Specific Pharmacological Profile of A$_{2A}$ Adenosine Receptor Predicts Reduced Fractional Flow Reserve in Patients With Suspected Coronary Artery Disease

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Background—The rapid and reliable exclusion of myocardial revascularization is a major unmet clinical need in patients with suspected coronary artery disease (CAD) and non-contributive electrocardiography and troponin. Non-invasive tests have high rates of false positives and negatives, and there is no biomarker to assess myocardial ischemia. The presence of spare adenosine A$_{2A}$ receptors (A$_{2A}$R)—characterized by a high dissociation constant/half maximal effective concentration (K$_D$/EC$_{50}$) ratio—expressed on peripheral blood mononuclear cells (PBMC) has been associated with ischemia during exercise stress testing in patients with CAD. In this work, we investigated the diagnostic accuracy of spare A$_{2A}$R versus fractional flow reserve (FFR) in patients with suspected CAD.

Methods and Results—Sixty patients with suspected CAD, but non-contributive electrocardiography and troponin, were consecutively enrolled in this prospective study. The binding (K$_D$), functional response (cyclic adenosine monophosphate [cAMP] production; EC$_{50}$) on PBMC A$_{2A}$R were compared with FFR results. Patients were divided into 3 groups: 17 (group 1) with normal coronary angiography (n=13) or stenosis <20% (n=4); 21 with CAD and non-significant FFR (group 2); and 22 with CAD and significant FFR (group 3). Median K$_D$/EC$_{50}$ was 6-fold higher in group 3 (4.20; interquartile range: 2.81–5.00) than group 2 (0.66; interquartile range: 0.47–1.25) and 7-fold higher than group 1 (0.60; interquartile range: 0.30–0.66).

Conclusions—In patients with suspected CAD and non-contributive electrocardiography and troponin, the absence of spare A$_{2A}$R on PBMC may help to rule out myocardial ischemia.

Clinical Trial Registration—URL: http://www.clinicaltrials.gov. Unique identifier: NCT03218007. (*J Am Heart Assoc*. 2018; 7: e008290. DOI: 10.1161/JAHA.117.008290.)

Key Words: adenosine receptor • coronary artery disease • diagnosis

In the emergency department, the management of patients who present with suspected coronary artery disease (CAD)—but with non-contributive electrocardiography and troponin—remains a clinical challenge. Stable or low/intermediate-risk chest pain is a common clinical presentation that often requires costly non-invasive and invasive investigations.[1] Current guidelines recognize the use of many forms of non-invasive diagnostic tests, but they are associated with high rates of false-positive and false-negative results and markedly increase time and total treatment cost.[1]

Invasive coronary angiography (ICA) with functional parameters has become increasingly important for patient management. Fractional flow reserve (FFR) testing can accurately measure blood pressure and flow through a specific part of the coronary artery and is performed through a standard diagnostic catheter during ICA. FFR is currently regarded as

the reference for the assessment of the hemodynamic severity of CAD,[2] but this invasive strategy may increase the rate of inappropriate ICA and can be associated with rare —but potentially fatal—complications such as death, stroke, and iatrogenic acute myocardial infarction.

Biomarkers—such as high-sensitive troponin, heart-type fatty acid binding protein, copeptin, or ischemia-modified albumin—constitute an important diagnostic advance among patients with chest pain.[3] However, some biomarkers lack specificity (copeptin, ischemia-modified albumin) and specific ones (eg, high-sensitive troponin) are markers of myocardial injury. The accuracy of diagnostic strategies is a key priority to prevent inappropriate hospital admissions and minimize avoidable morbidity and costs. A reliable method for the diagnosis of minimal cardiac ischemia is highly desired, particularly for the sensitive diagnosis of CAD in patients with chest pain but non-contributive electrocardiography and/or negative conventional biomarkers. Recently, it has been shown that adenosine A_{2A} receptors ($A_{2A}R$) expressed on peripheral blood mononuclear cells (PBMC) may be a promising biological marker to screen patients with CAD.[4,5]

$A_{2A}R$ are strongly expressed in the coronary system and their activation—by adenosine (an adenosine triphosphate derivative)—leads to an increase in coronary blood flow,[6] partly through the production of cyclic adenosine monophosphate (cAMP) in target cells.[7] We previously developed Adonis —an agonist-like monoclonal antibody to human $A_{2A}R$—that can evaluate the $A_{2A}R$ pharmacological characteristics, ie, expression, binding capacity (as measured by the dissociation constant [K_D]), and functional response (half maximal effective concentration [EC_{50}]) in terms of cAMP production in PBMC.[5] We showed that patients with CAD had low expression of $A_{2A}R$,[4,5] while having the pharmacological profile of spare receptors, ie, $EC_{50} < K_D$[6] in patients with positive exercise stress testing. Low expression of $A_{2A}R$ and low production of cAMP consequently seemed to be associated with myocardial ischemia, documented by positive exercise stress testing.[5]

Because ICA with FFR is a useful tool for detecting hemodynamic obstructive CAD that could benefit from revascularization,[2] we investigated the diagnostic accuracy of $A_{2A}R$ pharmacological profiles compared with FFR evaluation of patients with suspected CAD in a blinded prospective study.

