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Acceleration of cardiovascular disease by a dysfunctional prostacyclin receptor mutation, potential implications for COX-2 inhibition

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Abstract

Recent increased adverse cardiovascular events observed with selective cyclooxygenase-2 (COX-2) inhibition led to the withdrawal of rofecoxib (Vioxx) and valdecoxib (Bextra), but the mechanisms underlying these atherothrombotic events remain unclear. Prostacyclin is the major endproduct of COX-2 in vascular endothelium. Using a naturally occurring mutation in the prostacyclin receptor, we report for the first time that a deficiency in prostacyclin signaling through its G protein coupled receptor contributes to atherothrombosis in human patients. We report that a prostacyclin receptor variant (R212C) is defective in adenylyl cyclase activation in both patient blood and in an *in vitro* COS-1 overexpression system. This promotes increased platelet aggregation, a hallmark of atherothrombosis. Our analysis of patients in three separate Caucasian cohorts reveals that this dysfunctional receptor is not likely an initiating factor in cardiovascular disease, but that it accelerates the course of disease in those patients with the greatest risk factors. R212C was associated with cardiovascular disease only in the high cardiovascular risk cohort (n=980), with no association in the low risk cohort (n=2263). In those at highest cardiovascular risk, both disease severity and adverse cardiovascular events were significantly increased with R212C when compared to age and risk factor-matched normal allele patients. We conclude that for haploinsufficient mutants, such as the R212C, the enhanced atherothrombotic phenotype is likely dependent upon the presence of existing atherosclerosis or injury (high risk factors), analogous to what has been observed in the COX-2 inhibition studies or prostacyclin receptor knockout mice studies. Combining both biochemical and

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clinical approaches, we conclude that diminished prostacyclin receptor signaling may contribute in part to the underlying adverse cardiovascular outcomes observed with COX-2 inhibition.

Keywords

prostacyclin; eicosanoid; cyclooxygenase-2; G-protein coupled receptor; mutation

INTRODUCTION

The direct cost of treating cardiovascular disease is estimated at $431.8 billion annually for the 79,400,000 individuals who have cardiovascular disease (NHLBI Chart Book - 2007). The recent withdrawal of rofecoxib (Vioxx™), following the results of the VIGOR, APPROVe and other cyclooxygenase-2 (COX-2) inhibitor clinical outcomes trials[1-4], and the development of cardiovascular disease in predisposed prostacyclin receptor knockout mice[5, 6], underscores the necessity to better understand the effects of COX-2-derived metabolites on cardiovascular health. Endothelial prostacyclin synthesis requires the COX-2 enzyme [7], and may serve a role in protection from atherothrombosis[8, 9]. This cardioprotective role has been supported by recent prostacyclin receptor knock out mice studies, showing that the absence of the prostacyclin receptor (IP - International Union of Pharmacology Receptor classification) leads to intimal hyperplasia, atherosclerosis, and hypercoagulability [5, 6], as well as reperfusion injury [10]. and premenopausal atherogenesis [11]. Despite such accumulating information, controversy remains as to whether prostacyclin deficiency is the etiology of the cardiovascular events observed with COX-2 inhibition [12], particularly as no human studies have directly implicated defective prostacyclin signaling in the development of cardiovascular disease.

The human prostacyclin receptor gene (*PTGIR*) spans approximately 7,000 bases along chromosome 19 (locus 19q13.3), and is comprised of 3 exons separated by two introns, one intron lying upstream from the ATG start codon and the other at the end of the sixth transmembrane helix [13]. It encodes a G-protein coupled receptor (GPCR) composed of 386 amino acids and has a molecular mass ranging from 37-41 kDa, depending upon different states of glycosylation [14]. The hIP is most commonly associated with coupling to the Gαs subunit of the heterotrimeric G-protein, which upon receptor activation stimulates membrane-bound adenylyl cyclase to catalyze the formation of the second messenger, cAMP [15]16. Like other prostanoid receptors, the hIP has been categorized (based upon sequence homology, ligand structure, and overall receptor functionality) as a Class A rhodopsin-like GPCR, and shares many structural commonalities with rhodopsin --- the Class A representative and `prototypical' GPCR. These common traits can be divided into three major receptor domains: 1) the extracellular domain, consisting of a short amino N-terminal tail and three extracellular loops (exoloops), 2) a transmembrane (TM) domain, comprised of seven transmembrane-spanning α-helices, whose upper third house the putative binding pocket [17], and 3) the cytoplasmic or intracellular domain that is made up of three helix-joining intracellular loops (cytoloops), a fourth loop produced by lipid anchoring (palmitoylation) of intracellular cysteines, and a fairly lengthy carboxy-terminus (Figure 1). Genetic variants may therefore have differential effects on binding, expression and activation, dependent upon localization within the protein structure, analogous to the Retinitis Pigmentosa rhodopsin mutations [18].

We now report that defective prostacyclin signaling appears to accelerate atherothrombosis in human subjects leading to increased cardiovascular disease and events, analogous to COX-2 inhibition. Importantly, we conclude that the association of dysfunctional prostacyclin signaling with cardiovascular disease is cardiovascular risk factor-dependent, potentially explaining the variability in association with cardiovascular disease observed with COX-2

inhibition trials[19]. The mechanism arises from defective activation, inducing relative states of increased thrombosis, a hallmark of cardiovascular events.

METHODS

Whole blood assessment

Ten milliliters of whole blood (preserved in EDTA) was collected from patients. Ligand-binding characteristics for the platelet-expressed hIP and human thromboxane receptor (hTP) were determined through saturation binding assays, using 30μl of human blood (~1×10^7 platelets) and one of 6 different concentrations (1 nM to 200 nM) of [^{3}H] iloprost (human prostacyclin receptor agonist, specific activity 16 Ci/mmol, Amersham) or [^{3}H] SQ-29548, human thromboxane receptor antagonist, specific activity 48 Ci/mmol, PerkinElmer) to detect the hIP and hTP, respectively[20, 21]. Human IP receptor activation was determined through the [^{3}H]-cAMP radio-receptor competition assay (Amersham), using 100μl of human blood (~3.3×10^7 platelets) at maximal doses of iloprost stimulation (10nM to 1μM). Data was analyzed using GraphPad Prism® software (GraphPad Software, Inc. --- San Diego, CA). We believe that whole blood analysis is more physiological and directly pertinent to the patients. Patient variability from the use of medications and other concomitant diseases are important considerations, and thus the addition of COS-1 cell experiments (expressing the wild type or mutant receptors) were used to further support or refute our observations.

Functional assays of platelet aggregation were performed using a Chronolog aggregometer to comparing platelet-rich plasma with platelet-poor plasma (control) generated by differential centrifugation. Dose response of prostacyclin agonists (10 pM to 1 mM) was determined in parallel with standard controls (e.g., ADP). cAMP production was determined for each response (% inhibition) and correlated to the expected response for the reduced cAMP observed with R212C. Direct R212C patient blood analysis could not be performed as all patients were on multiple antiplatelet therapy including aspirin (and clopidogrel). Our aggregation analysis was therefore indirect, based upon the observation that reduced cAMP production leads to decreased inhibition of aggregation (a relative state of hyperaggregation).

