFlow, Novartis, Opsens, Philips, and Siemens Healthcare. K.G.O. has received consultant and speaker fees from Abbott Vascular, Biosensors and Boston Scientific. P.R. has received consultant and speaker fees from AstraZeneca. None of these companies have had any involvement with this study except Astra Zeneca supplied zibotentan for laboratory studies. None of the other authors have any potential conflicts of interest.

Address for correspondence: * Professor Colin Berry. British Heart Foundation Glasgow Cardiovascular Research Cntre, Institute of Cardiovascular and Medical Sciences, University of Glasgow, UK. Telephone: +44 141 330 3325; Email: colin.berry@glasgow.ac.uk

Dr Anthony Davenport, Experimental Medicine and Immunotherapeutics, University of Cambridge, UK; Email: apdd10@medschl.cam.ac.uk

Twitter: @TomJFord, @ColinBerryMD, @UofGICAMS

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Abbreviations: ET-1 - Endothelin-1; IHD - ischaemic heart disease; ACh – acetylcholine, CFR – coronary flow reserve, CMD - coronary microvascular dysfunction, CMR - cardiac magnetic resonance, FFR - fractional flow reserve, INOCA - ischaemia with no obstructive coronary artery disease, IMR – index of microcirculatory resistance

Abstract

Background: Endothelin-1 (ET-1) is a potent vasoconstrictor peptide linked to vascular diseases through a common intronic gene enhancer [(rs9349379-G allele), chromosome 6 (PHACTR1/EDN1)]. We performed a multimodality investigation into the role of ET-1 and this gene variant in the pathogenesis of coronary microvascular dysfunction (CMD) in patients with symptoms and/or signs of ischaemia but no obstructive coronary artery disease (INOCA).

Methods and Results: 391 angina patients were enrolled, 206 (53%) with obstructive CAD were excluded leaving 185 (47%) eligible. 109 (72%) of 151 subjects who underwent invasive testing had objective evidence of CMD (COVADIS criteria). rs9349379-G allele frequency was greater than in contemporary reference genome bank control subjects (allele frequency 46% (129/280 alleles) v 39% (5551/14380); P=0.013). The G allele was associated with higher plasma serum ET-1 (LS mean 1.59pg/mL v 1.28pg/mL; 95% CI 0.10 to 0.53; P=0.005). Patients with rs9349379-G allele had over double the odds of CMD (OR 2.33; 95% CI 1.10 – 4.96; P=0.027). Multimodality non-invasive testing confirmed the G allele was associated with linked impairments in myocardial perfusion on stress cardiac magnetic resonance imaging at 1.5 Tesla (N=107; GG 56%, AG 43%, AA 31%, P=0.042) and exercise testing (N=87; -3.0 units in Duke Exercise Treadmill Score; -5.8 to -0.1; P=0.045). ET-1 related vascular mechanisms were assessed *ex vivo* using wire myography with ET_A receptor (ET_A) antagonists including zibotentan. Subjects with rs9349379-G allele had preserved peripheral small vessel reactivity to ET-1 with high affinity of ET_A antagonists. Zibotentan reversed ET-1-induced vasoconstriction independently of G allele status.

Conclusion: We identify a novel genetic risk locus for coronary microvascular dysfunction. More research is needed however these findings implicate ET-1 dysregulation and support

the possibility of precision medicine using genetics to target oral ET_A antagonist therapy in patients with microvascular angina.

Keywords: Endothelin-1, single nucleotide polymorphism, stable angina pectoris, coronary microvascular dysfunction, microvascular angina, precision medicine

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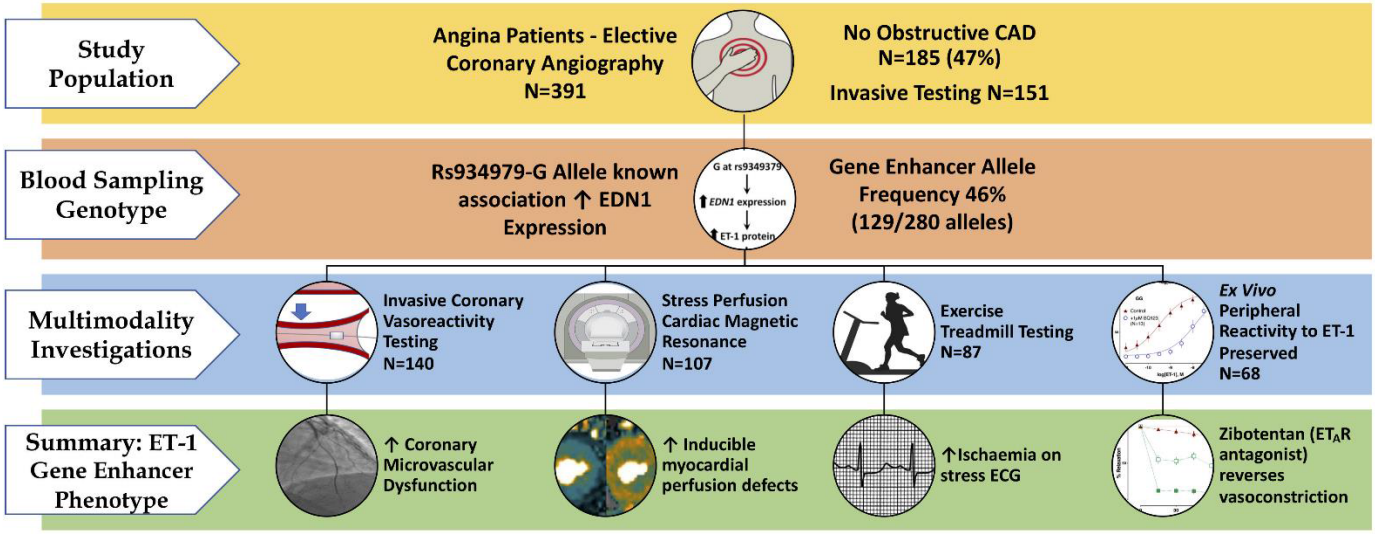

One sentence Summary

A common genetic polymorphism previously linked with increased endothelin gene activity (rs9349379-G allele) is associated with increased ET-1 in microvascular angina patients and correlates with both invasive and non-invasive markers of coronary microvascular dysfunction including ischaemia on exercise treadmill testing and stress perfusion cardiac magnetic resonance imaging.

Introduction

The coronary microcirculation has been implicated in the pathogenesis of angina for over fifty years, however disease mechanisms remain incompletely understood.[1] Coronary microvascular dysfunction (CMD) is associated with adverse outcomes in angina and a plethora of other cardiovascular disorders.[2-5] Standardised diagnostic criteria for microvascular dysfunction[6] underpin recent studies which have identified the disease prevalence affecting two thirds of angina patients without obstructive epicardial coronary artery disease.[7-10] These patients present a diagnostic and therapeutic challenge with up to one in four experiencing a major adverse cardiac event after five years of follow up.[11, 12] The syndrome of ischemia and no obstructive coronary artery disease (INOCA) is particularly important in women,[13] whose elevated cardiac risk is mostly driven by impaired coronary flow reserve (and not obstructive coronary disease).[11]

Endothelin-1 (ET-1) is a highly potent endogenous vasoconstrictor of human coronary arteries[14] and has been implicated in the pathogenesis of microvascular dysfunction.[15, 16] ET-1 mediated activation of the G protein-coupled ET_A receptor on vascular smooth muscle cells induces endothelial dysfunction, inflammation and vasoproliferative effects. Circulating concentrations of serum ET-1 are inversely associated with coronary flow responses in patients with CMD.[14, 16] Recently, a common (39%) genetic locus in chromosome 6p24 (PHACTR1/EDN1) has been shown to be a distal regulator of endothelin gene expression.[17] The allele, rs9349379-G, is associated with an increased risk for atherosclerotic epicardial coronary artery disease and myocardial infarction.[18] This functional single nucleotide polymorphism (SNP: rs9349379-G) is associated with increased endothelin gene expression resulting in a lifetime's exposure at least 20% higher ET-1 precursor levels in the plasma.[17]

ET-1 dysregulation is implicated in coronary vascular disease, however, the role of rs9349379 in the pathogenesis of CMD has not been examined.

We determined association of the rs9349379-G allele with coronary microvascular dysfunction in angina patients undergoing invasive coronary function testing. Our secondary objectives were to investigate whether the G allele associates with non-invasive parameters of myocardial ischaemia. Our final objective was to examine vascular mechanisms using isometric tension recordings in small peripheral resistance vessels isolated from patients according to genotype. We evaluated ET_A receptor mediated vasoconstriction in subjects according to rs9349379-G allele status. These included zibotentan, an ET_A receptor-selective antagonist, that is available for repurposing following neutral results in phase 3 oncology trials.

Methods

Study population

We prospectively enrolled patients with stable angina. We screened elective adult referrals to two hospitals serving a population of ~2.5 million in the West of Scotland. Patients were scheduled to undergo clinically indicated invasive coronary angiography for the investigation of suspected coronary artery disease. The participants were enrolled into the Coronary Microvascular Angina (CorMicA) study (ClinicalTrials.gov: NCT03193294), which is a randomized, controlled, strategy trial of stratified medicine in angina patients without obstructive CAD[19]. The Rose-Angina questionnaire was administered on the day of the angiogram and only patients with definite or possible angina were eligible to participate.[20] Exclusion criteria included a non-coronary indication for invasive angiography e.g. valve disease, severe renal dysfunction (GFR<30 mL/min), inability to give informed consent and obstructive coronary disease determined during invasive coronary angiography (≥50% diameter stenosis and/or fractional flow reserve (FFR) ≤ 0.80). All coronary vasodilating drugs were discontinued at least 24 hours before the procedure. Pooled control genotype frequencies were ascertained from a contemporary medical genome reference cohort.[21]

Definitions: coronary microvascular dysfunction

We defined CMD using invasive coronary function testing and the Coronary Vasomotion Disorders International Study Group (COVADIS) diagnostic criteria.[20] These physiological criteria included response to abnormal microvascular response to adenosine (raised IMR [≥25]) and/or abnormal CFR [<2.0]). In addition, CMD also included subjects with microvascular spasm during ACh provocation (reproduction of angina symptoms, ischaemic ECG changes (≥1mm ST segment deviation), but < 90% epicardial spasm during ACh

testing).[22] CMD is frequently associated with epicardial vasospasm and hence patients with abnormal vasoreactivity during adenosine assessment (abnormal IMR and/or CFR) *and* coexistent epicardial vasospasm during ACh provocation were included within the CMD group. FFR was measured to rule-out flow limiting coronary artery disease as an alternative explanation for myocardial ischaemia (INOCA subjects had FFR >0.8 in target artery).

Measurement of coronary vascular function *in vivo*

We used an interventional diagnostic procedure (IDP) that combined guidewire-based direct measurement of coronary vascular function followed by pharmacological vasoreactivity testing. Specifically, the IDP included a guidewire-based measurement of coronary vascular function (FFR, coronary flow reserve [CFR], and the index of microvascular resistance [IMR]) followed by pharmacological vasoreactivity testing with acetylcholine (ACh) and glyceryl trinitrate (GTN) and has been previously described.[19, 23]

In brief, an intravenous infusion of adenosine (140 $\mu g \cdot kg^{-1} \cdot min^{-1}$) was administered via a large peripheral vein to induce steady-state maximal hyperaemia for a period of at least 90 seconds with a target time of 180 seconds. A pressure-temperature sensitive guidewire was placed into the distal third of a major epicardial coronary artery (typically the left anterior descending [LAD]). The myocardial FFR was calculated by the ratio of mean distal coronary pressure to mean aortic pressure at maximal hyperaemia. A FFR $\leq$0.80 was taken as abnormal and indicative of flow-limiting coronary artery disease.[24] CFR was calculated using thermodilution as resting mean transit time divided by hyperaemic mean transit time.[25] A CFR <2.0 was defined as abnormal representing impaired vasodilator reserve.[26] The IMR was calculated as the product of mean hyperaemic transit time and mean distal coronary pressure at hyperaemia.[27] An IMR $\geq$25 was defined as abnormal and indicative of increased

microcirculatory resistance.[28] These invasive parameters were simultaneously derived in real-time using dedicated software (Coroventis, Uppsala, Sweden). We assessed endothelium-dependent coronary vasomotor function using intra-coronary infusions of ACh via the guiding catheter at concentrations of 0.182, 1.82, and 18.2 µg/mL (10^{-6}, 10^{-5}, and 10^{-4} mol/L, respectively) at 1 mL/min for 2 minutes via a mechanical infusion pump.[29] Patients who had CMD (e.g. abnormal CFR and/or IMR) but co-existent epicardial vasospasm during acetylcholine bolus (100µg bolus of ACh; 5.5 mL of 10^{-4} mol/L over 20 seconds) were considered in the CMD group.[30] In order to assess non-endothelial dependent vasodilatation, 300 µg of GTN was administered by manual intra-coronary bolus injection. Detailed methods are reported in the online appendix.

Blood and tissue analysis

Serum ET-1 was determined using blood obtained on the day of coronary function testing (Quantikine ® ELISA, R&D Systems® Europe, Abington [UK]). Blood was obtained from participants following an overnight fast in a recumbent position.

Ex vivo pharmacological assessment of peripheral vascular function was performed on patients who volunteered to undergo a gluteal skin fat biopsy within 4 weeks of the invasive coronary function assessment. The biopsy was obtained under sterile conditions using local anaesthesia with lidocaine (2%). Small peripheral resistance vessels (< 400µm) were carefully dissected from fresh biopsies using a light microscope. 2mm length vessels were mounted on 40-µm stainless steel wires for isometric myography in multi-channel myograph chambers (DMT, Denmark) filled with physiological saline solution. Isometric tension recordings followed-on directly using the technique of wire myography to study small peripheral resistance arteries with paired cumulative concentration response curves (CCRCs) to ET-1 in the presence or absence of an ET_A receptor antagonist, either BQ123 or zibotentan

(AstraZeneca, U.K.; Open Innovation). This vascular biology sub study was an extension of previous work in INOCA subjects that was previously published in this journal.[31] The detailed methods are described in the study appendix. The peripheral vascular sensitivity to ET-1 (pEC_{50}) and maximum vasoconstriction to ET-1 (E_{max}) were determined.

For the antagonist studies the affinity (K_B) of BQ123 was first determined in paired vessels from individuals and calculated using Schild regression. The pK_B ($-\log_{10} K_B$) values were compared between each genotype as an indicator of whether or not patients of different genotypes are likely to respond equally well to an ET_A antagonist used clinically. A final series of experiments involved paired vessel experiments using ET-1 CCRCs in the presence and absence of a highly selective ET_A receptor antagonist, zibotentan to determine a pK_B value and assess whether zibotentan could reverse an established ET-1 mediated vessel constriction.