Methods

Patient Selection

We conducted a blind prospective study at the emergency room or cardiac department on patients with suspected CAD based on a clinical assessment including chest pain, risk factors, and physical examination and had non-contributive electrocardiography and troponin at admission and negative ECG-troponin monitoring. We excluded patients with: ST-segment elevation of ≥1 mm in 2 contiguous leads on the presenting ECG; any significant rise in cardiac troponin (>9th percentile); pretest probability <15%; Global Registry of Acute Coronary Events (GRACE) score >140[8] known CAD or awaiting revascularization; history and clinical examination suggesting non-cardiac chest pain; abnormal resting echocardiography and/or chest X-ray; hemoglobin <13 g/dL; or thyroid disorder. No sex-based or race/ethnicity-based differences were addressed. The study was approved by the ethics committee (Reference number A01836-47, Boulogne, France); and written, informed consent was obtained from all patients.

Routine Clinical Assessments

This study included patients on average 4 days after the index period (delay for performing non-invasive tests). Serial ECGs were recorded according to hospital protocol (directly on admission, after the administration of sublingual nitrates, and during any new episode of chest pain while the patient remained under observation). Resting echocardiography and chest X-ray were performed for all patients. As the European Society of Cardiology[9,10] considers the pretest probability >15% as a fundamental component to select the most appropriate diagnostic test in patients with stable angina, all such patients were considered for further non-invasive cardiac investigations, eg, stress echocardiography, exercise stress testing, myocardial perfusion scintigraphy, and multi-slice computed tomography coronary angiography (MSCTA). Use of these non-invasive cardiac investigations was at the discretion of the attending physician, but ICA and FFR were performed in all patients. As the transition from stable to unstable syndromes is a continuum—without a clear boundary—a pretest risk of CAD was determined based on individual GRACE scores.[8]

Laboratory investigations (fasting plasma glucose, glycated hemoglobin, and fasting lipid profile) were used to identify possible cardiovascular risk factors. High-sensitive cardiac troponin I was measured at patient admission (ie, within 6 hours of chest pain onset), then 6 and 12 hours later.

Pretest probability, GRACE score, laboratory investigations, non-invasive cardiac investigations, ICA, and catheter-based FFR were carried out during the patient's hospital stay according to local protocols and the relevant guidelines of the European Society of Cardiology.[9,10]

ICA and FFR

ICA was performed according to a standard clinical method using a visual quantitative scoring system for image analysis, with CAD defined as a luminal diameter narrowing between 20% and 90% in one or more epicardial arteries or their major branches. Vessels with a luminal diameter <2 mm were excluded. Intracoronary glyceryl trinitrate (200 µg) was injected to minimize vasospasm. When arteries with stenosis >20% were visually perceived, an FFR pressure wire (Certus, St. Jude Medical, St. Paul, MN) was positioned distal to the stenosis of interest to determine vessel FFR using RadiAnalyzer (St. Jude Medical) under steady-state hyperemia (intravenous adenosine: 140 µg/kg per minute for 3–6 minutes). FFR ≤0.80 was considered as an evidence-based physiological threshold indicative of obstructive CAD in clinical practice to perform percutaneous coronary intervention.[11,12]

Adjudicated Final Diagnosis

To establish the final diagnosis at discharge for each patient, 2 independent cardiologists—blinded to the results of A$_{2A}$R pharmacological characteristics—reviewed all available medical records (including patient history, physical examination, results of laboratory tests, exercise stress testing, ICA, and FRR) from the time of emergency/cardiac department presentation to discharge. In case of diagnosis disagreement, data were reviewed and adjudicated in conjunction with a third cardiologist.

High-Sensitive Cardiac Troponin I Measurement

High-sensitive cardiac troponin I testing was performed on Centaur® (SIEMENS, Munich, Germany) apparatus. This immunodosage uses one polyclonal and 2 monoclonal antibodies and detection was by a chemiluminescent signal. The 99th percentile value in a healthy population given by the manufacturer was 0.047 µg/L and the detection threshold was 0.006 µg/L. The coefficient of variation was <10% in the measuring range, 0.006 to 50 µg/L.

Adenosine Measurement

The adenosine plasma level assay has been previously described.[13] In brief, blood (3 mL) was collected using an ice-cold syringe containing 2 mL of the cold stop solution to prevent adenosine degradation. Samples were immediately centrifuged ($1500g$, 10 minutes, 4°C) and the supernatant was pipetted off and deproteinized with perchloric acid 70% (v/v). After centrifugation, the supernatants were analyzed by chromatography using a modular system with a diode array detector (Chromsystem®, Munich, Germany). Adenosine was identified by its elution time and spectra, and quantified by comparison of peak areas with those given by known quantities of adenosine. The sensitivity threshold was 3 pmol/mL of plasma matrix. The intra- and inter-assay coefficients of variation ranged from 3% to 5%.