In vitro assessment in COS-1 cell system

Details of the mutagenesis procedure including random mutagenesis at the 212 position can be found in the Supplementary Materials. Details of saturation binding, cAMP activation and confocal microscopy can be found in our previous publication [22].

Dartmouth-Hitchcock Medical Center (DHMC), Nurses Health Study (NHS) and Health Professional Follow-Up Study (HPFS) Cohorts

Details about each of the cohorts studied and genomic analysis are found in the Supplementary Material.

Statistical analysis

Baseline characteristics were compared between groups using Student *t*-tests and chi-square tests as appropriate. The association of the R212C with coronary heart disease was analyzed using the Mantel-Haenszel test and an estimate of the odds ratio, which aggregated the odds ratio from each of the three study cohorts. The homogeneity of the odds ratio across the three studies was tested using the Q statistic. To adjust for risk factors present in the R212C group a control group was randomly selected (n = 20) from the DHMC cohort. The percent of adverse events occurring in patients found to be positive for the R212C mutation compared to risk-matched control patients was analyzed using a Wilcoxon Rank-Sum test for two independent samples. The analysis was limited to three years, analogous to the time periods observed in the

COX-2 inhibition studies. Analysis of variance (ANOVA with post test Newman-Keuls) was used for multiple group comparisons and unpaired Student *t*-tests were used to directly compare two sets of samples.

Analysis for the DHMC cohort was stopped at 1036 (980 used in the study) after clear statistical significance was reached. The HPFS cohort was powered greater (n=2293, double that of the DHMC cohort) to establish whether there was truly a lack of association in a low cardiovascular risk factor group.

RESULTS

We recently screened a multiracial population to detect novel hIP genetic variants [23]. An R212C was found in low frequency in both Caucasian (1/125 samples, 0.8%) and Asian (1/127 samples, 0.8%) cohorts. The R212C (rs4987262) mutation (Figure 1A and 1B), located in the critical third intracellular loop, represented a significant change in both size and charge. Upon analysis of our three-dimensional homology model of the hIP (based upon the 2.8Å crystal structure of rhodopsin and site-directed mutagenesis data[24]), the R212C appears to be located at the C-terminal end of an alpha-helical conformation within the critical third intracellular loop, which is known in other G-protein coupled receptors to interact with G-protein[25, 26] (Figure 1C).

R212C exhibits defective function in patients blood (*ex vivo*)

We have also detected this R212C variant in a human cardiology patient population. We were fortunate in obtaining a limited number of whole blood samples from heterozygote R212C patients (n=4, CGC/TGC), and from a single homozygote patient (n=1, TGC/TGC, described in detail in Supplementary Results). No such mutant hIP structure/function analysis has ever been performed on human tissue. Our studies revealed significant defects in both agonist binding (K_D) (Figure 2A and Table 1) and receptor activation (cAMP production) (Figure 2B), with all mutant patient samples exhibiting clear defects. Figure 2A shows saturation binding from individual patients and Table 1 summarizes the results. The human thromboxane receptor (hTP), also found in abundance in human blood was used as an internal control for both the assessment of binding affinities and hIP receptor expression. Binding affinities (K_D) were 28.7 ±5.2nM and 196.5±23.4nM for the WT hIP and R212C, respectively (p=0.0004, Student's *t*-test) (Table 1). Upon further detailed studies it appears that this binding defect is at least in part related to age and disease severity (Supplementary Results). Paradoxically, hIP receptor numbers were increased in relation to human thromboxane receptors (hTP, also found on platelets) in the R212C heterozygous group (p=0.007, Student's *t*-test) (Table 1). This was reflected in the reversed hIP/hTP receptor ratio (WT = 0.44 ± 0.10, R212C = 1.36 ± 0.15, p=0.003, Student's *t*-test). Despite the increased receptor expression, receptor activation (cAMP production) remained severely impaired for the R212C variants (100nM iloprost WT=1.53±0.63pmol versus R212C=0.07±0.12pmol cAMP, p=0.03, Student *t*-test) (Figure 2B). Both heterozygote and homozygote patient blood samples exhibited defective function and increased receptor expression compared to age and cardiovascular risk factor matched control patients.

R212C exhibits defective function in COS-1 cells (*in vitro*)

As samples from patients were limited, we sought to biochemically validate the effects of the R212C mutation in a COS-1 cell overexpression system. As with patient samples, functional assessment of the R212C variant in COS-1 revealed a consistent reduction in cAMP production compared to wild type receptor when expressed at equivalent levels (1.0 pmol/mg membrane protein). A significant defect was observed for agonist potency (WT EC_{50} = 0.8 ± 0.1nM n=6, R212C = 2.6 ± 0.7 nM n=7, p=0.035, Student *t*-test) (Figure 2C). The reason for this could be

further explored with a combination of saturation binding (Bmax), Western analysis, and confocal microscopy. R212C showed a significant reduction in cell surface expression using saturation binding (WT = 1.05 ± 0.06 pmol/mg membrane protein vs R212C = 0.52 ± 0.03 pmol/mg membrane protein, p= 0.01 Student t-test). Western analysis of cell surface membrane preparations also showed a marked reduction in protein levels for R212C (Figure 3A). Confocal analysis staining for receptor (red, anti 1D4 antibody) endoplasmic reticulum (green, anti calnexin antibody) and nucleus (blue, DAPI) showed that there was a marked reduction in cell surface expression for R212C in comparison to wild type, as seen from the reduced red surface staining (white arrows) on the R212C versus the wild type (Figure 3B). There was significant endoplasmic reticulum retention for R212C (Figure 3B, red surrounding nucleus). Because in overexpression systems it is usually the endogenous signal transduction effectors that are limiting and not receptor numbers, our results suggest that the R212C that is expressed at the surface is greatly impaired given 3.25 fold reduction in potency. Thus, the *in vitro* results confirm the *ex vivo* studies showing that the R212C is functionally defective. The acute expression of R212C results in endoplasmic reticulum retention and reduced cell surface expression (reduced Bmax), however, with patient blood samples, there is an interesting paradoxical increase in receptor expression (increased Bmax), most probably secondary to a compensatory response. Although both *in vitro* and *ex vivo* analyses showed reduced function, these studies highlight the importance of using human patient tissue with the mutation of interest in order to observe pathophysiologically relevant results.