Cardiac magnetic resonance imaging and ischaemia testing protocol

Patients were prospectively invited to undergo quantitative perfusion cardiac magnetic resonance (CMR) imaging at 1.5 Tesla using pharmacological stress testing with intravenous adenosine (140 µg/kg/min) within 6 weeks of the index coronary angiogram. CMR studies were performed using a standardized CMR protocol (Siemens MAGNETOM Avanto, Erlangen, Germany). The CMR scans were interpreted by two experienced observers (Level III accreditation, European Society of Cardiovascular Imaging) blind to diagnostic findings and genotype. The raw stress and rest perfusion images were qualitatively assessed for inducible or fixed perfusion defects. The perfusion was classified as either normal, abnormal, or equivocal. If a perfusion defect was present, it was reported as having and epicardial, microvascular or equivocal pattern. Perfusion defects were then reported on a segmental basis

according to the American Heart Association 16-segment model[32], and were classified according to the transmurality of the perfusion defect (<50 % or >50%), and the number of segments with qualitatively abnormal perfusion was defined. Dark rim artefact was adjudicated based on standardised criteria.[33]

The first-pass perfusion images were then post-processed to derive quantitative pixel perfusion maps to derive absolute myocardial blood flow (MBF) and myocardial perfusion reserve (MPR) (further detail in Supplementary information).[34]

Treadmill exercise stress electrocardiography using the Bruce protocol was analysed from the sub-group of patients who had been pre-selected for this procedure on clinical grounds prior to invasive coronary angiography. We used the Duke treadmill score (DTS) which is a validated metric with established prognostic cardiovascular utility.[35] The exercise treadmill test analysis included (1) exercise duration and (2) the Duke Treadmill Score[36] by a cardiology researcher (EY) blinded to genotype and invasive physiology. The DTS is based on the occurrence of angina during treadmill exercise testing, ST-segment depression during the test and peak exercise duration (or METS achieved). Specifically, the DTS equals the maximum exercise time in minutes – (5 × the maximal net ST-segment deviation in mm during or after exercise) – (4 × the treadmill angina index (where 0 = no angina, 1 = non-limiting angina, 2 = exercise limiting angina).

All subjects were asked to abstain from caffeine-containing beverages or foodstuffs for 24 hours, and vasoactive medications for 48 hours prior to the CMR examination. All scan acquisitions were spatially co-registered. All CMR analyses were performed by a blinded analyst with Level 3 EACVI accreditation.

Statistical analysis

The main hypothesis in our study was that regulation of ET-1 gene expression reflected by the presence of the intronic ET-1 gene enhancer, rs9349379 G, associates with invasive tests of CMD. We tested the association of genotype (SNP rs9349379 G-A allele status) with CMD on invasive coronary vasoreactivity testing by calculating the odds ratio (OR) and its 95% confidence intervals (CI). Multivariable logistic regression was used to determine whether genotype was independently associated with CMD (as defined by abnormal response to intracoronary ACh and/or systemic adenosine) adjusting for overall cardiac risk (ASSIGN score) including previous cardiac events.[37]

Categorical data are presented as percentages and continuous parameters are shown as means with SD values or medians with interquartile ranges. For secondary analyses, subjects were divided into three genotype groups. Kruskal-Wallis test was used to test whether distribution of non-parametric variables is the same between the groups. Subgroup analysis of A versus G genotypes was determined a priori to evaluate any differences between the two most differentiated groups. The least-squares (LS) mean of serum ET-1 levels was compared between the groups derived using analysis of co-variance with serum ET-1 as dependent variable and adjusted for age, sex, BMI, genotype and cardiovascular risk as covariates and possible confounders. Linear associations with invasive and non-invasive metrics of microvascular disease were performed by analysis of variance [ANOVA] with P for linear trend for continuous parameters and $\chi 2$ test with P for linear-by-linear test for categorical variables. Statistical analyses were performed with Prism 7.0 (GraphPad, La Jolla, CA) and SPSS 25.0 (SPSS, Chicago, IL).

Results

We prospectively enrolled three hundred and ninety-one patients with angina between 25/11/2016 - 11/12/2017 at two hospitals serving a population of ~2.5 million in the West of Scotland (CorMicA: ClinicalTrials.gov NCT03193294).[19] Invasive coronary angiography revealed obstructive disease in 206 (53.7%) participants who were then excluded from further study. One hundred and fifty-one participants with no obstructive coronary disease continued in the study (Figure 1, Table 1). Evidence of CMD was found in 109 (72%) of 151 subjects undergoing invasive coronary vasoreactivity testing (Table 2). An overview of the study and investigations is illustrated in Figure 1. Genetic analysis was completed in 140 subjects (93%) using baseline venous blood samples. The mean age of patients in this analysis 61.1 ±10.1 years. There was a predominance of women (103 [74%]) and the estimated 10-year risk of cardiovascular events (ASSIGN) was appreciable at 25% (± 20).

The genotype distribution of rs9349379 was AA (N=50, 36%), AG (N=51, 36%), GG (N=39, 28%). This SNP did not fulfil Hardy Weinberg equilibrium (P=0.0015) reflecting biologic ascertainment of genotypes. One hundred and forty subjects underwent genetic analysis for (rs9349379 G allele) with an allele frequency of 46% (129/280 alleles). The allele frequency was increased in our angina cohort compared to that of genome bank control subjects (rs9349379-G allele frequency 39% [5551/14380]; Chi squared = 6.15, P=0.013).[21] The rs9349379-G allele was associated with over double the odds of CMD (OR 2.33; 95% CI 1.10 – 4.96; P=0.027; Figure 2A). Subjects with G allele had higher circulating serum ET-1 concentration (LS mean 1.59pg/ml versus 1.28pg/ml; difference 0.31pg/ml; 0.10 to 0.52; P=0.005; Figure 2B). Each additional G allele was linearly associated with CMD on invasive interrogation (Figure 3A; P=0.021). On multivariable analysis, the G allele remained associated with CMD (OR per G allele 2.31; 1.08 - 4.91; P=0.030; Supplementary Table 1).

Considering diagnostic subtypes of microvascular dysfunction, the vast majority had CMD during adenosine interrogation (73% abnormal CFR and/or IMR) and only 27% of the genotyped population had isolated microvascular spasm (isolated CMD to ACh only). There was a statistically significant relationship between genotype and coronary microvascular dysfunction, as reflected by an impaired coronary vasodilator reserve (abnormal CFR: AA 20%, AG 35%, GG 41%; Figure 3B; P=0.030). A similar relationship was noted for prevalence of abnormal microvascular resistance in each genotype (abnormal IMR: AA 24%, AG 33%, GG 46%; Figure 3C; P=0.029). CFR decreased linearly with each additional rs9349379-G allele (AA 3.0 (2.1, 3.7); AG 2.7 (1.8, 3.5); GG 2.1 (1.7, 3.2); overall P=0.046; Figure 3D; Table 2). The highest risk group (GG) had a significantly lower CFR than the AA group (median difference 0.84, 95% CI 0.1 – 1.1). The prevalence of abnormal invasive acetylcholine response was not statistically different between the groups (any G allele 36% versus no G allele 30%, P=0.463). Patients with isolated CMD to ACh (microvascular spasm) had similar ET-1 levels to those without (1.33ng/ml v 1.28ng/ml; P =0.769). The highest serum ET-1 levels were seen in subjects with concordant abnormalities in both CFR and IMR with linear stepwise reduction compared to those with only one index of CMD and lowest in those without any abnormalities (mean 1.67ng/ml (both) v 1.39ng/ml (one) v 1.31ng/ml (none); P trend = 0.041).

The Gensini angiographic score reflecting the extent (or burden) of coronary atherosclerosis was higher in the rs9349379-GG group (median score 1.0, [0.0, 6.0]) compared to the AA group (median score 0.0, [0.0, 2.0]; P=0.037; Table 2). As might be expected in this population of INOCA patients, the physiological burden of epicardial coronary artery disease was similar between the groups (myocardial fractional flow reserve (FFR), AA 0.88 (±0.05); AG 0.88 (0.06); GG 0.88 (±0.05); P=0.977).

One hundred and seven subjects underwent an adenosine stress perfusion cardiac MRI within 6 weeks of the invasive angiogram. Forty-six (43%) patients had evidence of a sub-endocardial circumferential abnormality of myocardial perfusion attributable to CMD (Table 2). The rs9349379-G allele was associated with abnormal myocardial perfusion disclosed by stress perfusion MRI (AA 31%, AG 43%, GG 56%; P=0.042, Figure 4A). The association of genotype with CMD was more robust when considering subjects with either a circumferential subendocardial perfusion defect disclosed by MRI or invasive evidence of CMD, (AA 65%, AG 85%, GG 91%; P<0.001; Figure 4B). The absolute global and subendocardial perfusion reserve (MPR) was numerically lower with each G allele however the differences were not statistically significant (Table 2; Figures 4C & 4D).

We then assessed relationships between exercise treadmill testing, invasive measures of coronary vascular function and genotype. Ninety subjects prospectively completed exercise treadmill testing during standard care diagnostic work up prior to invasive coronary angiography, eighty-four of these subjects were included in the study with the remainder being excluded due to lack of genotype data. The mean exercise duration was 367 (± 156) seconds and similar between the groups (Table 2). The mean Duke Treadmill Score (DTS) was -1.0 (± 5.3) units. The presence of CMD was associated with reduced DTS (CMD -2.3 v No CMD +3.5; Difference -5.8 units, 95% CI -8.2 to -3.3; P<0.001; Figure 4E). Overall, there was a moderate inverse correlation between presence of CMD and the DTS (Spearman's Rho = -0.42; P<0.001). Considering the cohort of eighty-four patients in whom genotype and DTS were both available, there was a lower DTS for each additional G allele consistent with increasing ischaemia with ET-1 gene enhancement. *A priori* analysis of high-risk subjects (homozygous for the minor G allele) compared to the AA group revealed a mean difference of -3.0 units in DTS (95% CI -5.8 to -0.1; P=0.045; (Figure 4F). There was a modest correlation between the continuous Duke Treadmill Score (DTS) and genotype

(Spearman's rho -0.21; P=0.055), that was not statistically significant. The angina index during exercise was linearly associated with G allele status (non-limiting or limiting angina AA 59% v AG 68% v GG 87%; P trend =0.036). The exercise time was not significantly lower amongst subjects with the G allele (365 v 392 seconds; P=0.423).

Sixty-eight genotyped subjects agreed to participate in a vascular biology sub study, providing written informed consent for a gluteal subcutaneous biopsy within 4 weeks of coronary angiography. Subjects who volunteered to have a biopsy were of similar age and cardiac risk to those who declined to participate in the sub study (biopsy participants mean age 62 ± 9 years v 61 ± 11 years (P=0.134), ASSIGN score 23% ± 18 v 28% ± 23 (P=0.198). Forty-four (65%) of these patients had biopsies with a sufficient number of small arteries to undergo paired cumulative concentration response curves (CCRCs) to ET-1 in the presence and absence of an ET_A receptor antagonist, either BQ123 or zibotentan (ZD4054; AstraZeneca, Cambridge, UK). Grouping according to genotype (AA, n=16; AG, n=14; GG, n=14), vasodilator responses to ACh (ACh E_{max}) were similar (Table 3). Similarly, vessels had similar potency for ET-1 (pEC_{50} AA 9.34, AG 9.45 and GG 9.32; P=0.533) and maximum vasoconstriction to ET-1 (E_{max} AA 122.3%, AG 115.5%, GG 129.7%; P=0.533; Figure 5A; Table 3).

Notably, the selective ET_A receptor antagonist, BQ123, caused a parallel rightward shift of the CCRC with comparable pK_B values between groups AA, AG and GG (pK_B values of 7.07 [± 0.23], 7.79 [± 0.35] and 7.41 [± 0.26] respectively; P=0.209; Figure 5B). Zibotentan, ahighly selective orally active ET_A receptor antagonist, attenuated the constrictor response to ET-1 with pK_B of 7.54 (95% CI 7.27 – 7.82), comparable to that of BQ123 pK_B 7.53 (95% CI 7.37 – 7.69).

Crucially, these studies confirmed that zibotentan produced a concentration-dependent inhibition of an established constrictor response to ET-1 and was still efficacious in subjects with G allele ($P<0.001$; Figure 5C). Figure 6 shows representative investigations from a female subject with few traditional cardiovascular risk factors but high-risk ET-1 enhancer genotype (GG).

Discussion

We identify a novel genetic risk locus for coronary microvascular dysfunction. Our study extends a report from the WISE investigators on genotype associations with arterial vasomotion.[13] Our results support the hypothesis that dysregulation of the ET-1/ ET_A receptor system underpins abnormalities in the coronary microcirculation leading to myocardial ischaemia. Firstly, rs9349379-G allele status is associated with higher serum ET-1 and the presence and extent of CMD in patients with angina but without obstructive coronary disease. Secondly, the genetic polymorphism associates with ischaemia testing using distinct, non-invasive modalities including exercise stress electrocardiography and stress perfusion CMR. Thirdly, we demonstrate in *ex vivo* human small peripheral resistance vessels isolated from affected patients, that the ET_A vasoconstrictor response is not downregulated in the presence of increases in endothelin gene expression and ET-1 activity in patients with the rs9349379 G allele. Finally, we provide proof-of-concept mechanistic data supporting a role for zibotentan, an orally active highly selective ET_A receptor antagonist, in reversing established ET-1 mediated vasoconstriction. These findings have potential clinical relevance since zibotentan is available for repositioning as a novel, disease-modifying therapy in this patient population. The results of our study support the rationale for the 'Precision Medicine with Zibotentan in Microvascular Angina (PRIZE)' trial involving gene testing for the SNP rs9349379 and linked therapy (ClinicalTrials.gov Identifier: NCT04097314).