A$_{2A}$R Expression Assay

The data, analytic methods, and study materials (Adonis is available via CliniSciences, France) will be available to other researchers for purposes of reproducing the results or replicating the procedure. The A$_{2A}$R expression procedure has been described previously.[5,14] In brief, blood samples were collected at the beginning of the ICA from the brachial vein in Vacutainer CPT tubes (Beckton Dickinson, Franklin Lakes, NJ) that provide a single-step, standardized method for the isolation of PBMC. After washing and counting, PBMC (0.25×10^6/test) were lysed by sonication in the loading buffer and submitted to 12% polyacrylamide gel electrophoresis under reducing conditions, followed by transfer onto a polyvinylidene difluoride (Thermo Fisher®; Waltham, MA) membrane. The filter was incubated with an anti-A$_{2A}$R monoclonal antibody (Adonis, 1 µg/mL)[14]; staining was performed using horseradish peroxidase–labeled anti-mouse antibodies and enhanced chemiluminescence substrate. The 45 kDa bands corresponding to A$_{2A}$R were submitted to densitometry analysis using the Image J 1.42q software (National Institutes of Health) and results are expressed as arbitrary units (AU; ratio of pixels generated by the A$_{2A}$R band to pixels generated by the background signal). The method for background evaluation involves determining the pixel density of an open area of blot (a rectangle) that is the same size as the area of blot that is used for the determination of A$_{2A}$R. Total protein load per line was also further estimated by monitoring an irrelevant band always present below A$_{2A}$R.

Spare A$_{2A}$R Determination (K$_D$/EC$_{50}$ Ratio)

We developed Adonis, an immunoglobulin M, κ mouse monoclonal antibody directed against a linear epitope localized in the second extracellular loop of the human A$_{2A}$R that demonstrates agonist properties.[5] Adonis binding to the

PBMC surface triggers cAMP production[14] and therefore allows determination of both binding capacity (K_D) and functional response (EC_{50}) parameters of $A_{2A}R$.[14] For both analyses, PBMC were prepared using the vacutainer CPT tubes (as described above) and 0.75×10^6 cells were incubated with increasing concentrations of Adonis (7-point curve: 0.028, 0.056, 0.112, 0.225, 0.45 and 0.9 μmol/L) in 0.5 mL culture medium for 90 minutes at room temperature with shaking. PBMC were then either washed once with phosphate buffered saline (pH 7.3) to eliminate unbound Adonis or centrifuged without washing for K_D or EC_{50} determination, respectively.

K_D was defined as the concentration of ligand (here Adonis) at which 50% of the binding sites (here $A_{2A}R$) were occupied.[15,16] For K_D determination, we used Western blotting to establish the binding curve of Adonis to $A_{2A}R$ on PBMC and to determine the K_D value. As described above, PBMC (0.25×10^6/test) previously incubated with increasing concentrations of Adonis were solubilized by sonication in lysis buffer containing 5% mercaptoethanol before sodium dodecyl sulfate polyacrylamide (12%) gel electrophoresis using 60×90 mm, 1.5-mm thick minigel (Biorad, Hercules, CA), transfer onto a 0.45 μmol/L polyvinylidene difluoride membrane and saturation with non-fat dried milk. The reducing conditions led to the dissociation of Adonis into its heavy and light chains.[5,17] Only the blotted kappa light chain (25 kDa) was visualized using an image station (Kodack Rochester, NY) after incubation of the membrane with peroxidase-labeled anti-mouse μ-chain antibodies and a chemiluminescent substrate. The bands were submitted to densitometry analysis (NIH Image software) and values expressed as AU (ie, pixels generated by the light chain band versus pixels generated by the background signal). K_D was evaluated via densitometry analysis of the $A_{2A}R$ band obtained following the use of the 6 increasing concentrations of Adonis.

EC_{50} was defined as the concentration of Adonis that led to half of the maximal cAMP production when incubated with PBMC.[15,16] cAMP production measurement has been previously described.[13] Briefly, we addressed the cAMP production level of PBMC (0.75×10^6 cells/test) induced by incubation of increasing doses of the agonist-like Adonis in the presence of 3-isobutyl-1-methylxanthine 500 μmol/L as a phosphodiesterase inhibitor using the Amersham Biotrak Kit (GE healthcare Bio-Sciences, Upsala, Sweden). Dodecyl-trimethyl-ammonium bromide acetate buffer was used to stop the incubation step. The competitive enzyme immunoassay was carried out in microplate according to the manufacturer's instructions and optical density (OD; 450 nm) was measured. Wells without cells were used to determine nonspecific binding OD. Results were expressed as percent of standard or sample OD−nonspecific binding OD versus zero standard OD−nonspecific binding OD. A standard curve from 0 to 6400 pg/well was generated to quantify cAMP production.