R212 stabilizes the critical third intracellular loop

We then performed random mutagenesis (Supplementary Methods) at the R212 position to determine the structural role played by the native Arg at this position. In addition to the three naturally occurring mutations at the R212 position (R212C, TGC, R212H, CAC and R212R CGT) which we had previously identified[23], random mutagenesis led to the production of an additional five mutations (R212R, CGA, R212L, CTC, R212S, AGC, R212P, CCC and R212T ACT) (Online Table 1 & Supplementary Figure 1). All synonomous mutations (no change in amino acids) showed no difference in binding and activation in comparison to wild type protein. In contrast all nonsynonomous mutations exhibited an activation deficiency with evidence of normal binding. Interestingly, R212L had a significant proportion of binding deficient receptor. These data when combined with molecular modeling suggested that the 212 position is critical, by virtue of both its size and charge, in stabilizing the third intracellular loop through interaction with S205 (Figure 1C). Additional mutation of S205 to alanine displayed defective activation with an EC_{50} of 19.1 ± 8.1 nM (WT = 0.8 ± 0.1 nM, Online Table 1) further supporting an R212-S205 hydrogen bonding stabilizing interaction.

R212C defective signaling leads to an increased thrombotic state

We proceeded to address the question as to whether the defective signaling could affect platelet function leading to a state of increased thrombosis. Because R212C patients from which blood samples were obtained were all on aspirin therapy, it was necessary to conduct these experiments on wild type platelets from human volunteers. Dose-response experiments (agonist-induced inhibition of thrombosis) were performed on human platelet-rich plasma. A sigmoidal dose-response was achieved with the addition of increasing concentrations of iloprost (EC_{50} = 7.1 ± 1.4 nM, n=4). Corresponding cAMP production was determined and plotted against percent (%) inhibition of aggregation (Figure 4). We superimposed the ranges of cAMP production previously determined in multiple samples from R212C and wild type patients as bars along the x-axis of this graph. These experiments indicate that the very low levels of hIP-stimulated cAMP generated by a defective receptor correspond to a pronounced decrease in inhibition of aggregation, which would result in a relative hyper-thrombotic state (Figure 4), a critical component in the development of atherothrombosis.

R212C is associated with coronary heart disease (CHD) in a risk factor dependent manner

We demonstrated that the R212C leads to defective hIP signaling promoting key components in the development of atherothrombosis. To determine whether these defects are associated with clinical disease, we examined three separate Caucasian cohorts (total n=3970) (the R212C was found initially in only Caucasians and Asians). The prevalence of the R212C genotype (heterozygote or homozygote) was comparable between the three cohorts (2.14% in DHMC, Dartmouth Hitchcock Medical Center n = 980; 1.72% in NHS, Nurses Health Study n = 697; and 1.92% in HPFS, Health Professional Follow-up Study n = 2293). Overall, the R212C was significantly associated with an increased risk for CHD (OR = 1.68 [1.03-2.76], p=0.04) (Online Table 2). Interestingly, analogous to the COX-2 inhibition studies, there was considerable variability in the Odds Ratio for individual cohorts with only the DHMC cohort being significantly associated with CHD (OR = 4.71 [1.09-20.36], p=0.022). An initially surprising observation was the lack of association with the HPFS, which had no association with CHD (OR 1.15 [0.61-2.17], p=0.66). Of importance, however, we noted that the prevalence of cardiovascular risk factors was much greater in the DHMC group in comparison to the HPFS group (Figure 5 and Online Table 3). Furthermore, a group with intermediate risk (NHS) exhibited a trend towards CHD (OR 2.02 [0.65-6.28], p=0.21). The three cohorts followed the same rank order: DHMC > NHS > HPFS for both R212C associations with CHD and risk factors (Figure 5 and Online Table 3). In the high risk DHMC cohort there are significantly more R212C in the CHD cases (19/660 = 2.9%) compared to the controls (2/320 = 0.6%). Thus the association of R212C with coronary artery disease appears to be risk factor-dependent suggesting that development of atherothrombosis with R212C was dependent upon existing underlying disease (promoted by the large number of risk factors). This observation is strongly supported by the prostacyclin knockout mouse studies which also demonstrated that injury or increased risk factors (concurrent LDL receptor knockout, balloon injury to artery, or precipitation of thrombosis) was required in order to observe a cardiovascular phenotype [5, 6]. Additionally with COX-2 inhibition (leading to reduced levels of prostacyclin), cardiovascular events appeared to be most evident in those patients most predisposed to cardiovascular disease (highest cardiovascular risk factors)[9]. This led to our hypothesis that reduced prostacyclin signaling was accelerating existing disease, rather than causing cardiovascular disease de novo.

R212C increases CHD severity, both disease burden and cardiovascular events

To address the hypothesis (existing underlying disease was accelerated by R212C) it was mandatory to compare age-and risk factor matched controls with and without R212C in the DHMC Cardiology population for which we had coronary angiographic results. We assessed both disease severity (number of major vessels score of 1 to 3 with significant obstructions) and clinical cardiovascular events (MI, PTCA, stroke, documented PVD, unstable angina, and cardiac bypass surgery). For the DHMC cohort, there was a mean ± SE of 1.5±0.04 vessels occluded per patient, in comparison to 2.1 ± 0.2 vessels occluded per patient in the R212C cases (p = 0.019, Student's *t*-test) (Figure 6A). The R212C cohort had a lower incidence of patients with zero disease and a higher incidence of triple vessel disease (Figure 6B). The number of adverse events was significantly higher for the R212C group (4.6±0.7 events) versus control (2.7±0.4 events) (p=0.026 Student *t*-test, Figure 6C), despite the use of aspirin in all patients except one (allergic to aspirin, on clopidogrel). To determine if the R212C variant predisposed patients to a higher frequency of adverse events, we employed a Wilcoxon Rank-Sum test where we limited the assessment of events to the number of follow-up years available for all patients (first 3 years from the initial event, 60 patient years in total for both groups). We found a significant difference between the R212C and risk factor matched control cohort with regard to adverse event frequency (p=0.016, Wilcoxon Rank-Sum test) (Figure 6D). Again, these results are supported by the knockout mice studies where the exaggerated atherothrombotic response in these knockout mice studies required vascular injury initiation

(e.g. concurrent LDL receptor knockout[11] or balloon injury[5]). Similarly, the hazards associated with COX-2 inhibition are most apparent in patients with the greatest cardiovascular risk factors (i.e. predisposition to atherothrombosis)[9]. The most dramatic example of coronary artery disease acceleration could be observed in our single homozygote patient (Supplementary Results and Supplementary Figure 2).

DISCUSSION

Only recently have prostacyclin receptor polymorphisms been reported and characterized[20, 23, 27]. All characterizations, however, have been in *in-vitro* COS-1 overexpression systems. Our current studies in human patient tissues highlight the importance of such analysis, as we are able to detect functional defects, in addition to potential compensatory mechanisms for the defects (increase in hIP receptors on platelets). Furthermore, the mechanism which leads to clinical association can be deciphered, and the reason for lack of association in other populations may be determined. There has already been some evidence in the literature from COX -2 inhibition trials that the reduction in prostacyclin may be associated with systemic hypertension in human subjects[28], however no study has directly linked such prostacyclin signaling deficiencies with human cardiovascular disease. Consistent with these early observations, 80% of the R212C patients in the DHMC population were hypertensive in comparison to the DHMC cardiology population overall which had a 67% incidence of hypertension. Our goals herein were to assess whether defective prostacyclin signaling is associated with cardiovascular disease in human subjects, as was suggested from both the selective COX-2 inhibitor trials[1-3], and genetic knockout mice[5, 6, 10].