Endothelin dysregulation

Pre-clinical studies in experimental models of CMD implicate increased cardiac ET-1 production leading to endothelial dysfunction, enhanced vascular expression of rho-kinases and reactive oxidant species such as superoxide and enhanced ET-1-mediated vasoconstriction.[38] In patients with angina but no obstructive CAD, microvascular

dysfunction is a systemic phenomenon characterised by peripheral endothelial dysfunction and enhanced peripheral small vessel vasoconstriction.[31, 39] Further, impaired coronary microvascular function and the propensity to myocardial ischaemia may increase longer term-risk of major adverse cardiac events (MACE).[40, 41] Our study is distinct and builds on our prior vascular studies of ET-1 in microvascular angina as we used zibotentan which has more potential for clinical translation requiring future phase II studies.[31] In addition, subjects were analysed by ET-1 rs9349379-G allele status rather than presence or absence of CMD. We observed that chronic exposure to increased circulating concentrations of ET-1, as reflected by rs9349379-G allele status, did not lead to downregulation to ET_A mediated ET-1 vasoconstriction in patients with microvascular angina. The converse SNP (rs9349379-A) was recently found to be associated with spontaneous coronary artery dissection (SCAD) which typically occurs in patients without atherosclerosis.[21] This finding is consistent with our work, particularly given that microvascular function is typically normal in SCAD.[42]

We showed that rs9349379-G allele was associated with higher serum ET-1 levels which is consistent with previous studies whereby the SNP associates with higher levels of ET-1 and its precursor (Big ET-1) in healthy subjects. Interestingly, the ET-1 plasma concentration in our INOCA population is comparable to ET-1 plasma concentrations in other conditions including pulmonary artery hypertension[43] but lower than in other INOCA cohorts.[44] We acknowledge that abluminal secretion of ET-1 away from endothelial cells toward underlying vascular smooth muscle means circulating concentrations of ET-1 are an imperfect measure of ET-1 activity in vascular tissues.[45] Chronic elevation of circulating ET-1 may lead to adaptive down-regulation of its endogenous G-protein coupled receptors. This phenomenon has been described for ET_A receptors in mice in which the clearing ET_B receptor has been knocked out.[46] The baseline blood pressure was similar between the groups in our study analysis rs9349379 G allele and cardiovascular risk factors in large data sets have confirmed

an inverse association with systolic blood pressure.[17] This is particularly interesting given its association with atherogenesis and CAD. It is thought that excess ET-1 effects healthy populations mediate hypotension via hypotension via ET_B -induced nitric oxide and prostacyclin production, resultant vasodilation, diuresis, and natriuresis.[47] Our study was underpowered to determine significant differences between baseline blood pressures which may also be obfuscated by previous treatment for hypertension in the groups.

Microvascular angina is a chronic, debilitating condition of unmet therapeutic need. The vascular pharmacology findings in our clinical study indicate that despite a genetic predisposition to enhanced endothelin gene expression based on the rs9349379-G allele status, potentially leading to lifelong enhanced exposure to circulating concentrations of ET-1, the net effect on ET-1 response or sensitivity to ET_A antagonists was similar between the groups by rs9349379 allele status. This result indicates that the ET_A receptor may not be downregulated in affected patients raising the potential for health gain by treatment with a selective ET_A receptor antagonist, such as zibotentan. Importantly, BQ123 fully blocked the constrictor responses in all of the groups. Our vascular pharmacology study was specifically focused on the relationships between the rs9349379-G allele status, ET-1 vasoactive responses and ET_A receptor blockade. Patients with microvascular angina may have similar tissue responses to oral ET_A receptor blocker therapy – this important possibility merits further (NCT04097314).

In a mechanistic, randomized, controlled trial in patients with microvascular angina, Johnson and Gould reported that ET_A receptor antagonism increased (improved) the homogeneity of resting myocardial perfusion.[48] Their study used cardiac positron emission tomography (PET) to quantify the homogeneity index (a visual notion of homogeneity derived from PET).[49] Kaski *et al* showed that patients with microvascular angina were exposed to increased

circulating concentrations of ET-1 which in turn was associated with increased coronary vascular resistance and impaired coronary blood flow.[50] Recently, Theuerle et al have shown that plasma ET-1 is associated with invasive CMD in a 32 INOCA patients, however the relationship was driven by elevated microvascular resistance and not coronary flow reserve.[51]

Limitations

We describe compelling mechanistic evidence for a functional SNP being linked to CMD. We have followed accepted guidelines for CMD classifications, but it is recognized there are caveats with any classification system and acknowledge these are also relevant to this study. Firstly, we adopted binary cut-offs for the IDP test. It is possible that indeterminate (grey-zone or borderline) test results may have misclassified some patients. Furthermore, patients with CMD were heterogenous and we aggregated patients with different types of microvascular dysfunction e.g. impaired flow reserve, increased microvascular resistance, abnormal acetylcholine response. Nonetheless, the vascular phenotype of affected patients was of coronary vascular dysfunction based on consensus guidelines for abnormal coronary microvascular response during systemic adenosine, an abnormal vasomotor response to intracoronary Ach, or both.[6] In support of this approach, we observed a strong linear relationship between CMD and non-invasive ischaemia testing on the exercise treadmill (Figure 4F). In addition, heterogeneity is the rule rather than exception when considering many similar cardiovascular disorders, for example heart failure with preserved ejection fraction.[52] Our stratified sensitivity analysis by CMD type i.e. structural microvascular disease (i.e. raised IMR) and impaired vasodilator reserve (reduced CFR) (Table 2) lend further support to the design of our translational study. Secondly, not all patients underwent all testing and the exercise treadmill tests were not performed in a core-lab and were indicated as part of standard care. Nevertheless, they were performed according to the Bruce

protocol and the results were determined in a standardised manner, blinded to rs9349379 allele status. Treadmill exercise testing is an imperfect measure of ischaemia and hence it is plausible that the known association of the rs9349379-G allele with epicardial CAD is a confounding factor. Johnson and Gould recently highlighted how flush ostial branch vessel occlusion may account for ischaemia despite a visual "normal" angiogram without stenosis.[53] On the other hand, the Duke Treadmill Score has a mature associated literature with proven utility in CMD patients.[54, 55] The relatively small sample size and possibility of unmeasured baseline differences increases the possibility of type I error. Thirdly, we administered intra-arterial doses of short acting GTN (100-200 micrograms) to facilitate procedure safety relating to transradial access, coronary arteriography and invasive coronary vasoreactivity testing. Theoretically, GTN may affect the vascular responses to ACh however the half-life of GTN is around two minutes. Hence, after 10 minutes, only 3% of the GTN dose is bioavailable and we think the potential for confounding and a false negative test for microvascular vasospasm is unlikely. Conversely, a positive ACh test confounds assessment of true resting flow and may lead to falsely lowered CFR and hence we support ACh testing after adenosine assessment. Finally, we compared the allele prevalence within our cohort from Scotland with a pooled multicentre contemporary medical genome reference group of controls. Our study would have been strengthened by a control comparator group from the same area and ethnic background as our subjects. Further, although the SNP did not fulfil the Hardy-Weinberg equilibrium for the population as a whole, the control group from this study without CMD was consistent with the equilibrium (Chi square 2.99, P=0.084). It is plausible that HW was not met in the CMD group due to its association with the rs9349379 G allele of interest. This study is a cross-sectional analysis of a single genetic locus and provides associative findings of clinical interest but may overlook other important genetic risk determinants.

Clinical translation

These observations hypothesis generating particularly given the small sample size and heterogeneous patient population. The findings require external validation in other CMD cohorts whilst future work in populations from different regions would provide helpful context.

Overall, our study supports the case for selective ET_A blockade distinct from ET_B modulation in patients with microvascular disease in the heart. Oral ET_A-selective blockade has therapeutic potential by attenuating the propensity to microvascular vasospasm, increasing coronary blood flow, and further improving coronary endothelial function through NO-mediated release.[56] Zibotentan is one compound that holds promise as the most ET_A selective of all orally active ET_A receptor antagonists, which makes it particularly suited to use in microvascular angina. A targeted approach using selective ET_A receptor antagonist therapy in patients based on genotype is being assessed in the PRIZE trial (NCT04097314).

Conclusion

We identified a genetic risk locus for coronary microvascular dysfunction. The common genetic polymorphism (SNP rs9349379-G allele) was associated with higher ET-1 and both invasive coronary microvascular dysfunction and non-invasive tests for ischaemia in subjects with angina but no obstructive CAD. Mechanistic *ex-vivo* studies confirmed subjects with this functional allele have preserved response to ET_A receptor blockade. Zibotentan, an orally active ET_A receptor antagonist, reversed an established ET-1 mediated vasoconstriction. This study offers hope for angina patients although future trials are needed to determine whether CMD represents a potential new disease subtype for ET_A antagonist therapy.

Online content

Additional methods and supplementary information are available online.

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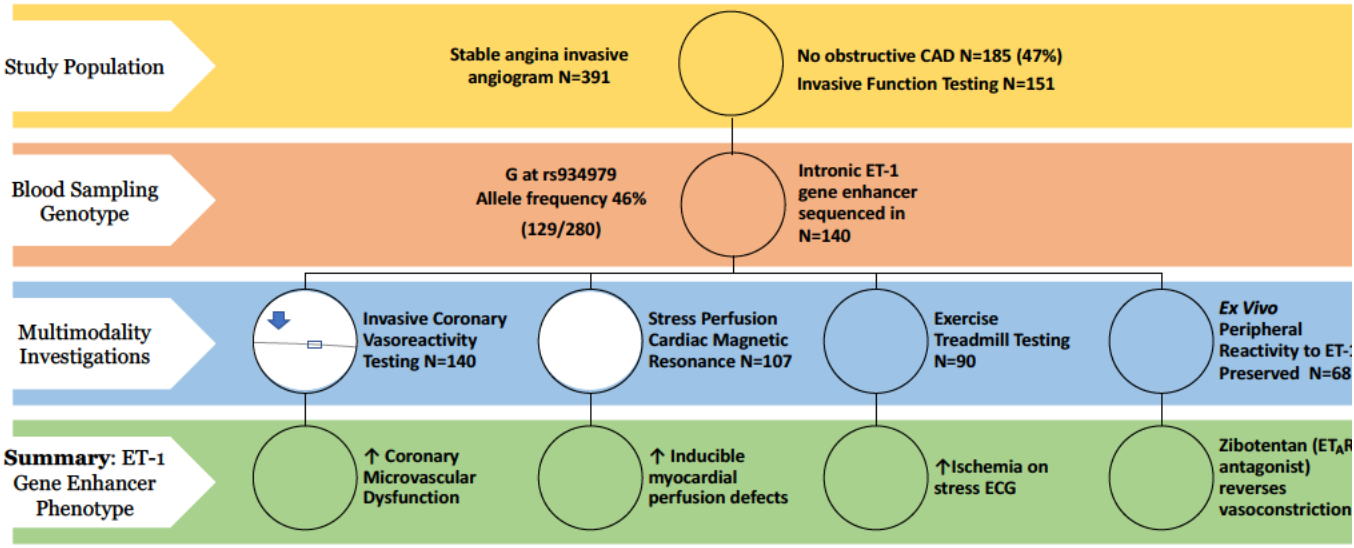
Author Contributions

TJF contributed to the study design and grant, recruited the patients, obtained the angiographic, laboratory and MRI data, and contributed to statistical analysis. DC & RC analysed and helped obtain MRI scans. EY performed blinded analysis of study data and helped edit the manuscript. AA performed the genotyping, SP supervised the genotype analysis and contributed to the manuscript, PR supervised biopsy work, recruited patients and edited the manuscript. RG, MME, SH, KR, MML, HE, KGO, RMcG and RMcD recruited patients and edited the manuscript, JJM provided extensive support, contributed to the statistical analysis of myography data and editing of the manuscript, LYH and AEA both applied novel MRI analysis techniques and edited the manuscript, KGO recruited patients and edited the manuscript, RMT contributed to grant funding, provided guidance on study design, data interpretation and edited the manuscript. APD contributed to conceiving and designing the research, provided guidance on scientific analysis of the data and critique of the manuscript at all stages of production, CB devised and obtained the study funding, PI of the CorMicA study, recruited patients and edited the manuscript.

Additional information

Supplemental information and methodology appended.

Fig. 1 – Study overview: Endothelin-1 gene enhancer in microvascular angina

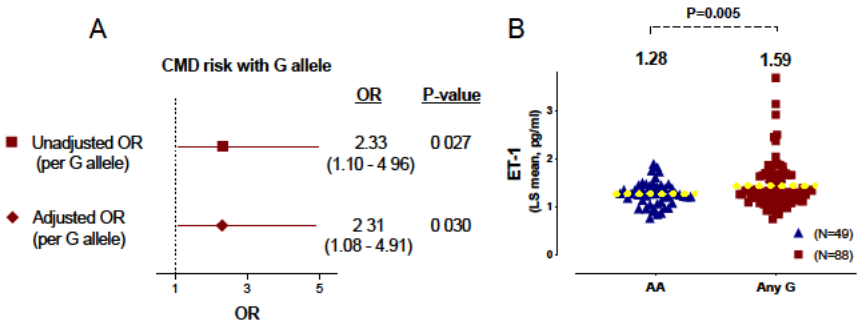


Three hundred and ninety-one patients with stable angina were prospectively enrolled without prior knowledge of coronary anatomy. 185 (47%) had no obstructive coronary artery disease and thus eligible for invasive coronary vasoreactivity testing and further sub studies. 151/185 (82%) were able to undergo adjunctive invasive tests for coronary microvascular dysfunction (CMD).

One hundred and nine (72%) of subjects tested had evidence of CMD. One hundred and forty subjects underwent genetic analysis for (rs9349379 G allele) with an allele frequency of 46% (129/280 alleles). The frequency of detrimental G alleles was higher than reference genome bank control subjects (46% v 39%; P=0.013). Patients with rs9349379-G allele had higher serum ET-1 and over double the odds of CMD (OR 2.33; 95% CI 1.10 – 4.96; P=0.027). In addition, subjects were more likely to have impaired myocardial perfusion (P=0.04) and exercise tolerance (-3.0 units in Duke Exercise Treadmill Score; P=0.045). Peripheral small artery reactivity to ET-1 and affinity of ET_A receptor antagonists were preserved in the rs9349379 G allele group (P=0.209). Crucially, zibotentan tested at clinically relevant concentrations, fully reversed an established ET-1 vasoconstriction, indicative of efficacy in

conditions associated with vasospasm. This suggests that ET_A receptor antagonism in this group of patients may have therapeutic benefit.