K_D and EC_{50} values were calculated using non-linear regression analysis (Prism® software; GraphPad Software, San Diego, CA). The presence of spare $A_{2A}R$ was evaluated using the K_D/EC_{50} ratio.

Statistical Analysis

A descriptive analysis was first performed according to the 3 groups of interest, ie, group 1: patients with a normal angiogram; group 2: CAD patients with non-significant FFR; group 3: CAD patients with significant FFR. Categorical variables are reported as numbers and percentage, and quantitative variables as means and standard deviations (SDs) or as medians and interquartile ranges (IQRs). The characteristics were compared between the 3 groups using the Chi-square test or the Fisher test for categorical variables, and the Kruskal–Wallis test for quantitative variables when considering the 3 groups or the Mann–Whitney test when considering 2 groups (groups 1+2 versus group 3). The number of diseases vessels was compared between groups 2 and 3 using the Chi-square test.

Receiver operating characteristic curves were established to define the best threshold value for expression and for K_D/EC_{50} ratio to discriminate patients from group 3 from patients from groups 1 and 2. The areas under the curve and their 95% confidence intervals were estimated. Sensitivity and specificity associated with the best threshold were estimated according to the Youden method,[18,19] which allows the maximization of both values.

All tests were 2-sided and $P<0.05$ was considered statistically significant. All analyses were performed using R software (version 3.4.1).

Technicians performing biological analysis and the medical staff participating to the study were blinded to the clinical and biological results, respectively.

Results

Study Population

Sixty patients who met the inclusion criteria were consecutively enrolled (September 1, to October 29, 2016) in the study and completed the study protocol without adverse effects (Figure 1). Clinical characteristics are given (Table). Among the patients, 36/60 (60%) had high blood pressure, 33 (55%) had hyperlipidemia, 24 (40%) had diabetes mellitus and 21 (35%) were current smokers. Hypertension was defined as a systolic pressure >140 mm Hg or a diastolic pressure >90 as measured in both arms and by a 24-hour ambulatory blood pressure monitoring.[20]

Diabetes mellitus was defined as a fasting plasma glucose level >7.0 mmol/L, a 2-hour plasma glucose value in a 75 g
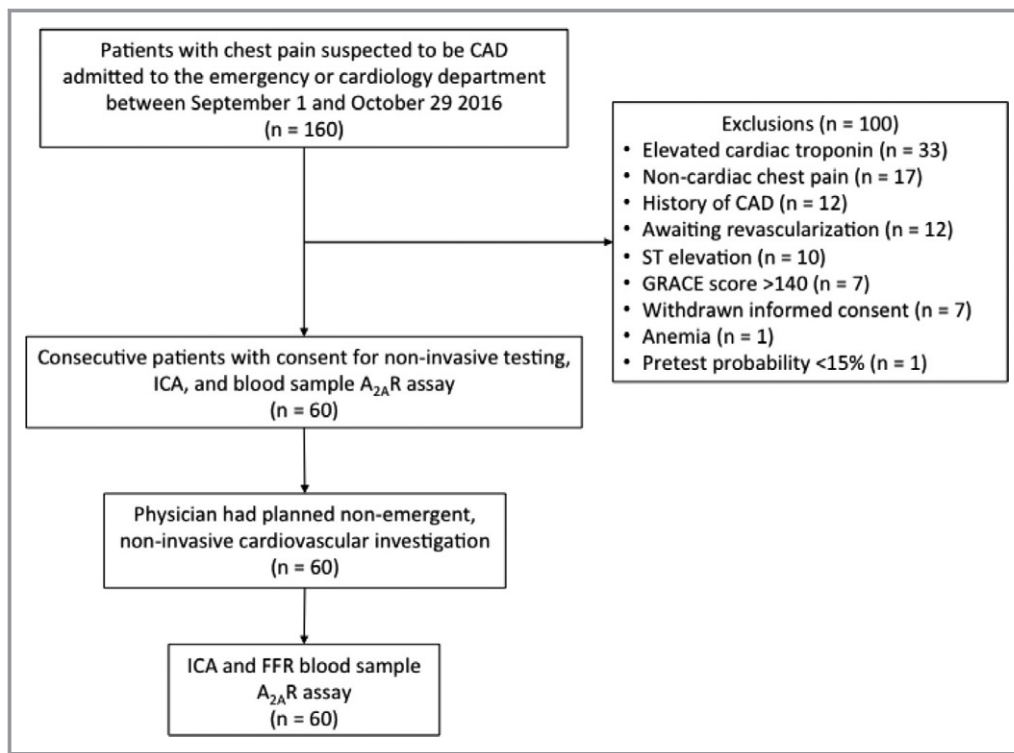
Figure 1. Study flow chart. CAD indicates coronary artery disease; FFR, fractional flow reserve; GRACE, Global Registry of Acute Coronary Events; ICA, invasive coronary angiography.