Underlying disease or injury is required for the R212C phenotype

We are rapidly becoming aware that genetic variants may remain silent under normal physiological conditions, or throughout childhood and early adulthood, with the underlying functional abnormalities becoming apparent only in the diseased state, or under times of pathophysiological insult[29-31]. For the prostacyclin signaling pathway, this was demonstrated most dramatically with the prostacyclin receptor knockout mice, where underlying stress or injury was required to reveal that the lack of this receptor promotes increased thrombosis or intimal hyperplasia [5, 6, 10]. We now demonstrate in patients, that cardiovascular risk factors likely provide the analogous underlying injury. In this context, the R212C accelerates cardiovascular disease. In the absence of significant risk factors there appears to be no significant R212C phenotype. This is in parallel to COX-2 inhibition where reduction in prostacyclin production appears to increase cardiovascular events in high cardiovascular risk patients, but shows a lack of association in other study populations. This important principle may explain why many high profile manuscripts reporting polymorphism-disease associations are not uniformly confirmed by parallel studies. For such association studies, separate populations with differential risk factor profiles may now be necessary.

Mechanism for R212C modulation of CHD

The mechanism for the accelerated atherothrombosis appears to be at least in part due to a combination of reduced ability to inhibit thrombosis from reduced cAMP signaling in platelets, and an inability to reduce human coronary VSMC proliferation and dedifferentiation[32], also from reduced cAMP production in VSMC. For the R212C variant this appears to arise from the disruption of a critical interaction between R212 and S205, required for stabilizing conformation in the critical G-protein interacting third intracellular loop. A decrease in the effective dose of prostacyclin, due either to receptor signaling defects or to inhibition of prostacyclin production (COX-2 inhibition), would also promote thrombosis and promote dedifferentiation and proliferation in human coronary VSMCs. These critical components of atherothrombosis were most clearly defective in our homozygote patient who had significant

disease acceleration at the age of 70, on a background of only statin controlled hyperlipidemia. Aspirin (predominant COX-1 inhibitor) itself appears insufficient to oppose the atherothrombotic state in our CHD R212C patients, as all the CHD R212C patients (except one) were on low-dose aspirin therapy.

Conclusion

Our combination of in vitro tissue culture analysis, patient blood analysis (defective R212C activation), and the case-controlled study of R212C patients (showing increased disease and cardiovascular events), together with the published observations from COX-2 inhibition trials (increased cardiovascular events) and prostacyclin receptor knockout mice studies (increased cardiovascular disease), all support the conclusion that defective prostacyclin signaling promotes cardiovascular disease in human subjects. Analogous to the knockout mice, we have now shown for the first time that prostacyclin signaling appears to contribute to cardiovascular phenotype in humans in a risk factor-dependent manner. While the R212C hIP polymorphism is observed at low frequency (2% in our three cohorts), with over 60 million cardiovascular patients in the USA, 2% represents over one million patients. In addition to mandatory control of risk factors, the therapy of choice for R212C may ultimately be potent, stable prostacyclin analogues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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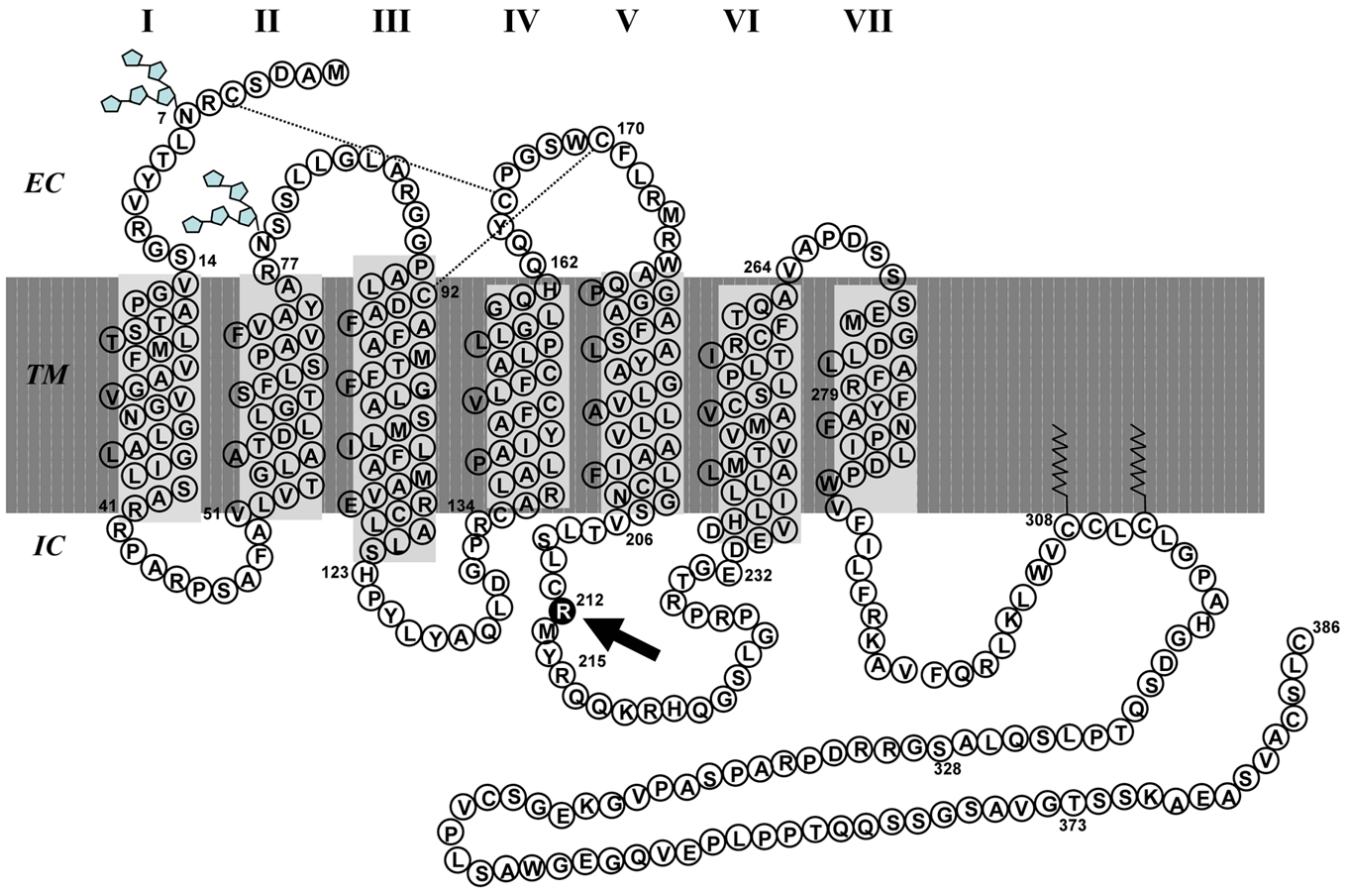