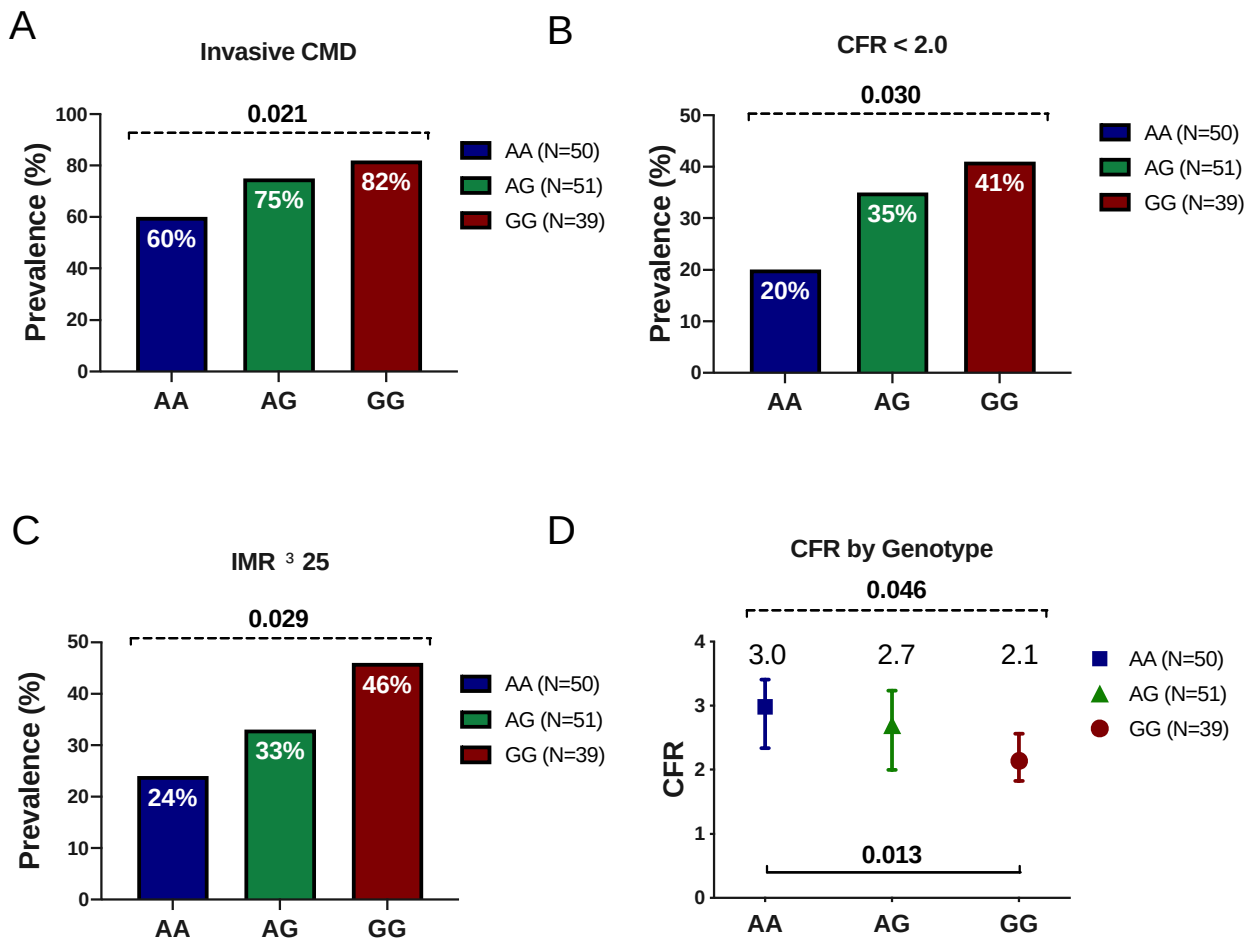
Fig. 2 – Detrimental effects of rs9349379-G allele on coronary microvascular function and ET-1



A – Patients with G allele were over twice as likely to have underlying microvascular dysfunction (OR per G allele 2.33; 95% CI 1.10 – 4.96; P=0.027) Even after adjustment for other risk factors the G allele was predictive of microvascular disease (OR 2.31; 95% CI 1.0 – 4.91). This finding supports a detrimental impact on the coronary microcirculation of a lifetime of increased Endothelin gene expression.

B – In a multivariable regression model adjusting for baseline group differences, patients with rs9349379-G allele had higher plasma ET-1 (LS mean 1.59pg/mL v 1.28pg/mL; 95%CI 0.10 to 0.53; P=0.005).

Fig. 3 – Genotype: phenotype association of G allele with invasive coronary microvascular dysfunction

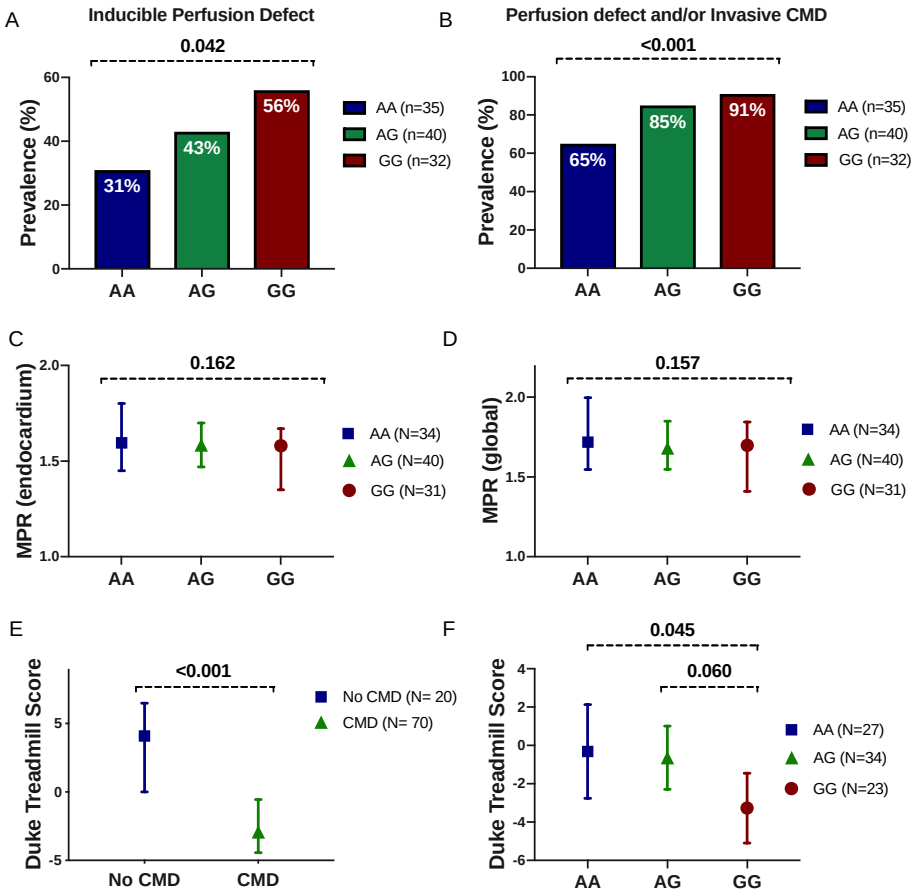


A - C The prevalence of microvascular dysfunction detected during invasive coronary testing was associated with genotype status (AA 60%, AG 75%, GG 83%; P=0.021). Presence of abnormal coronary flow reserve and microcirculatory resistance were linearly associated with each additional G allele. P-value represents Pearson-Chi square test for linear trend (categorical data).

D - Coronary flow reserve (CFR) was lower amongst subjects with two high risk G alleles (rs9349379) consistent with detrimental effects of increased Endothelin gene expression on the coronary microcirculation (Kruskal Wallis between groups dotted line P=0.046). A priori subgroup

analysis (AA v GG group – solid line) showed lower CFR in the GG group (P=0.013). Data is median CFR plus error bars represent 95% confidence intervals for the median.

Fig. 4 - Genotype: phenotype association of G allele with non-invasive ischaemia testing



A - Cardiovascular Stress Magnetic Resonance Imaging at 1.5 Tesla (N=107). There was a linear relationship between the G allele and presence of an inducible perfusion defect on CMR (χ^2 test for linear trend P=0.042).

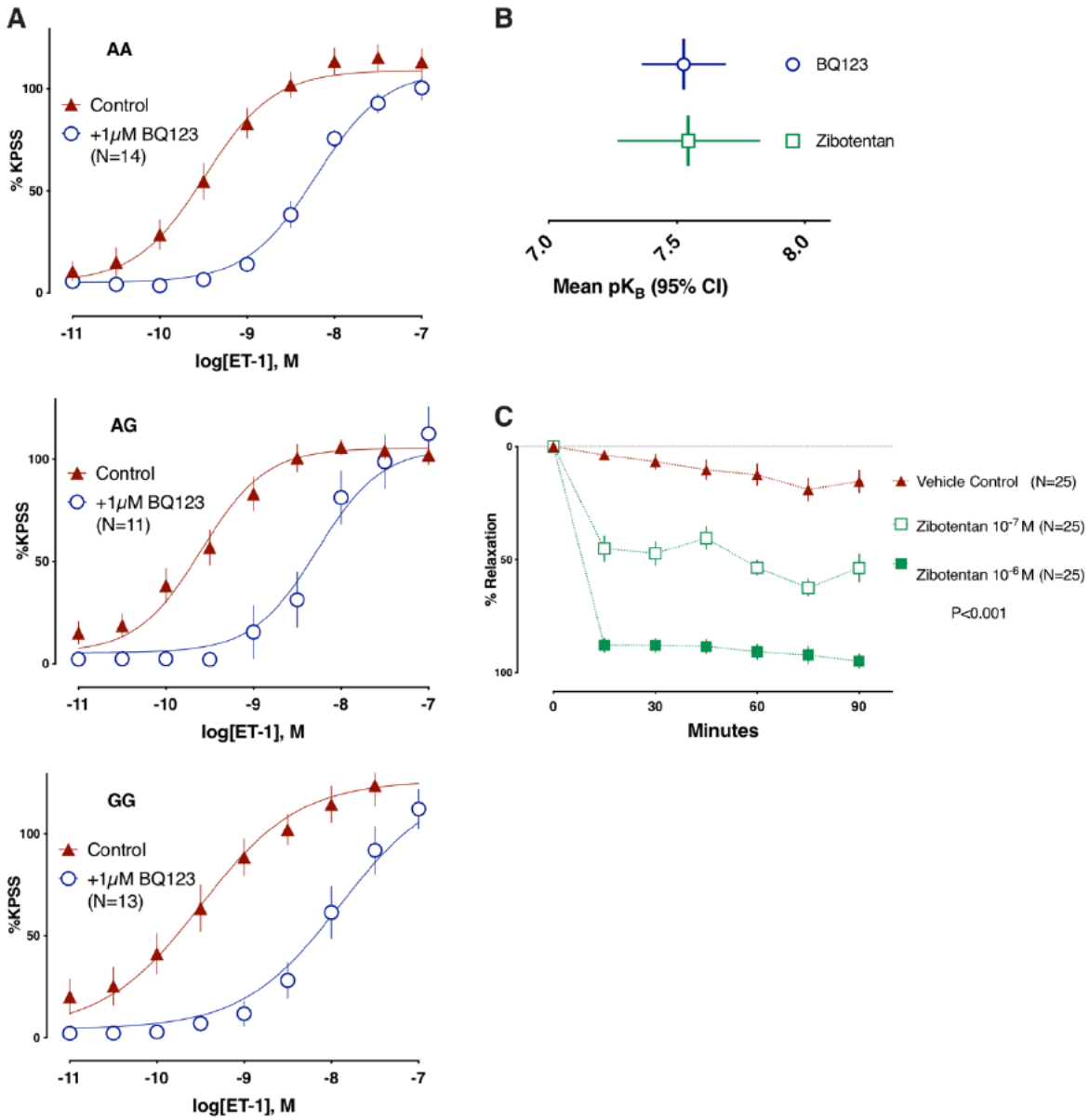
B – The relationship was more robust when considering with invasive evidence of CMD and/or inducible perfusion defect. Over 90% of GG subjects had at least one abnormality compared with only 65% of AA subjects (P<0.001)

C & D – Myocardial perfusion reserve was numerically reduced in AG and GG subjects compared to AA subjects however this was not statistically significant (P value represents ANOVA test for trend). Error bars represent 95% confidence intervals for the mean.

E – Invasive evidence of microvascular dysfunction (defined by abnormal response to intracoronary ACh and/or systemic adenosine) was functionally significant and associated with ischaemic burden on symptom limited exercise treadmill testing (CMD -2.3 v No CMD +3.5; Difference -5.8 units; -8.2 to -3.3; P<0.001).

F - Exercise Treadmill Testing (n=84) – There was a relationship between genotype group and worsening ischaemia on stress testing (ANOVA P trend=0.045). The mean difference in ischaemia by DTS between group GG and group AA was -3.0 units (95% CI -5.8 to -0.1; P=0.045). Error bars represent 95% confidence intervals for the mean.

Fig. 5 – Endothelin-1 *ex vivo* vascular biology by genotype

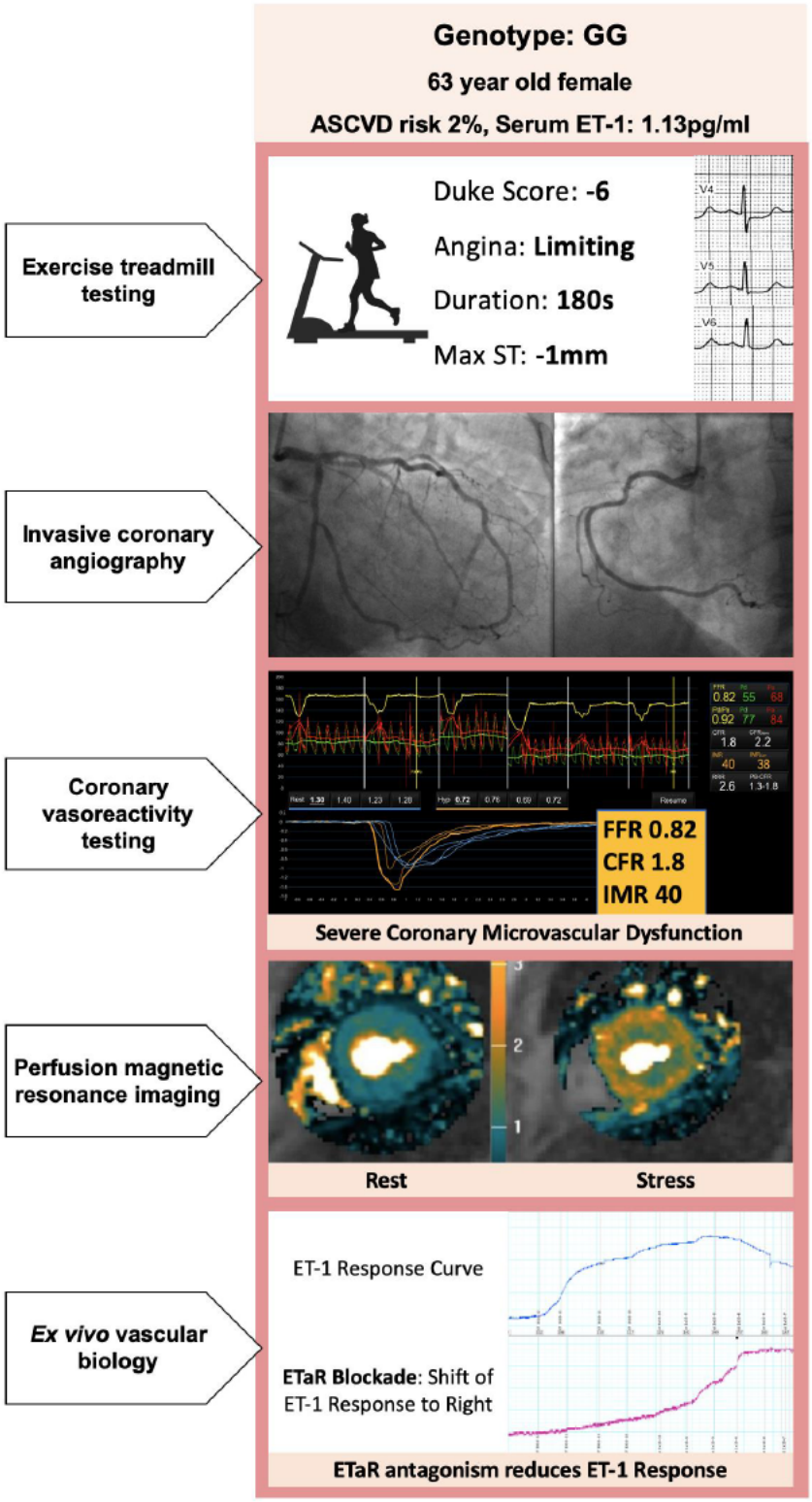


A - CCRC to ET-1 in the three groups in the presence and absence of ET_A antagonist BQ123 (n=44). Similar antagonist potency (rightward curve shift) for each group suggesting firstly that the ET_A receptors are the dominant effectors of the ET-1 vasoconstrictor response and secondly that the ET_A receptor pathway is not downregulated in spite of the elevated endothelin-1 gene expression and known increase in ET-1 activity in the G allele SNP patients.