oral glucose tolerance test >11.1 mmol/L, or a glycated hemoglobin (A1C) value >6.5%.[21]

The smoking history status was self-reported on the basis of a questionnaire submitted at the time of hospitalization. There were categories: non-smoker, ex-smoker (smoking cessation since at least 3 years) and current smoker. Only current smoker was recorded.[22]

ICA and FFR Test

All the 60 patients were investigated by ICA but 17 patients (group 1) were not selected for FFR testing because their arteries were without identifiable atheroma plaque (n=13) or with minimal disease (stenosis <20%; n=4). No completely occluded or sub-totally or heavily calcified arteries were found in the remaining 43 patients. FFR testing was performed successfully in all patients with visually perceived diameter stenosis >20%, involving 77 subtended territories.

The 43 patients all underwent FFR interrogation in every vessel territory, so one FFR was performed in all 69 coronary arteries with readings ranging from 0.67 to 0.99 (mean±SD; 0.84±0.15). Twenty-one patients (group 2) were considered to present non-hemodynamically significant coronary artery stenosis despite 5 patients having visually perceived diameter stenosis >70%. At least one hemodynamically significant coronary lesion (FFR <0.80) was found in the remaining 22 patients (group 3). Using this approach,

FFR interrogations of 36 arteries in group 3 were classified as significant in: 12/12 vessels in patients with single-vessel disease, 8/12 vessels in patients with double-vessel disease, and 8/12 vessels in patients with triple-vessel disease. All culprit lesions had FFR regarded as significant in the territory suspected of ischemia. Table shows the results of the patients in each group. There were no significant differences regarding angiographic findings between CAD patients in group 2 and group 3 (*P*=0.24).

In group 1, we performed 14 non-invasive diagnostic tests in 9 patients and 5 MSCTAs. The remaining 3 patients were aged >80 years, and we decided to evaluate their CAD status by angiography because of their pretest probability at >85%. In group 2, 15 patients had a positive non-invasive diagnostic test. Myocardial perfusion scintigraphy was inconclusive in 7 patients so MSCTA was undertaken. In group 3, 4 exercise ECG tests were considered inconclusive and ICA was performed. Myocardial perfusion scintigraphy was abnormal in 13 patients, and showed myocardial ischemia (n=6) and positive dobutamine stress echocardiography (n=7). MSCTA was positive in the remaining 5 severe angiographically-documented patients with CAD.

High-Sensitive Troponin Assay

High-sensitive cardiac troponin I was <0.047 ng/mL (99th percentile) in admission, 6-, and 12-hour tests in all groups

Table. Demographics, Risk Factors, Angiographic Data and Positive Non-Invasive Diagnostic Testing

	Group 1 (n=17)	Group 2 (n=21)	Group 3 (n=22)	P Value*
Age, y	75±4	74±9	73±9	0.66
Male	10 (59)	14 (67)	16 (73)	0.66
Cardiovascular risk factors				
Hypertension	10 (59)	13 (62)	13 (59)	0.98
Hyperlipidemia	9 (53)	13 (62)	11 (50)	0.72
Diabetes mellitus	7 (41)	7 (33)	10 (45)	0.71
Family history of CAD	4 (24)	3 (14)	5 (23)	0.78
Current smoking	6 (35)	9 (43)	6 (27)	0.58
Treatment				
Calcium channel blockers	10 (59)	13 (62)	13 (59)	0.97
Angiotensin converting enzyme inhibitor	8 (47)	10 (47)	11 (50)	0.98
Angiotensin receptor blocker	4 (23)	7 (30)	6 (27)	0.55
Beta-blockers	9 (52)	11 (52)	10 (45)	0.70
Diuretics	7 (41)	9 (42)	8 (36)	0.57
Statins	11 (64)	15 (71)	15 (68)	0.95
Metformin	5 (29)	6 (28)	8 (22)	0.66
DPPIV inhibitors	6 (35)	5 (24)	6 (27)	0.71
Angiographic findings				
Number of diseased vessels				0.24[†]
0	17 (100)	0	0	
1	0	10 (48)	12 (55)	
2	0	10 (48)	6 (27)	
3	0	1 (5)	4 (18)	
Culprit vessel				0.25
Left anterior descending coronary artery	…	15 (71)	10 (45)	
Right coronary artery	…	2 (10)	5 (23)	
Circumflex coronary artery	…	4 (19)	7 (32)	
Positive non-invasive diagnostic testing				
MSCTA	5 (29)	6 (29)	5 (23)	0.87
Stress echocardiography	2 (12)	5 (24)	7 (32)	0.35
Exercise stress testing	1 (6)	2 (10)	0	0.38
Myocardial perfusion scintigraphy	6 (35)	8 (38)	6 (27)	0.74

Values are n (%) or mean±SD. Group 1: normal angiogram. Group 2: CAD with FFR 0.8 to 1.0. Group 3: CAD with FFR <0.8. CAD indicates coronary artery disease; DDPIV, dipeptidyl peptidase IV; FFR, fractional flow reserve; MSCTA, multi-slice computed tomography coronary angiography.
*Three-way comparison unless otherwise indicated.
[†]P value for group 2 vs group 3.