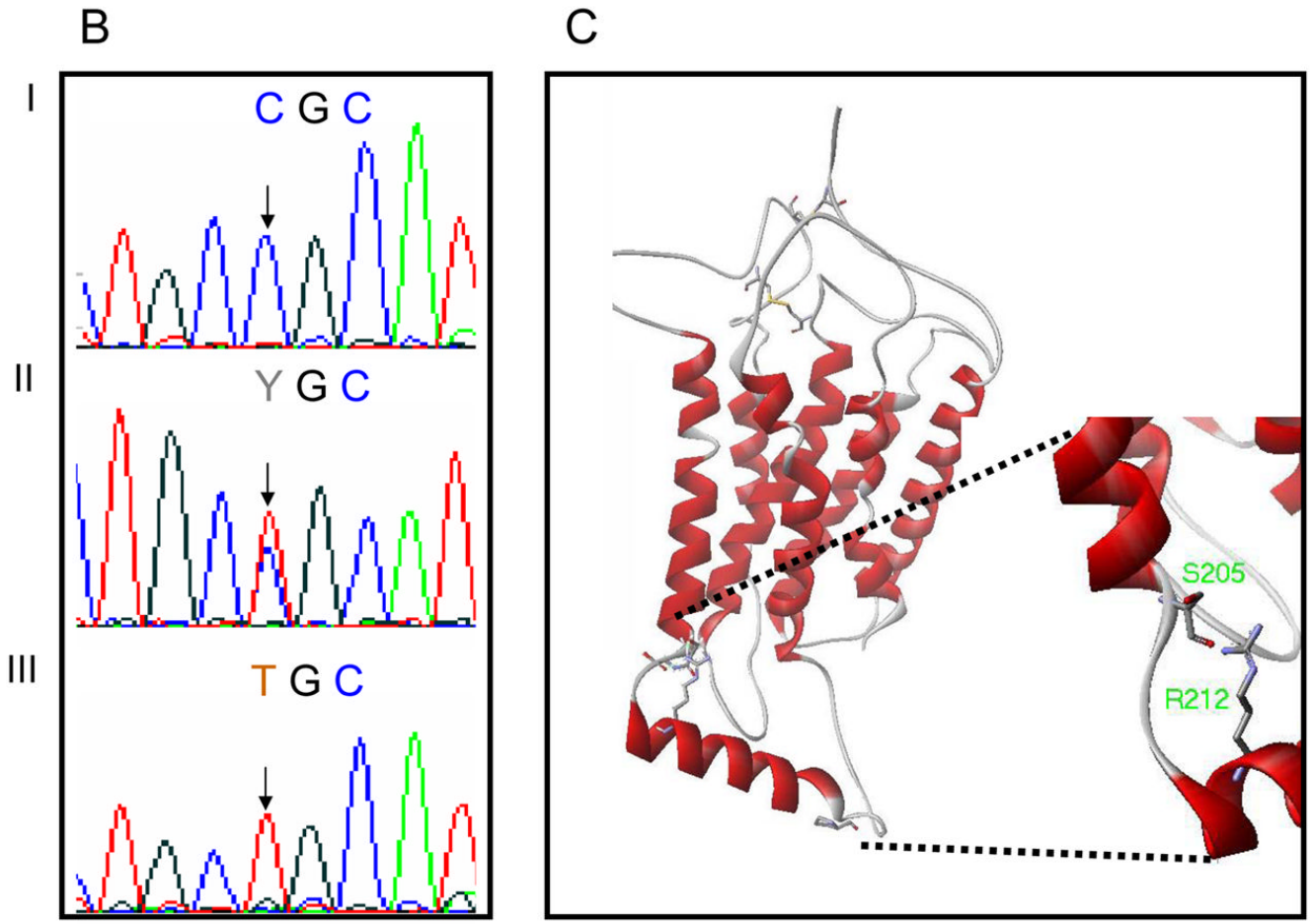

Figure 1. Detection, localization and function of hIP receptor mutant R212C

PANEL A: Amino acid sequence of the human prostacyclin receptor placed in a secondary structure (snake-plot) format with the seven transmembrane (TM) helices. The N-terminus is located extracellular (EC), with two sites of glycosylation indicated by pentagonal chains. The dual disulfide bonds are shown by the dashed line. The C-terminus is intracellular (IC) with putative sites of palmitoylation-isoprenylation indicated (serrated lines). The position of the R212C is highlighted with a black arrow. **PANEL B:** Chromatogram from genomic DNA sequencing of cardiology patients showing the nucleotide changes at position 212 wild-type codon (CGC - Panel I), heterozygote mutation (T/CGC - Panel II) and homozygote mutation (TGC - Panel III). **PANEL C:** Computer derived 3D-model (energy-minimized) of hIP receptor, showing TM helices (red) and EC and IC loops (gray). Enlarged region highlights third intracellular loop and location of Arg residue at position 212, at the C-terminal end of the putative alpha-helix; the position disrupted upon conversion to a cysteine (R212C).