B – Antagonist potency of novel therapeutic oral ET_A receptor antagonist zibotentan (N=8, mean 7.54 [95% CI 7.27-7.82]) is similar to peptide antagonist BQ123 (N=27, mean 7.53 [95% CI 7.37-7.69]). Higher pK_B represents a higher antagonist potency.

C - Zibotentan: reversal of established ET-1 vasoconstriction. Proof of concept dose dependent reversal of potent and established ET-1 mediated peripheral arteriolar vasoconstriction. Crucially, the highest concentration tested which is also the plasma concentration achieved by a clinically relevant dose of 10 mg/day rapidly and fully reversed the established ET-1 constrictor response, indicative of efficacy in conditions of vasospasm. Comparison using ordinary two-way ANOVA including time and dose both significant factors ($P<0.001$ after adjustment for multiple testing).

Fig. 6 – Illustrative cases: GG (high risk ET-1 gene enhancer)



Illustrative case from a patient with stable angina including representative images from invasive and non-invasive work up are shown in relation to clinical presentation and ET-1

enhancer genotype. Maximum ST represents the maximum planar or down sloping ST segment depression during the exercise treadmill test. Invasive coronary angiography of both subjects is near identical showing only minimal luminal irregularities. White arrows represent subendocardial inducible ischaemic myocardium during adenosine stress MRI in a patient with severe coronary microvascular dysfunction. *Ex vivo* vascular biology (bottom panel) shows typical ET-1 mediated vessel constriction during wire myography. Increasing vessel tension corresponds to the rising curve at each dose titration. A paired identical vessel experiment is performed after incubation with BQ123, an ET_A receptor antagonist. This curve is marked in blue, the curve of ET-1 response is shifted to the right indicating that the ET_A receptor mediates vasoconstriction. Despite the ET-1 gene enhancer, the GG subject does not appear to have ET_A receptor downregulation with similar levels of antagonist potency. This supports that ET_A receptor antagonism in this group of patients may have therapeutic benefit.

CFR: coronary flow reserve, FFR: fractional flow reserve, IMR: index of microcirculatory resistance, ET_A: Endothelin A receptor

Table 3 – Pathophysiology: vascular biology of ET-1.

	SNP (rs9349379) genotype (n=44)			
	AA (N=16)	**AG (N=14)**	**GG (N=14)**	**P-value***
Vessel Diameter (um)	344 (±88)	342 (±89)	347 (±125)	0.851
Vessel Length (mm)	1.85 (±0.12)	1.87 (±0.10)	1.82 (±0.11)	0.276
ACh E_{max} (%)	77.7 (52.9 – 97.8)	80.2 (59.9 – 97.6)	92.5 (57.8 – 99.1)	0.696
ACh pEC_{50}	7.28 (6.88 - 7.82)	7.26 (6.82 - 8.00)	6.96 (6.84 – 7.44)	0.308
ET-1 E_{max} (%)	122.3 (115.7 - 134.7)	115.5 (107.5 - 125.2)	129.7 (115.8 - 151.2)	0.533
ET-1 pEC_{50}	9.34 (9.15 - 9.52)	9.45 (9.24 - 9.67)	9.32 (8.96 - 9.69)	0.533
BQ123 pK_B [± SEM]	7.07 [±0.23]	7.79 [±0.35]	7.41 [±0.26]	0.209

Forty-four (65%) of 68 patients who underwent invasive biopsies had a sufficient number of small arteries to undergo paired cumulative concentration response curves (CCRCs) to ET-1 in the presence and absence of an ET_A receptor antagonist. Data are mean (± SD) or mean (95% CI for pooled best fit CCRC). CCRC: cumulative concentration response curves were drawn with best-fit derived values. pK_B data involved paired vessels undergoing ET-1 CCRC in the presence or absence of BQ123 ET_A receptor antagonist (available in 37 out of the 44 subjects: AA N=14; AG N=10; GG N=13). *Significance determined using ANOVA for normally distributed means, Kruskal-Wallis test used for between group comparison of non-parametric variables and Extra-Sum of squares F test (for CCRC pooled best fit ET-1 data). There were no differences in between group baseline demographics in this vascular sub-study.

Table 1. Baseline demographics by genotype.

	SNP (rs9349379) genotype (n=140)			
	AA (N=50)	AG (N=51)	GG (N=39)	P-value*
Clinical Features				
Age, years	60.6 (±11)	61.1 (± 10)	61.6 (± 10)	0.649
Female	36 (72%)	36 (71%)	31 (80%)	0.607
ASSIGN score †	24 (±21)	27 (±23)	25 (±19)	0.811
Dyslipidaemia	12 (24%)	10 (20%)	8 (21%)	0.671
Hypertension	30 (60%)	32 (63%)	27 (69%)	0.382
Previous cardiovascular event‡	10 (20%)	10 (20%)	13 (33%)	0.239
Diabetic	9 (18%)	11 (22%)	6 (15%)	0.794
Smoker	6 (12%)	8 (16%)	9 (23%)	0.169
Family history	17 (34%)	13 (26%)	13 (33%)	0.886
Peripheral vascular disease	2 (4%)	3 (6%)	2 (5%)	0.789
Atrial fibrillation	5 (10%)	4 (8%)	1 (3%)	0.195
Pulse (rate / min)	69 (±11)	67 (±11)	71 (±11)	0.697
Systolic blood pressure (mmHg)	138 (±22)	136 (±31)	138 (±25)	0.951
Diastolic blood pressure (mmHg)	73 (±11)	74 (±15)	70 (±12)	0.260
Body mass index (kg/m²)	30.4 (±8)	30.4 (±6)	29.4 (±7)	0.515
Laboratory Investigations				
Cholesterol (mmol/L)	3.5 (±1)	3.5 (±1)	3.6 (±1)	0.904
Glucose (mmol/L)	4.6 (±1)	5.0 (±2)	4.7 (±2)	0.774
C-reactive protein (mg/L)	3.2 (±5)	3.2 (±5)	3.1 (±4)	0.920
N-terminal brain natriuretic peptide (pg/ml)	140 (±187)	157 (±197)	135 (±153)	0.937
Endothelin-1 (pg/ml)†	1.27 (0.42)	1.41 (0.63)	1.46 (0.56)	0.097

Data are mean (SD) or number (%). ACE-I = angiotensin converting enzyme inhibitor. ACh= Acetylcholine. BMI=body mass index. CCB = calcium channel blocker. CFR = coronary flow reserve. FFR = fractional flow reserve. LVEDP = left ventricular end-diastolic pressure. MI = myocardial infarction. IMR = index of microcirculatory resistance. * P-value represents between group ANOVA for linear trend (continuous data) or Pearson-Chi square test for linear trend (categorical data) or Kruskal-Wallis testing probability that the distribution of non-parametric variables are the same across the groups. † ASSIGN risk – predicted 10-year risk of cardiovascular event. ‡ denotes previous myocardial infarction or cerebrovascular event (including transient ischaemic attack). † Endothelin-1 levels were available in 137 genotyped subjects with significance determined using one-way ANOVA (linear trend).

Table 2 – Invasive coronary physiology and non-invasive stress testing.

	SNP (rs9349379) genotype			
	AA (N=50)	AG (N=51)	GG (N=39)	P-value*
Minor non-obstructive CAD‡	25 (50%)	30 (59%)	24 (62%)	0.265
Coronary atheroma burden (Gensini score)†	0 (0, 2)	2 (0,5)	1 (0, 6)	0.037
Left ventricular end-diastolic pressure (mmHg)	10 (±4)	10 (±5)	9 (±3)	0.520
Fractional flow reserve (FFR)	0.88 (0.05)	0.88 (0.06)	0.88 (0.05)	0.977
Coronary microvascular dysfunction (any)	30 (60%)	38 (75%)	32 (82%)	0.021
• *Abnormal CFR (<2.0)*	10 (20%)	18 (36%)	16 (41%)	0.030
Coronary flow reserve (CFR)	3.0 (2.1, 3.7)	2.7 (1.8, 3.5)	2.1 (1.7, 3.2)	0.046
• *Abnormal IMR (≥25)*	12 (24%)	17 (33%)	18 (46%)	0.029
Microcirculatory resistance (IMR)	18.9 (15.2, 24.2)	18.6 (14.2, 29.3)	22.1 (13.8, 29.3)	0.879
• *Abnormal CFR or IMR*	20 (40%)	26 (51%)	27 (69%)	0.007
• *Microvascular spasm (during acetylcholine)*	15 (30%)	21 (42%)	12 (31%)	0.385
Exercise treadmill testing (N=87)	28 (56%)	34 (67%)	25 (64%)	
Duration (seconds)	393 (±124)	352 (±157)	384 (±162)	0.827
METs	7.8 (±2.1)	7.4 (±2.6)	7.6 (±2.1)	0.786
Angina on treadmill	16 (59%)	23 (68%)	20 (87%)	0.036
Peak systolic blood pressure (mmHg)	178 (±30)	173 (±34)	182 (±25)	0.688
Duke Treadmill Score	-0.3 (±6.0)	-0.6 (±4.7)	-3.3 (±4.2)	0.045
Stress perfusion magnetic resonance imaging (N=107)				
Inducible myocardial perfusion defect	11 (31%)	17 (43%)	18 (56%)	0.042
Inducible myocardial perfusion defect with CMD	4 (13%)	14 (37%)	15 (47%)	0.016
Myocardial perfusion reserve (global)	1.8 (±0.4)	1.7 (±0.4)	1.6 (±0.4)	0.154
Myocardial perfusion reserve (endocardium)	1.7 (±0.4)	1.6 (±0.4)	1.5 (±0.4)	0.162
Left ventricular end diastolic volume (indexed, mL/m^2)	68.5 (±13.6)	70.1 (±13.2)	70.2 (±11.9)	0.591
Left ventricular end systolic volume (indexed, mL/m^2)	23.4 (±6.0)	25.4 (±8.8)	23.1 (±5.8)	0.848
Left ventricular ejection fraction (%)	65.9 (±4.4)	64.5 (±6.5)	67.3 (±5.2)	0.321
Stroke volume (indexed, mL/m^2)	45.0 (±8.8)	44.7 (±7.0)	47.1 (±8.2)	0.298

Left ventricular mass – (indexed, mL/m²)	42.0 (±7.0)	42.3 (±8.1)	42.1 (±7.8)	0.924

Data are mean (± SD), median (IQR) or N (%). CAD= coronary artery disease. CFR = coronary flow reserve. FFR = fractional flow reserve. LVEDP = left ventricular end-diastolic pressure. IMR = index of microcirculatory resistance. ♯ denotes core-laboratory adjudication of any angiographic evidence of coronary atherosclerosis including any minimal angiographic luminal irregularity. †Gensini angiographic score is a metric of angiographic disease severity incorporating lesion severity and location. METS: metabolic equivalent of task. Detailed MRI methodology available in online appendix. * P-value represents between group ANOVA for linear trend (continuous data) or Pearson-Chi square test for linear trend (categorical data), Kruskal-Wallis test of probability that the distribution of non-parametric variables are the same across the groups.

Figure 1 -

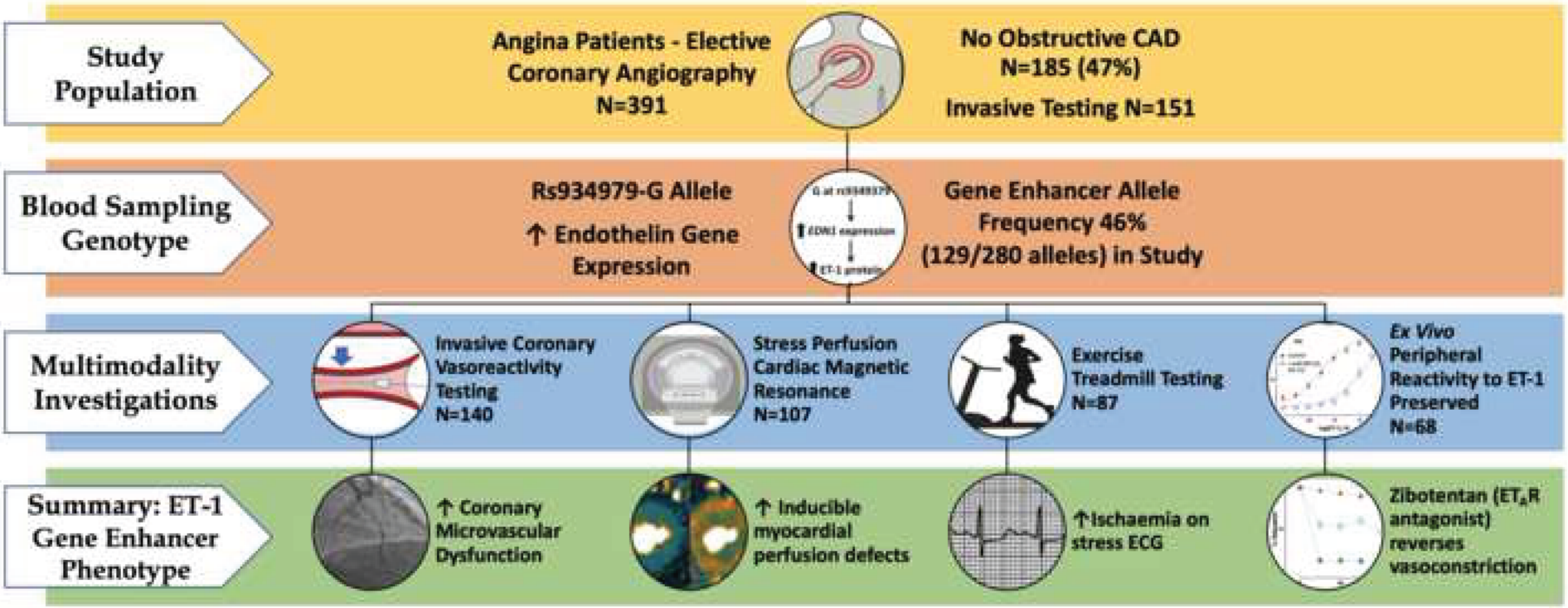

Study Population
Angina Patients - Elective Coronary Angiography N=391
No Obstructive CAD N=185 (47%)
Invasive Testing N=151
Blood Sampling Genotype
Rs934979-G Allele
↑ Endothelin Gene Expression
Gene Enhancer Allele Frequency 46% (129/280 alleles) in Study
Multimodality Investigations
Invasive Coronary Vasoreactivity Testing N=140
Stress Perfusion Cardiac Magnetic Resonance N=107
Exercise Treadmill Testing N=87
Ex Vivo Peripheral Reactivity to ET-1 Preserved N=68
Summary: ET-1 Gene Enhancer Phenotype
↑ Coronary Microvascular Dysfunction
↑ Inducible myocardial perfusion defects
↑Ischaemia on stress ECG
Zibotentan (ET_AR antagonist) reverses vasoconstriction