(data not shown). No rise in cTnI was observed during the 12-hour follow-up period.

Adenosine Plasma Level

There were no significant differences in basal adenosine plasma levels in the 3 groups. The mean±SD values for groups 1 to 3 were 0.59±0.11, 0.62±0.14, and 0.68±0.13 μmol/L, respectively (P>0.05).

A$_{2A}$R Expression

A$_{2A}$R expression was significantly lower in group 3 than in the 2 other groups (median [IQR] results from group 1–3: 1.15
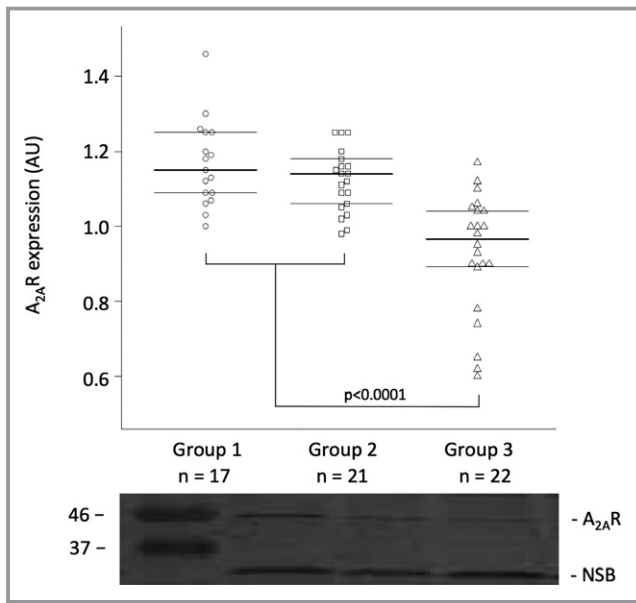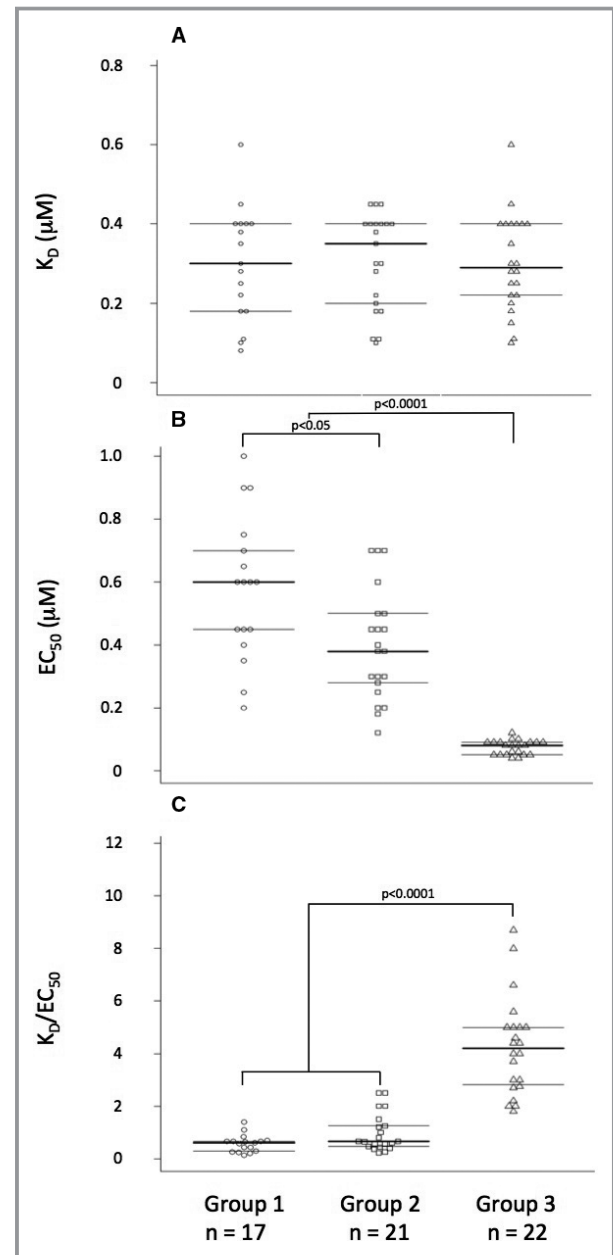
Figure 2. A$_{2A}$R expression evaluated on peripheral blood mononuclear cells in 60 patients with suspected coronary artery disease. Group 1: normal angiography (n=17); group 2: abnormal angiography but non-significant FFR (ie, 0.8–1.0) (n=21); group 3: abnormal angiography with significant FFR (ie, ≤0.8) (n=22). Expression was evaluated by Western blot; a representative blot from each group of patients is shown. The staining intensity was measured using densitometry analysis and data (medians [thick lines], IQRs [thin lines]) are expressed in AU (ie, ratio of pixels generated by the A$_{2A}$R band to pixels generated by the background signal). A$_{2A}$R indicates adenosine A$_{2A}$ receptors; AU, arbitrary units; FFR, fractional flow reserve; IQR, interquartile range. A representative Western blot with the molecular weight markers (left) and the specific A$_{2A}$R band (45 kDa) of a patient from each group is shown. The presence of a nonspecific band (NSB) demonstrates that equal amounts of protein were loaded in each lane.