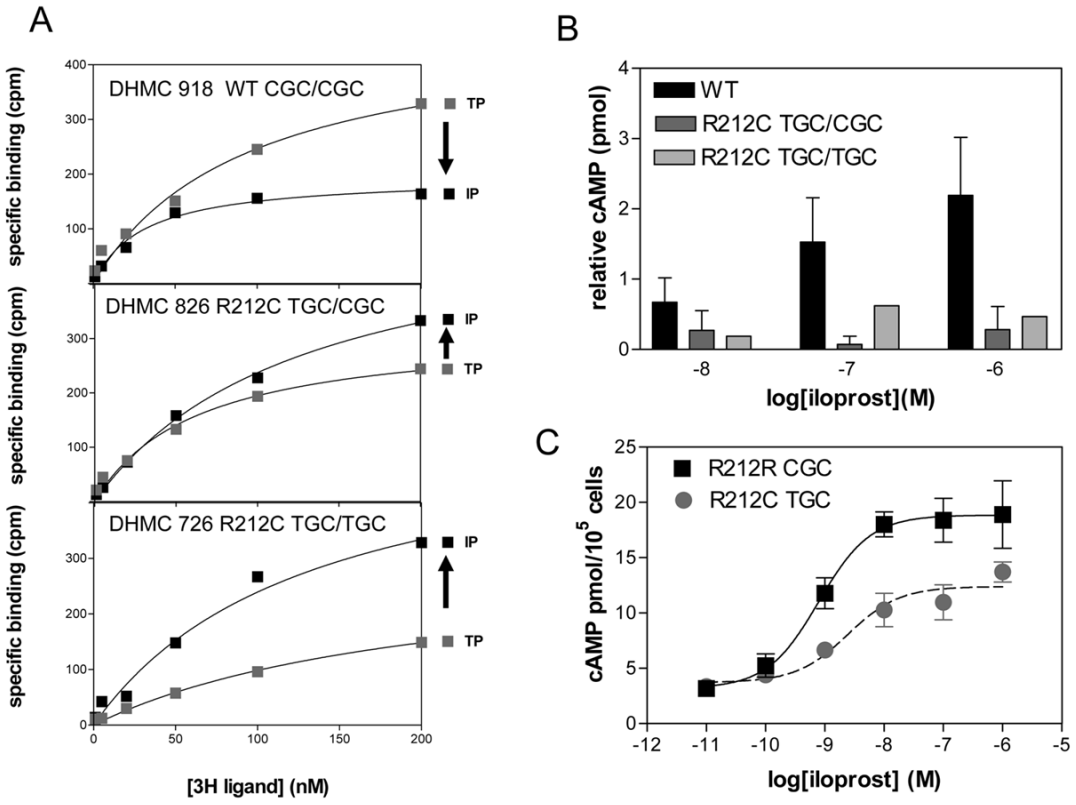

Figure 2. Patient blood analysis

PANEL A: Representative saturation binding curves on patient bloods for 3 individual patients DHMC 918 (wild-type), DHMC 826 (heterozygote R212C) and DHMC 726 (homozygote R212C) to demonstrate changes in binding and expression. [^{3}H] iloprost was used for detection of the human prostacyclin receptor (hIP) and [^{3}H]-SQ 29548 for the human thromboxane receptor (hTP). Arrows indicate relative changes in receptor numbers comparing hIP to hTP. **PANEL B:** Cyclic-AMP determination from the patient samples described in Panel B. Picomoles cAMP were corrected for changes in receptor numbers. **PANEL C:** Cyclic-AMP determination from COS-1 cell overexpression experiments for WT and R212C constructs. A dose response for iloprost was established (1μM, 0.1μM and 0.01μM).

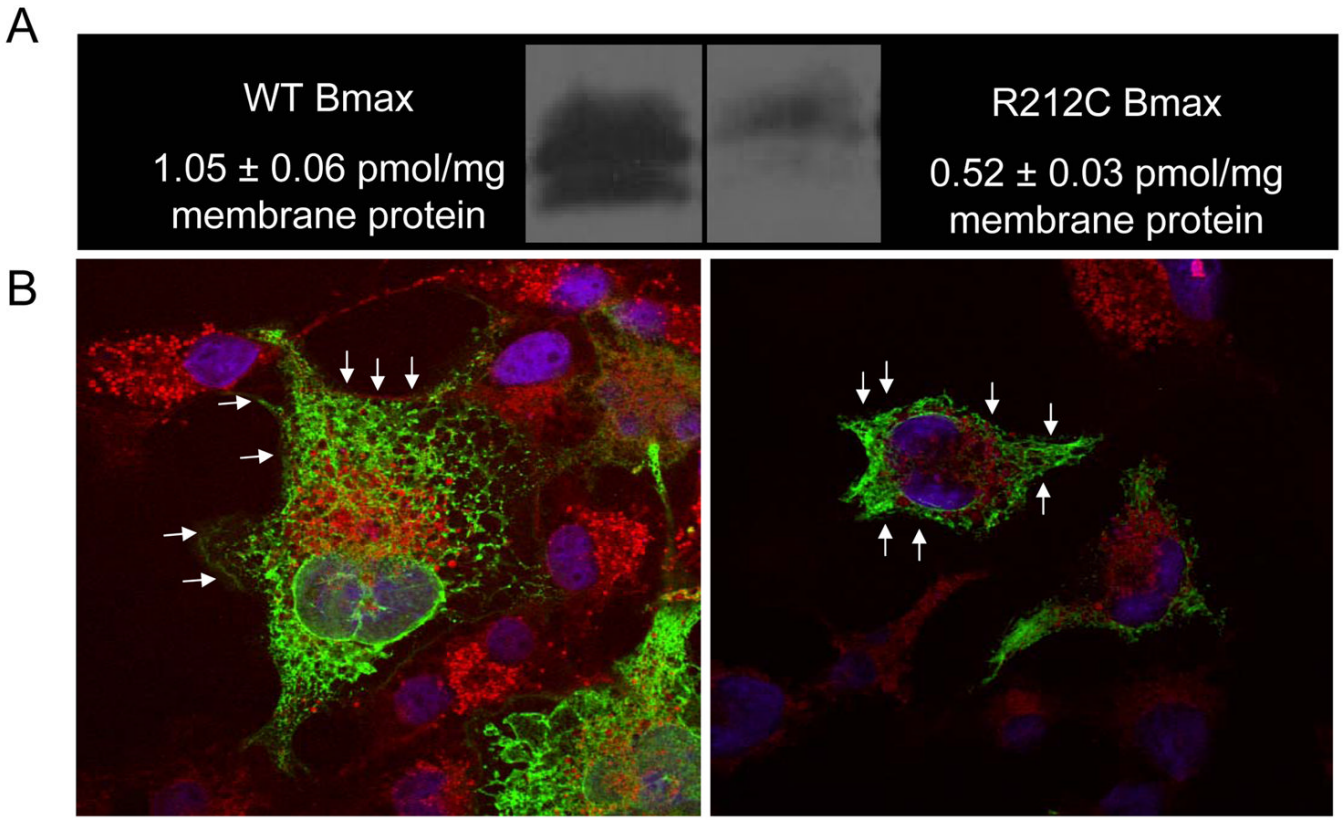

Figure 3. R212C expression in a COS-1 system

PANEL A: Results of saturation binding and western analysis performed on COS-1 membrane preparations containing R212C and wild type constructs. **PANEL B:** Corresponding confocal microscopy overlay images (63 x resolution) showing predominant membrane trafficking only for wild-type protein. The hIP receptors both wild type and mutants are labeled red (1D4 monoclonal antibody). The endoplasmic reticulum are labeled green (anti-calnexin antibody) and the overlay picture additionally has blue nuclear staining (DAPI) and a phase contrast microscopic image of the cell to localize the cells perimeter. White arrows are used to localize areas of cell surface membrane.