Figure 2

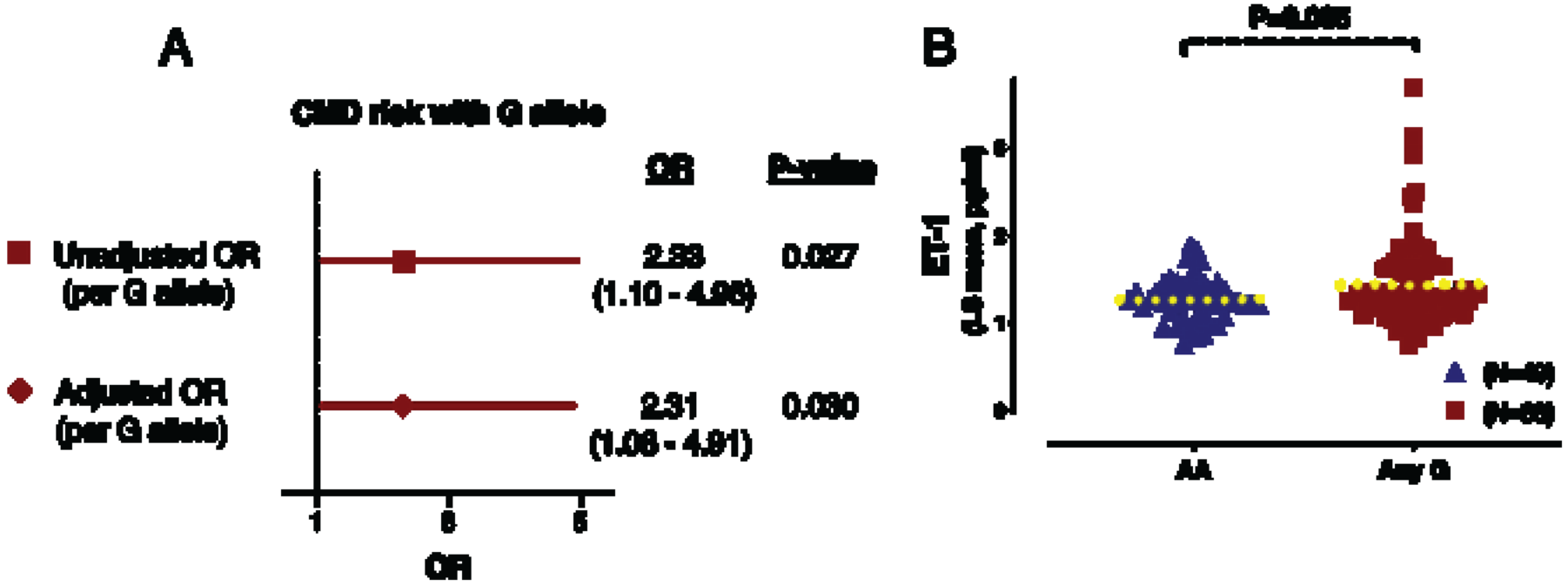

A
CHD risk with G allele
OR
P-value
Unadjusted OR
(per G allele)
2.33
(1.10 - 4.96)
0.027
Adjusted OR
(per G allele)
2.31
(1.06 - 4.91)
0.030
1
3
5
OR
B
ET-1
AA
Any G

Figure 3

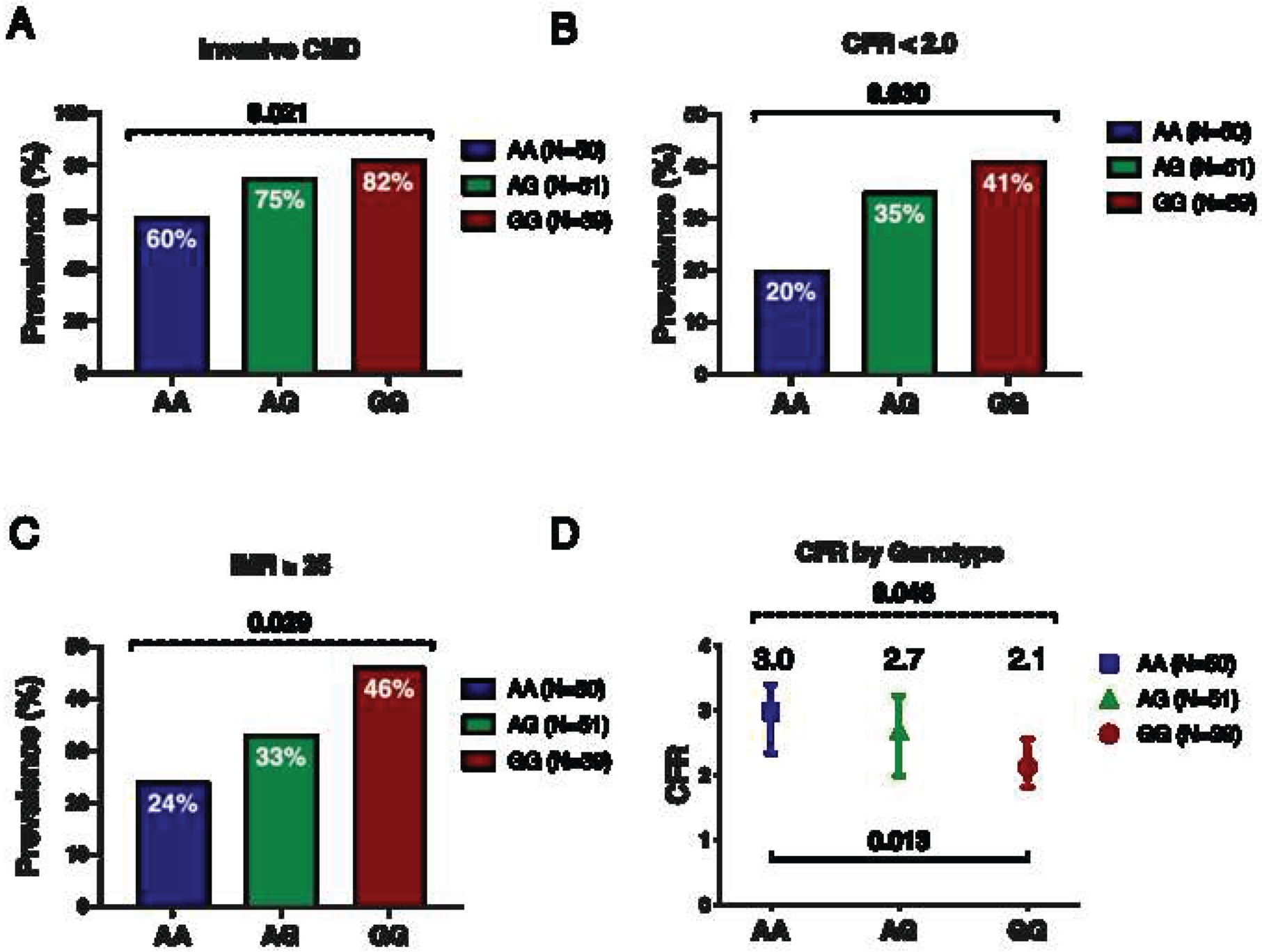

A
Invasive CMD
0.021
Prevalence (%)
60%
75%
82%
AA
AG
GG
B
CFR < 2.0
0.030
Prevalence (%)
20%
35%
41%
AA
AG
GG
C
0.029
Prevalence (%)
24%
33%
46%
AA
AG
GG
D
CFR by Genotype
0.046
CFR
3.0
2.7
2.1
0.013
AA
AG
GG

Figure 4

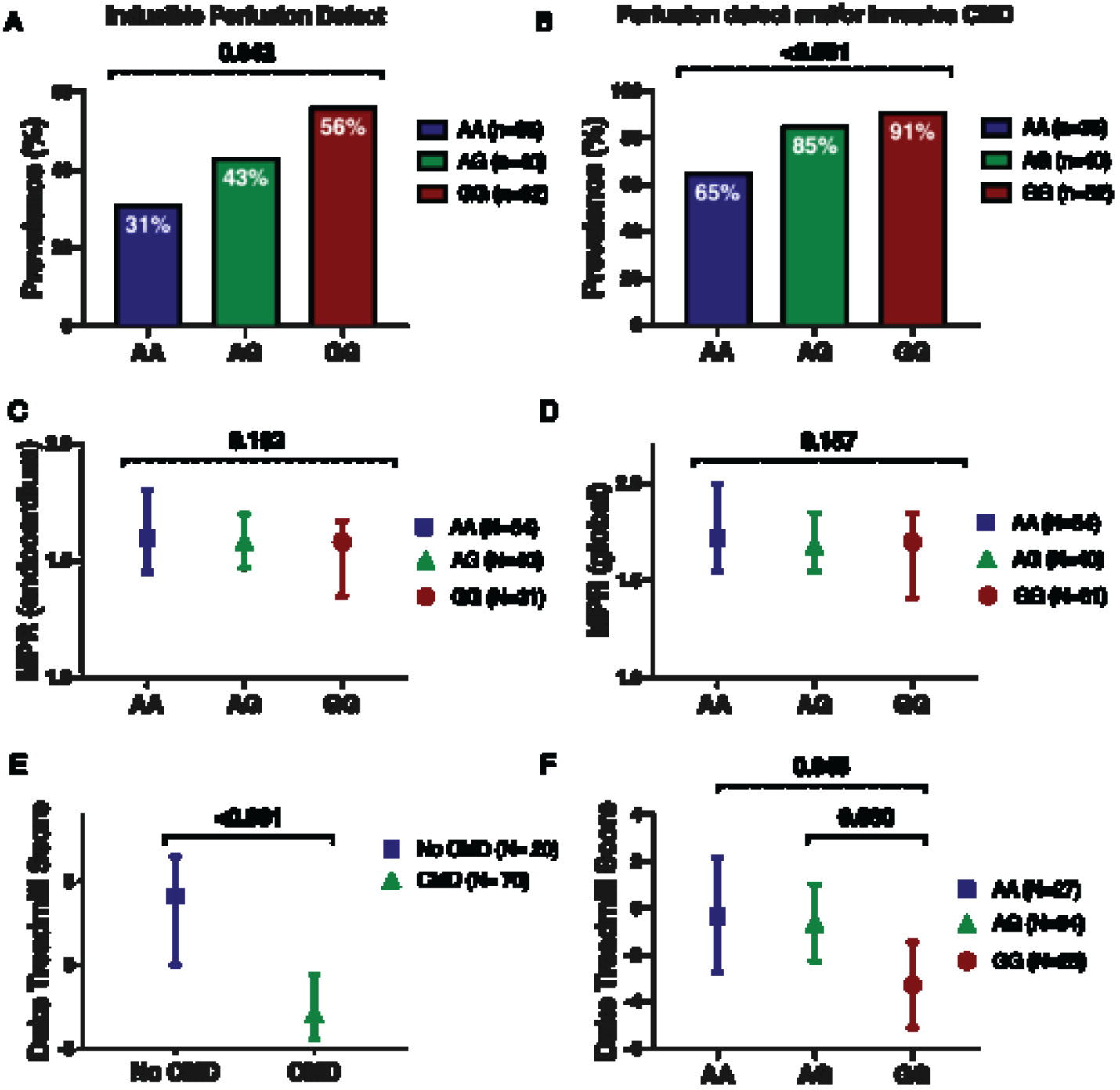

A
Inducible Perfusion Defect
0.043
Prevalence (%)
31%
43%
56%
AA
AG
GG
B
Perfusion defect and/or Invasive CAD
<0.001
Prevalence (%)
65%
85%
91%
AA
AG
GG
C
0.102
MPR (endocardium)
AA
AG
GG
D
0.157
MPR (global)
AA
AG
GG
E
<0.001
Duke Treadmill Score
No CAD
CAD
F
0.045
0.020
Duke Treadmill Score
AA
AG
GG