[1.09–1.25], 1.14 [1.06–1.18], and 0.97 [0.89–1.04] AU, respectively; *P*<0.01; Figure 2).

Spare A$_{2A}$R Identification

Median K$_D$ values were not significantly different between the 3 groups: median (IQR) values among Groups 1 to 3 were 0.30 (0.18–0.40), 0.35 (0.20–0.40), and 0.29 (0.22–0.40) μmol/L, respectively (Figure 3A). However, median EC$_{50}$ values decreased significantly from group 1 to group 3: median (IQR) values 0.60 (0.45–0.70), 0.38 (0.28–0.50), and 0.08 (0.05–0.09) μmol/L, respectively; *P*<0.01; (Figure 3B). The median K$_D$/EC$_{50}$ ratio in group 3 (4.20; IQR: 2.81–5.00) was 6-fold higher than in group 2 (0.66; IQR: 0.47–1.25) and 7-fold higher than in group 1 (0.60; IQR: 0.30–0.66) (Figure 3C).

Figure 4 shows the characteristic A$_{2A}$R patterns for the 3 groups. Interestingly, patients in group 3 had EC$_{50}$ values that were lower than the corresponding K$_D$ values; the opposite was seen in groups 1 and 2.

Figure 3. Peripheral blood mononuclear cells A$_{2A}$R characterization in patients with suspected coronary artery disease. Group 1: normal angiography (n=17); group 2: abnormal angiography but non-significant FFR (ie, 0.8–1.0) (n=21); group 3: abnormal angiography with significant FFR (ie, ≤0.8). K$_D$ (A) and EC$_{50}$ (B) were interpolated from the dose response curves obtained using increasing concentrations of Adonis (see Methods); K$_D$/EC$_{50}$ ratio (C). Data are expressed as median (thick lines) and IQRs (thin lines). A$_{2A}$R indicates adenosine A$_{2A}$ receptors; EC$_{50}$, half maximal effective concentration; FFR, fractional flow reserve; IQR, interquartile range; K$_D$, dissociation constant.

Receiver Operating Characteristic Curves

With a cut-off of 1.05 AU, the sensitivity and specificity of A$_{2A}$R expression level were both 82% (Figure 5A). The AUC was 0.89 (95% confidence interval: 0.81–0.97). With a cut-off of 1.8, the
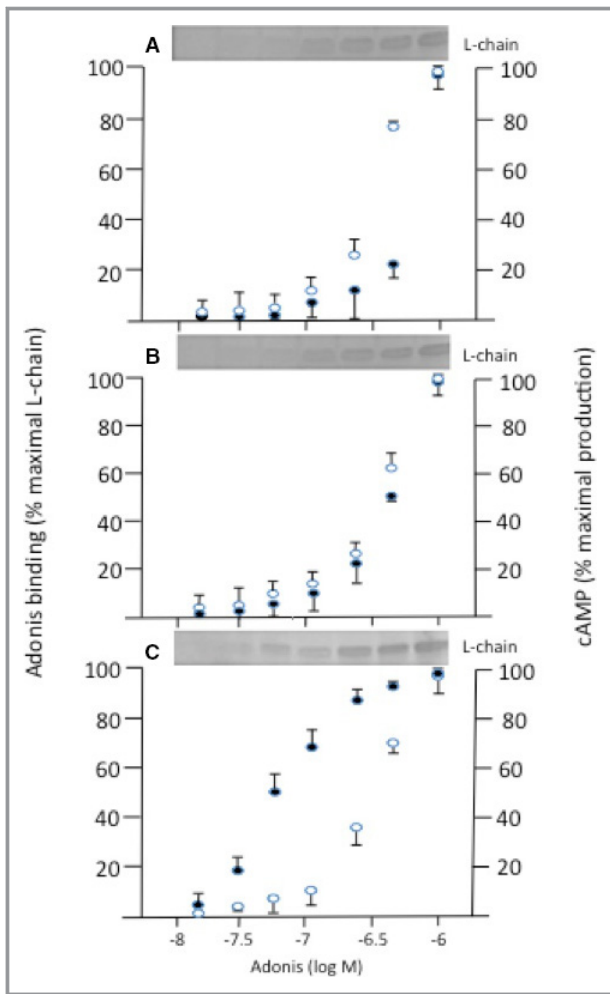
Figure 4. Identification of spare A$_{2A}$R in patients with suspected coronary artery disease. The dose–response curves resulting from Adonis binding to A$_{2A}$ adenosine receptors and the resulting cAMP production for each group of patients: (A) patients with normal angiography (n=17); (B) patients with stenosis but non-significant FFR (ie, 0.8–1.0) (n=21); (C) patients with stenosis and significant FFR (≤0.8) (n=22). White discs: K$_D$ (densitometry analysis of the 25 kDa band corresponding to the light [L-chain], see a representative Western blot at the top of the figure); black discs: EC$_{50}$ Results are mean±SD. cAMP indicates cyclic adenosine monophosphate; EC$_{50}$, half maximal effective concentration; FFR, fractional flow reserve; K$_D$, dissociation constant; SD, standard deviation.