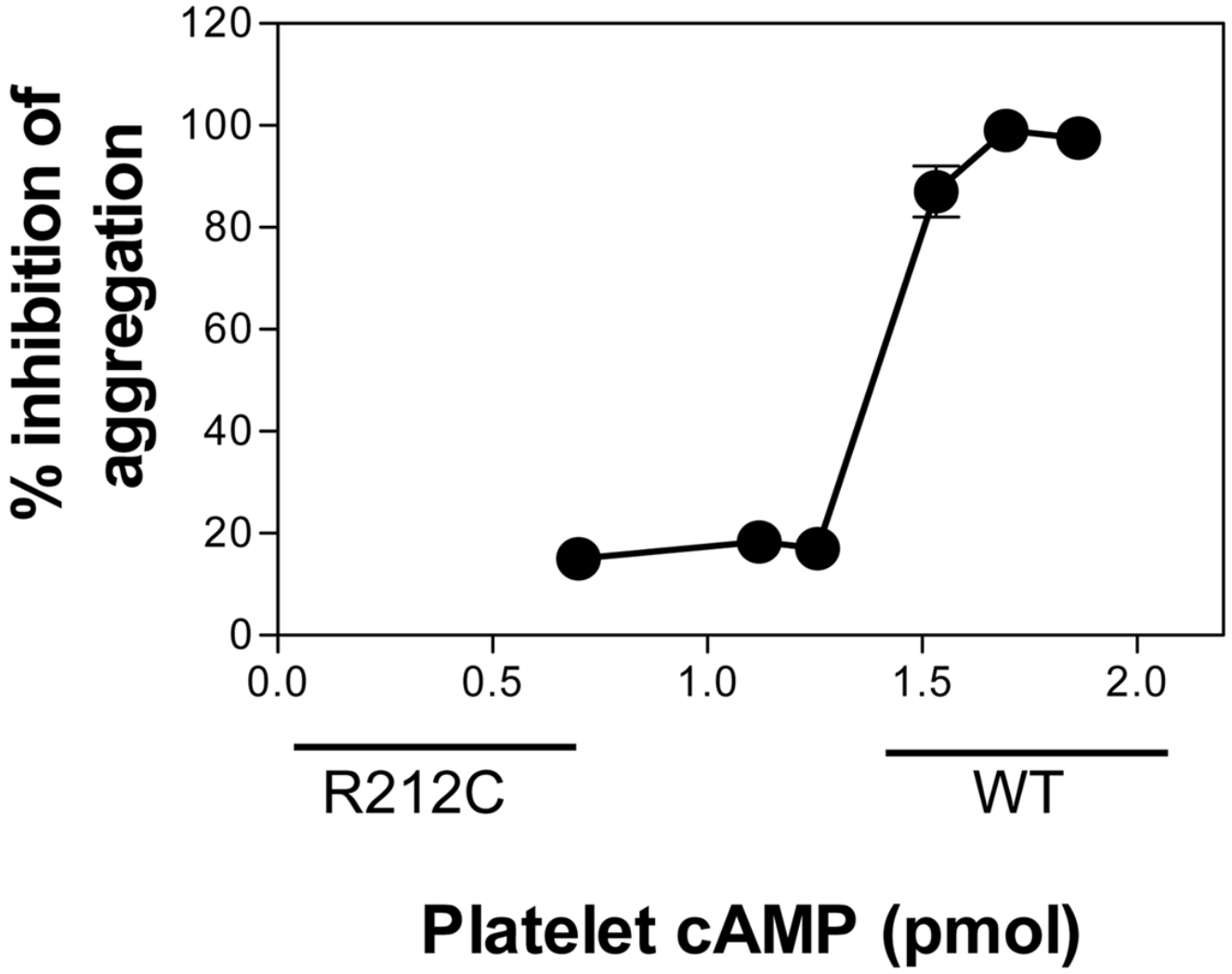

Figure 4. Platelet cAMP and inhibition of aggregation

Plot of percent (%) inhibition of aggregation versus platelet cAMP production (pM). Reduced cAMP promotes aggregation (observed with R212C) whereas increased cAMP inhibits aggregation (observed with wild type).