Figure 5

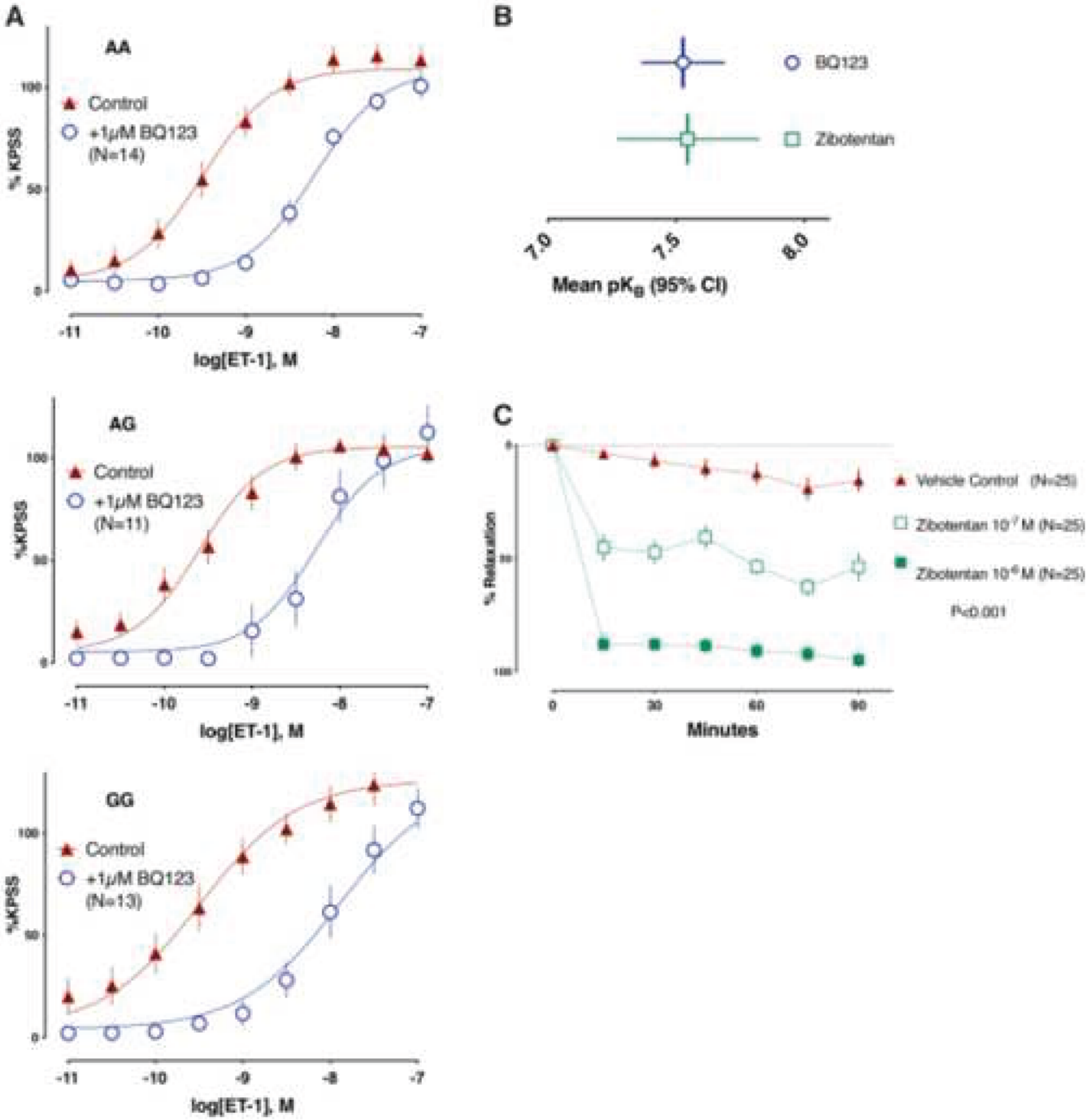
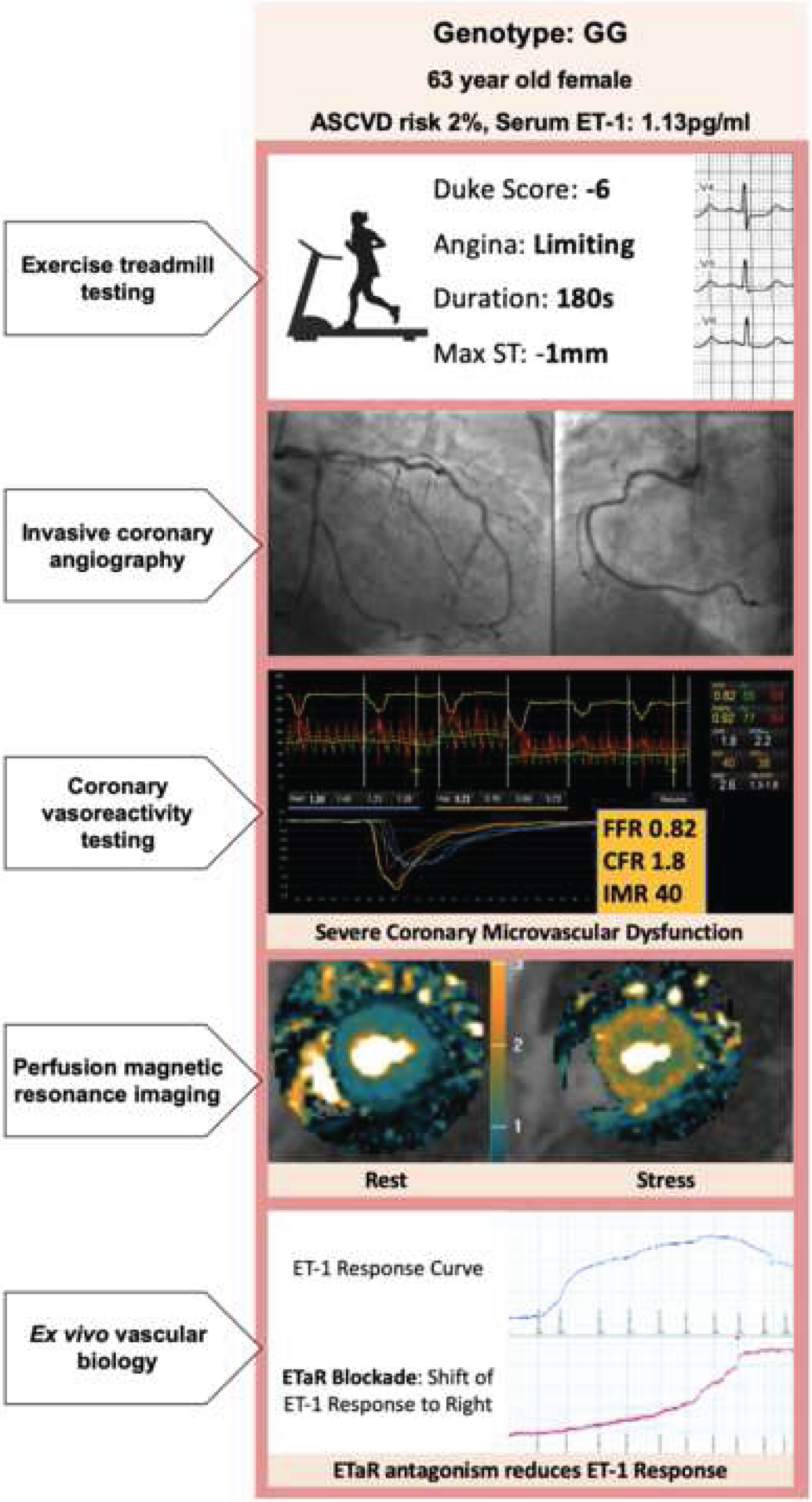

A
AA
Control
+1μM BQ123
(N=14)
% KPSS
log[ET-1], M
AG
Control
+1μM BQ123
(N=11)
%KPSS
log[ET-1], M
GG
Control
+1μM BQ123
(N=13)
%KPSS
log[ET-1], M
B
BQ123
Zibotentan
Mean pK_B (95% CI)
C
Vehicle Control (N=25)
Zibotentan 10^-7 M (N=25)
Zibotentan 10^-6 M (N=25)
P<0.001
% Relaxation
Minutes

Figure 6


Genotype: GG
63 year old female
ASCVD risk 2%, Serum ET-1: 1.13pg/ml
Exercise treadmill testing
Duke Score: -6
Angina: Limiting
Duration: 180s
Max ST: -1mm
Invasive coronary angiography
Coronary vasoreactivity testing
FFR 0.82
CFR 1.8
IMR 40
Severe Coronary Microvascular Dysfunction
Perfusion magnetic resonance imaging
Rest
Stress
Ex vivo vascular biology
ET-1 Response Curve
ETaR Blockade: Shift of ET-1 Response to Right
ETaR antagonism reduces ET-1 Response

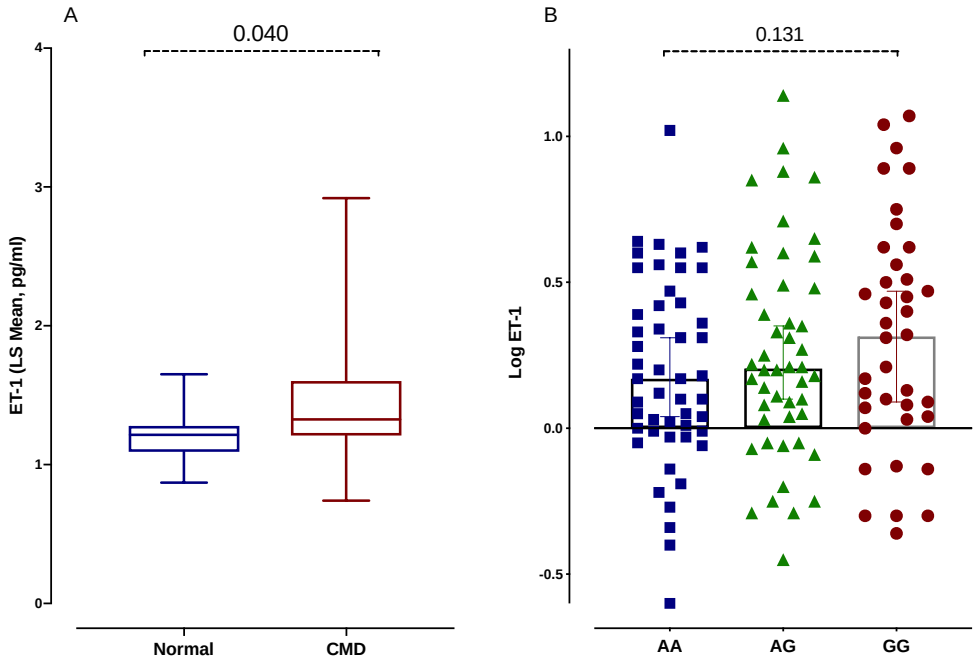
Supplementary Information (Appendix)

Genetic dysregulation of endothelin-1 is implicated in coronary microvascular dysfunction

Table of Contents

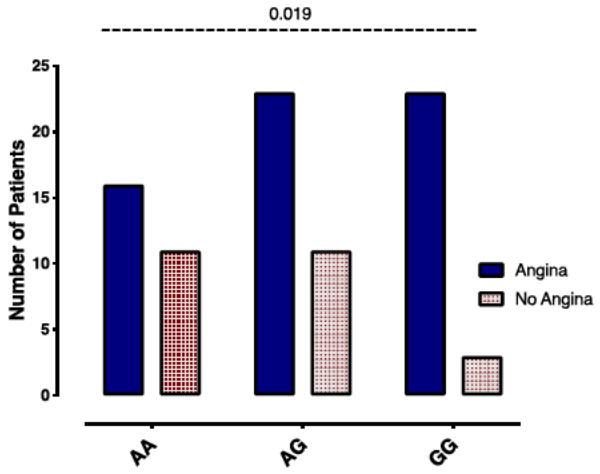
1. Supplementary Figure 1 – ET-1 in CMD subjects

A: Serum ET-1was higher in patients with coronary microvascular dysfunction than normal control subjects [P=0.040]. ET-1 was adjusted for baseline confounders in regression model outlined in study methods. B: Normalized serum ET-1 (Log transformed) showed a trend towards significance with increasing peptide linearly with each G allele [P linear trend = 0.131].



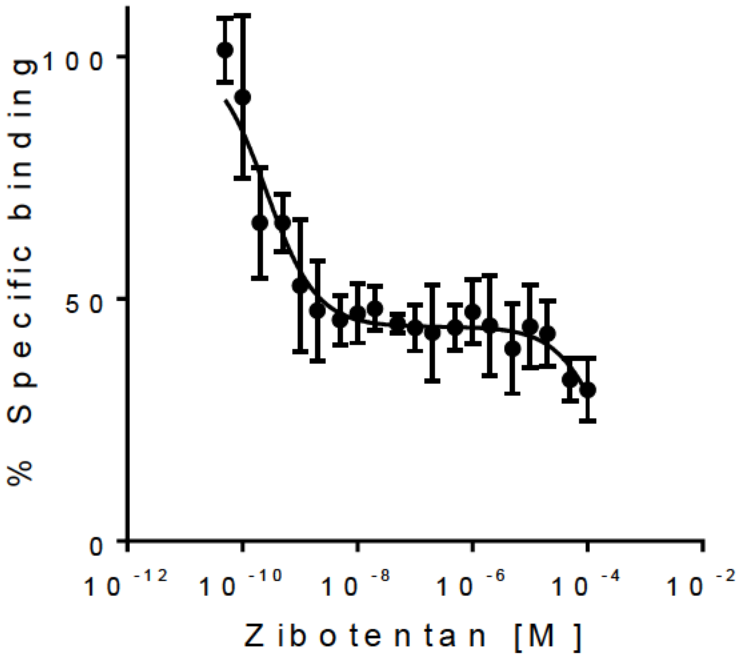
2. Supplementary Figure 2 – Angina on treadmill testing

Exercise treadmill testing of angina patients without obstructive CAD by genotype group according to presence or absence of angina during exercise. There was a significant linear association of angina status with rs9349379 genotype (P=0.019)



3. Supplementary Figure 3: Competition Binding Curve for zibotentan

Competition Binding Curve for zibotentan in human left ventricle confirms selectivity for the ET_A receptor



4. Supplementary Table 1. Genotype as multivariable predictor of CMD

	B	OR	Lower CI	Upper CI	P-value
Genotype (per G allele)	0.84	2.31	1.08	4.91	0.030
ASSIGN	0.00	1.00	0.98	1.02	0.970
Past CV event	0.23	1.26	0.51	3.15	0.619

Multivariable predictors of any CMD (N=109) with three baseline factors in model listed above. The fitted regression model showed moderate discrimination potential with an AUC of 0.647 (95% CI 0.544 – 0.750; P=0.007). ASSIGN refers to estimated 10-year risk of cardiovascular events incorporating social deprivation in a validated score within our geographical area.[1]

5. Supplemental Methods: Measurement of coronary vascular function *in vivo*

We used an interventional diagnostic protocol that combined guidewire-based direct measurement of coronary vascular function followed by pharmacological vasoreactivity testing. Specifically, the procedure included a guidewire-based measurement of coronary vascular function (FFR, coronary flow reserve [CFR], and the index of microvascular resistance [IMR]) followed by pharmacological vasoreactivity testing with acetylcholine (ACh) and glyceryl trinitrate (GTN) and has been previously described.[2, 3]

In brief, an intravenous infusion of adenosine (140 $\mu g \cdot kg^{-1} \cdot min^{-1}$) was administered via a large peripheral vein to induce steady-state maximal hyperaemia. A pressure-temperature sensitive guidewire was placed into the distal third of a major epicardial coronary artery (typically the left anterior descending [LAD]). The myocardial FFR was calculated by the ratio of mean distal coronary pressure to mean aortic pressure at maximal hyperaemia. A FFR $\leq$0.80 was taken as abnormal and indicative of flow-limiting coronary artery disease.[4] CFR was calculated using thermodilution as resting mean transit time divided by hyperaemic mean transit time.[5] A CFR <2.0 was defined as abnormal representing impaired vasodilator reserve.[6] The IMR was calculated as the product of mean hyperaemic transit time and mean distal coronary pressure at hyperaemia.[7] An IMR >25 was defined as abnormal and indicative of increased microvascular resistance.[8] These invasive parameters were simultaneously derived in real-time using dedicated software (Coroventis, Uppsala, Sweden). We assessed endothelium-dependent coronary vasomotor function using intra-coronary infusions of ACh via the guiding catheter at concentrations of 0.182, 1.82, and 18.2 µg/mL (10^{-6}, 10^{-5}, and 10^{-4} mol/L, respectively) at 1 mL/min for

2 minutes via a mechanical infusion pump.[9] We then immediately performed provocation testing for microvascular or epicardial coronary artery spasm using a 100μg bolus of ACh (5.5 mL of 10^{-4} mol/L over 20 seconds – reduced to 50μg for the RCA). In order to assess non-endothelial dependent vasodilatation, 300 μg of GTN was administered by manual intra-coronary bolus injection.

6. Angiographic analysis and quantitative coronary angiography (QCA)

Quantitative coronary analysis of the target coronary artery was performed using computer-assisted angiographic analysis (QAngio XA7.3, Medis, Leiden, Netherlands) by a trained cardiologist. Fluoroscopic images from two angles at least 30° apart were acquired. The coronary artery (typically left anterior descending artery) measurements were performed in the region where the greatest change had occurred during coronary reactivity testing.[10] End-diastolic cine frames that best show the segment were selected, and calibration of the video and cine images was performed. Coronary artery diameter change (% from baseline) was measured in response to both ACh and glyceryl trinitrate. Severe endothelial dysfunction was defined by ≥20% luminal constriction during ACh infusion (up to 10^{-4}M); this finding implies significant reduction in coronary artery blood flow with prognostic implications when compared with patients whose arteries were <20% constricted.[11] Coronary artery disease severity was assessed using the Gensini score.[12]A second trained observer (PM) performed QCA on a consecutive sample of 10% of cases, with high concordance for measurements of percentage lumen diameter vasoconstriction during ACh vasospasm assessment (intraclass correlation coefficient for average measures 0.96; 95% CI 0.88-0.99; p<0.001) and Gensini angiographic score (intraclass correlation coefficient for average measures 0.99; 95% CI 0.96-1.00; p<0.001).

7. Definitions: coronary microvascular dysfunction

We defined CMD using invasive coronary function testing and the Coronary Vasomotion Disorders International Study Group (COVADIS) diagnostic criteria.[13] These physiological criteria included raised IMR, abnormal coronary vasodilator capacity (CFR) and/or microvascular spasm during ACh provocation (reproduction of angina symptoms, ischaemic ECG changes (≥1mm ST segment deviation), but < 90% epicardial spasm during ACh testing).[14] FFR was measured to rule-out flow limiting coronary artery disease as an alternative explanation for myocardial ischaemia. Therefore, all participants had an FFR >0.8 in the target coronary artery and participants with an FFR ≤0.80 were excluded.

8. Blood and tissue analysis

Serum ET-1 was determined using blood obtained on the day of coronary function testing (Quantikine ® ELISA, R&D Systems® Europe, Abington [UK]). Blood was obtained from participants following an overnight fast in a recumbent position.

Ex vivo pharmacological assessment of peripheral vascular function was performed on patients who volunteered to undergo a gluteal skin fat biopsy within 4 weeks of the invasive coronary function assessment. The biopsy was obtained under sterile conditions using local anaesthesia with lidocaine (2%). Arterioles (< 400μm) were carefully dissected from fresh biopsies using a light microscope. 2mm length arterioles were mounted on 40-μm stainless steel wires for isometric myography in multi-channel myograph chambers (DMT, Denmark) filled with physiological saline solution. Isometric tension recordings followed-on directly using the technique of wire myography to study small peripheral resistance arteries with paired cumulative concentration response curves (CCRCs) to ET-1 in the presence or absence of an ET_A receptor antagonist, either BQ123 or zibotentan. The detailed methods are described in the study appendix. The peripheral vascular sensitivity to ET-1 (pEC_{50}) and maximum vasoconstriction to ET-1 (E_{max}) were determined.

For the antagonist studies the affinity (K_B) of BQ123 was first determined in paired vessels from individuals and calculated using Schild regression. The pK_B ($-\log_{10} K_B$) values were compared between each genotype as an indicator of whether or not patients of different genotypes are likely to respond equally well to an ET_A antagonist used clinically. A final series of experiments involved paired vessel experiments using ET-1

CCRCs in the presence and absence of a highly selective ET_A receptor antagonist, zibotentan to determine a pK_B value. More importantly the aim of these experiments was also to evaluate whether zibotentan could reverse an established ET-1 mediated vessel constriction.

9. DNA extraction and genotyping

Buffy coat was extracted from the whole blood of patients after centrifugation at 10,000g) to isolate genomic DNA using The PureLink® Genomic DNA Mini Kit (Invitrogen™). The samples were added to the lysis/binding buffer and digested with Proteinase K and RNase A for a minimum of 10 minutes at 50^{o}C. The samples were then brought to room temperature, followed by the addition of absolute ethanol. This was applied to the PureLink® Spin column and centrifuged, followed by subsequent washing and elution using Tris EDTA buffer. The quantity and quality of the DNA extracted was determined using NanoDrop™ Lite Spectrophotometer (ThermoFisher Scientific™).

To determine the genotype for rs9349379, the probes TaqMan® SNP Genotyping Assay ID C___1756707_10, with the context Sequence **[VIC/FAM]**: TCTATGCCCTTGAGATCATATAAAA**[A/G**]TAGCTTAAAATCATTGGCCATAGT T (Applied Biosystems™). DNA concentration of 5ng/uL was used with the TaqMan® probes and TaqMan® Universal Master Mix II, no UNG (Applied Biosystems™) to a total reaction volume of 5uL. This was amplified and read using QuantStudio™ 12K Flex (Applied Biosystems™) to determine the genotype.

10. Cardiac magnetic resonance imaging and ischaemia testing protocol

Patients were invited to undergo quantitative perfusion cardiac magnetic resonance (CMR) imaging at 1.5 Tesla using pharmacological stress testing with intravenous adenosine (140 µg/kg/min) within 6 weeks of the index coronary angiogram. CMR studies were performed using a standardized CMR protocol (Siemens MAGNETOM Avanto, Erlangen, Germany). Qualitative review for an inducible subendocardial perfusion defect consistent with microvascular dysfunction was independently performed by two cardiologists blinded to patient genotype. Quantitative measurement of the myocardial perfusion reserve ratio using a novel pixel-mapping technique was also performed (Supplementary information). Treadmill exercise stress electrocardiography using the Bruce protocol was analysed from the sub-group of patients who had been pre-selected for this procedure on clinical grounds prior to invasive coronary angiography. The exercise ECG parameters including (1) exercise duration and (2) the Duke Treadmill Score[15] were analysed by a cardiology researcher (EY) blinded to genotype and invasive physiology.

All subjects were asked to abstain from caffeine-containing beverages or foodstuffs for 24 hours, and vasoactive medications for 48 hours prior to the CMR examination. All scan acquisitions were spatially co-registered. All CMR analyses were performed by a blinded analyst with Level 3 EACVI accreditation.

Myocardial perfusion

Stress and rest first-pass perfusion imaging were performed using an echo planar imaging (EPI) dual-sequence investigational perfusion method, which consists of a low resolution arterial input function (AIF) image, followed by three short axis (base, mid, apex) myocardial images during each R-R interval.[16-18] First-pass perfusion images were obtained in 3 LV short-axis slices and one long-axis slice. Vasodilator stress was achieved with adenosine infusion 140-210 µg/kg/min for 3 minutes. Resting first-pass perfusion was performed at least 10 minutes later.

The raw stress and rest perfusion images were qualitatively assessed for inducible or fixed perfusion defects. The perfusion was classified as either normal, abnormal, or equivocal. If a perfusion defect was present, it was reported as having and epicardial, microvascular or equivocal pattern. Normal myocardial perfusion was depicted by homogeneous first pass perfusion as revealed by dynamic first pass perfusion imaging and the pixel maps. An inducible perfusion defect on dynamic first pass imaging during adenosine hyperaemia was reflected by a relative reduction in myocardial signal intensity notably in the sub-endocardium extending radially. The onset of the defect would occur with the arrival of the gadolinium contrast media in the left ventricular blood pool, it would persist beyond peak myocardial enhancement for 5 or more R-R intervals, and regresses over time towards the sub-endocardium. The defect would be present during stress but not resting conditions. The defect may conform to the myocardial blood supply of an epicardial coronary artery in a transmural distribution, or if the defect is primarily due to microvascular disease, the defect may be circumferential and restricted to the sub-endocardium. The perfusion defect should be 2 or more pixels wide.[19] An equivocal

perfusion abnormality would meet some but not all of these criteria, raising a suspicion of a perfusion abnormality but not clearly diagnostic. Perfusion defects were reported on a segmental basis according to the American Heart Association 16-segment model.[20] Dark banding artefact was adjudicated based on standardised criteria.[19]

Pixel-wise perfusion maps were generated and analysed to derive fully quantitative MBF estimates on a pixel-wise basis in ml/g/min of myocardium. The pixel-wise perfusion method used a series of automated post-processing steps on the raw Digital Imaging and Communications in Medicine (DICOM) images to generate fully quantitative pixel maps. The pixel-wise time-signal intensity curves were then quantified using model-constrained Fermi deconvolution.[21-23]

Extra-cardiac anatomy and LV volumes, function and mass

Fast gradient echo 'white-blood' images in the axial, coronal and sagittal planes were obtained, and were qualitatively assessed for extra-cardiac anatomy and pathology, and clinically-relevant incidental findings.

Steady-state free procession (SSFP) 'cine' imaging using a trueFISP sequence (multi-slice single-shot breath-hold true fast imaging) was performed in the 3 long-axis planes and short axis cine 'stack' for assessment of LV volumes, function and mass.

Myocardial tissue characterisation

Native T1 mapping was performed using a modified look-locker inversion-recovery (MOLLI) investigational prototype sequence. Images were obtained in three short-axis slices (base, mid, apex). T1 mapping was performed pre- and post-gadolinium contrast to

assess the myocardial native T1 relaxation time and estimate the myocardial extracellular volume (ECV) in both the mid-septum and globally.[24]

Late gadolinium enhancement imaging was performed using a segmented phase-sensitive inversion recovery (PSIR) turbo fast low-angle shot imaging sequence.[25] Images were obtained in the three long-axis planes and short-axis images covering the entire left ventricle. The pattern and burden of hyper-enhancement was both qualitatively and quantitatively assessed.

11. Peripheral vascular function assessment

Competition binding study in human heart to confirm ET_A selectivity of zibotentan

Initial reports on the pharmacology of zibotentan demonstrated that the compound had high affinity for the human cloned ET_A receptor (21 nmol/L) and no detectable affinity at human cloned ET_B receptors.[26] We have confirmed ET_A selectivity in human heart, a tissue that expresses both ET_A and ET_B receptors.

Briefly, human heart was collected with informed patient consent and local ethical approval. Competition binding experiments (n=3) were performed in cryostat-cut frozen heart sections (10μm) with [^{125}I]ET-1 (0.1nM) in the presence of increasing concentrations of zibotentan (2 pmol/L-100 μmol/L). Non-specific binding was determined using 1μol/L ET-1. Data were analysed to obtain affinity, pKi (the $-\log_{10}$of the equilibrium dissociation constant K_i determined in a competition binding assay), for zibotentan at ET_A and ET_B receptors using GraphPad Prism 6. Zibotentan competed in a

biphasic manner (Supplementary Figure 2) resulting in a $pK_i \pm SEM$ for the ET_A receptor of 9.88±0.13 and for the ET_B receptor of 4.02±0.04 indicating a >720,000 fold selectivity for the human ET_A compared to the human ET_B receptor in heart.

Experiments were designed to investigate whether patients with the SNP G allele, who had therefore been exposed to higher levels of endogenous endothelin-1 (ET-1), exhibited a change in responsiveness of vascular smooth ET_A receptors assessed *in vitro*. Initial studies determined whether there was evidence of endothelial dysfunction, indicated by a change in either potency or maximum response to the endothelium-dependent vasodilator acetylcholine (Ach) or whether the response to ET-1 was altered and if changes for either compound correlated to whether individuals expressed the SNP G allele or not. Finally, it was important to establish that, whatever the underlying genotype, responses to ET-1 could be blocked by ET_A antagonists. Two antagonists were tested. The very well characterised peptide antagonist BQ123 and importantly the orally active, highly selective ET_A antagonist zibotentan that has the potential to be rapidly repurposed for clinical use in this patient group.

Preparation of small resistance arteries

Vessels were dissected from a gluteal skin fat biopsy (approximately 3 x 2 x 2 cm) performed within 4 weeks of coronary angiography. Vasoactive medications were withheld for at least 24 hours prior to the surgical biopsy. Small resistance arterioles (normalised diameter <400 μm) were studied in a Mulvany-Halpern 4-channel wire

myograph (Danish Myotech, Aarhus, Denmark) with isometric tension recordings made as previously described.[27]

Experimental Protocol

After a standard normalisation and start-up protocol involving repeated washes with high potassium chloride solution (62.5 mmol/L KPSS), the arterioles were pre-constricted with the thromboxane-A2 analogue, U46619 (0.1 μmol/L). Previous work on human resistance arteries support its application in myography due to its consistent vasoconstriction with a steady plateau from which to assess arteriolar relaxation. Blood vessels with no responses were discarded. For viable tissue the integrity of the endothelium was determined by constructing a cumulative concentration-response curve (CCRC) to ACh (1n mol/L - 1μ mol/L). CCRCs were then obtained to the vasoconstrictor peptide ET-1(1 p mol/L - 1 μmol/L).

For relaxation data, responses to ACh were expressed as the percentage reversal of the constrictor response to U46619 (100nmol/L). Data for ET-1 were normalised as a percentage of the mean response to the last two responses to 62.5mol/L potassium chloride obtained during the set up procedure. CCRCs were fitted using four-parameter, non-linear regression curve fitting in Prism 7.0 (GraphPad Inc, La Jolla, CA, USA) to obtain values of potency (expressed as the pEC_{50}, that is the $-\log_{10}$ of the EC_{50} (the concentration producing half-maximal response)) and maximum response (E_{max}) for both agonists. Derived parameters were compared for patients with or without the SNP G allele.

For antagonist studies paired tissues from patients were used to construct CCRCs to ET-1 in either the absence (control) or presence of 1 µmol/L BQ123 (N=27) or 1µmol/L zibotentan (N=8). Data were expressed as % KPSS as described above and paired CCRCs were then analysed using the Gaddum/Schild EC_{50} equation (GraphPad Prism). The Hill and Schild slopes were constrained to 1 and therefore antagonist affinity was given as the pK_B (the $-\log_{10}$ of the equilibrium dissociation constant). pK_B values for BQ123 derived from individuals with different SNP alleles were compared.

Finally, vessels from some individuals were preconstricted with ET-1 and the constrictor response allowed to stabilise before addition of either no antagonist (time matched control) or 0.1µmol/L or 1µmol/L zibotentan was added. The contraction to ET-1 was monitored over the next 90 minutes to determine the extent of reversal by zibotentan over time.

12. Statistical Considerations

Pharmacology definitions: potency (pEC50) is the –log10 of the concentration of a drug that gives half-maximal response; Efficacy is given as Emax, the % contraction to KPSS; pKB is the –log10 of the antagonist affinity (KB the equilibrium dissociation constant of the antagonist for the ETA receptor). For wire myography, we analysed pKB between three groups using one-way ANOVA and adopted a two-tailed model with alpha of 0.05 and beta of 0.2 giving a planned power of 80%. An estimated effect size (f) of 0.544 was determined using estimated group mean pKB values of 7.7, 7.6 and 7.5 respectively (SD of 0.15). A minimum sample size of 36 subjects was determined using G*Power 3.1 (University of Melbourne, Parkville VIC, Australia).

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