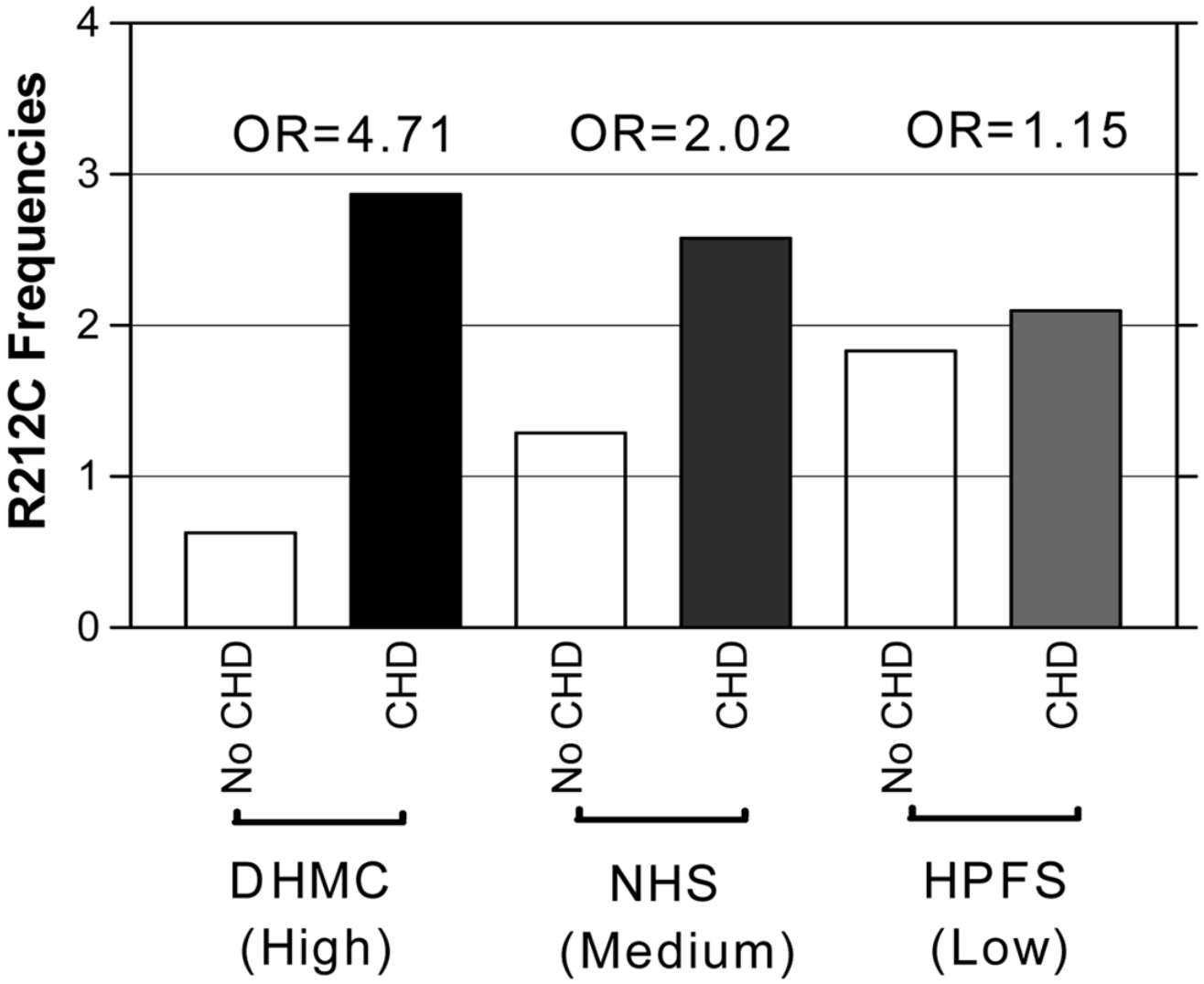

Figure 5. Clinical analysis of three Caucasian Cohorts

A graph representing the R212C frequencies in the 3 Caucasian cohorts, DHMC, NHS and the HPFS. The white bars represent no coronary heart disease (No CHD) and the shaded bars the coronary heart disease groups (CHD). The odds ratios (OR) are described and confidence limit observed in Online Table 2.

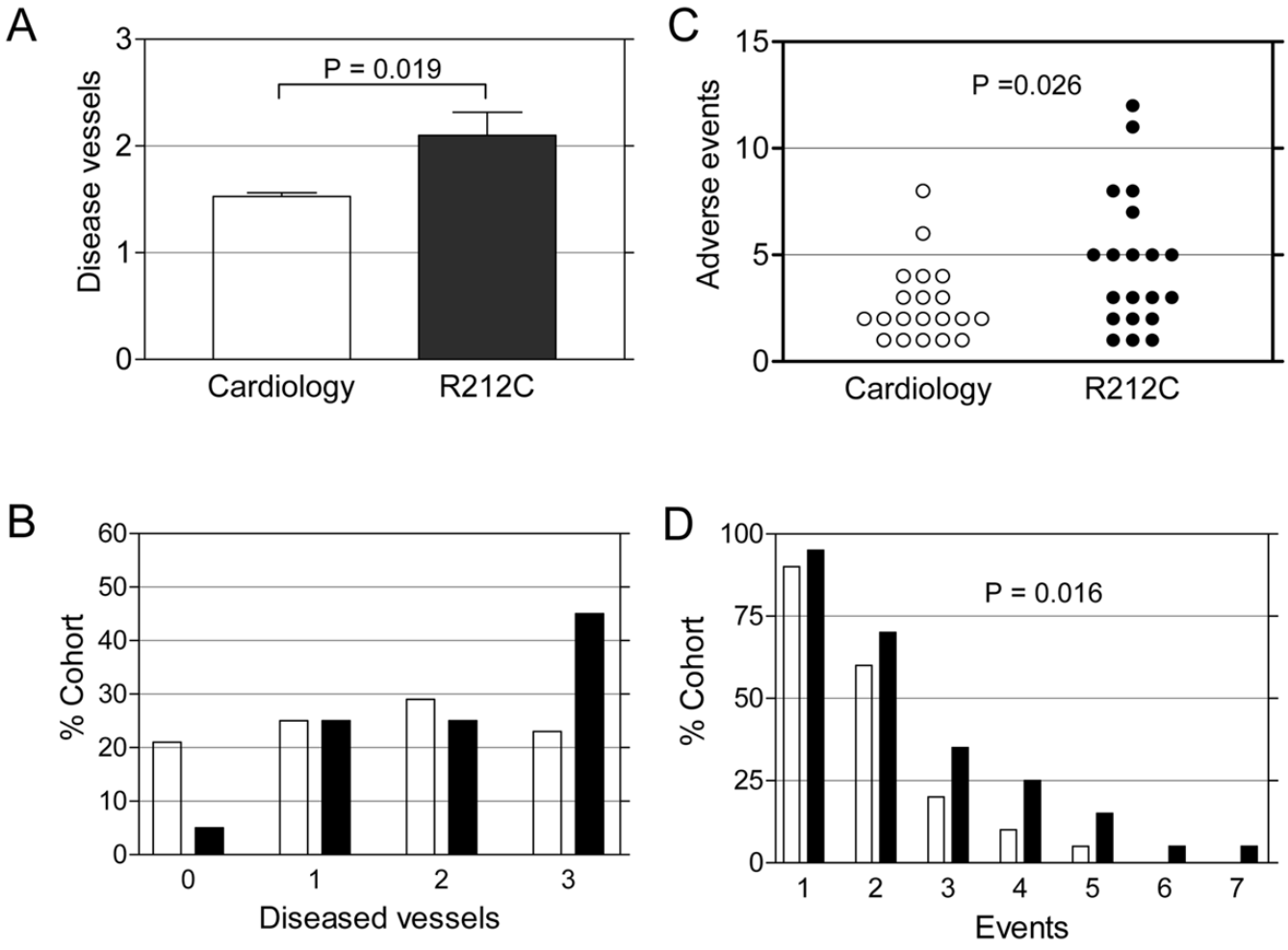

Figure 6. R212C association with accelerated disease

PANEL A: A comparison of disease severity (vessels with significant obstruction, left anterior descending, left circumflex and right coronary artery) in the cardiology cohort versus the R212C (both heterozygote and homozygote) patients. **PANEL B:** An analysis of disease severity in the cardiology control cohort versus the R212C cohort focusing on percent of cohort with the number of vessels obstructed. **PANEL C:** Total cardiovascular adverse events recorded in the risk-matched cardiology control patients (n=20) and the R212C patients (n=20). **PANEL D:** Number of cardiovascular events recorded for patients from either the risk-matched control patients or the R212C patients. Events were recorded for three years from the initial hospital visit/admission for a cardiovascular event.

Table 1

Table summarizing the results of the saturation binding for age and risk factor matched wild type patients (CGC/CGC, n = 5), diseased heterozygote patients (TGC/CGC, n = 4) and our single homozygote patient (TGC/TGC). Shown are the mean ± standard error dissociation constant (K_D, nM), the Bmax for each receptor and the ratio of prostacyclin to thromboxane ratio (Ratio). Unpaired Student t-tests were used to directly compare the wild type versus heterozygote group. [1] p=0.0004, [2] p= 0.007, [3] p=0.003, # POP726 single homozygote patient

	WT CGC/CGC	**R212C TGC/CGC**	**R212C TGC/TGC#**
KD hIP (nM)	28.7 ± 5.2	196.5 ± 23.4[1]	213.0
KD hTP (nM)	119.0 ± 34.1	67.8 ± 10.8	155.3
BmaxhIP (fmol/10^7plts)	63.0 ± 9.7	142.3 ± 17.5[2]	140
BmaxhTP (fmol/10^7plts)	156.3 ± 24.0	107.3 ± 17.5	65
Ratio (hIP/hTP)	0.44 ± 0.10	1.36 ± 0.15[3]	2.15