sensitivity and specificity of the K$_D$/EC$_{50}$ ratio were 100% and 89%, respectively (Figure 5B). The AUC was 0.99 (95% confidence interval: 0.97–1.00). Thus, receiver operating characteristic curve analysis showed that the K$_D$/EC$_{50}$ ratio was better than A$_{2A}$R expression for predicting significant obstructive CAD with FFR <0.8.

Discussion

The main result of this study is that in patients with suspected CAD and non-contributive ECG and troponin, FFR ≤0.8 was
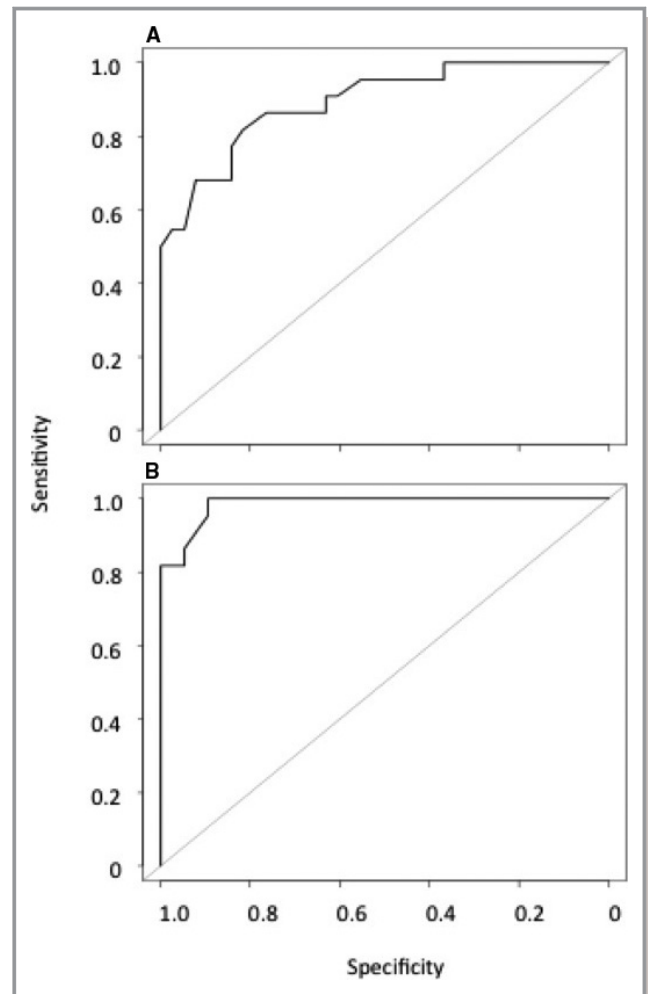
Figure 5. Receiver operating characteristic curves. For (A) A$_{2A}$R expression data and (B) K$_D$/EC$_{50}$ ratio data evaluated on peripheral blood mononuclear cells of patients with suspected coronary artery disease (n=60). A$_{2A}$R indicates adenosine A$_{2A}$ receptors; EC$_{50}$, half maximal effective concentration; K$_D$, dissociation constant.

associated with the presence of spare A$_{2A}$R. Thus, this prospective study in unselected patients confirmed the value of a high K$_D$/EC$_{50}$ ratio as a marker of severe obstructive CAD.

Various clinical trials demonstrated the benefits of using FFR measurement to more accurately identify stenosis that are obstructive and guide revascularization intervention successfully.[11,12,23] FFR is currently recommended in clinical guidelines only in stable CAD but the transition from stable to unstable syndromes is a continuum without a clear boundary. Recent studies have suggested however that FFR may be accurate in unstable angina. The FAMOUS NSTEMI CMR (Fractional Flow Reserve Versus Angiographically Guided Management to Optimise Outcome in Unstable Coronary Syndromes Cardiac Magnetic Resonance) sub-study showed excellent diagnostic accuracy of FFR <0.80 (92%) for predicting ischemia.[24] Furthermore, in the pivotal COURAGE (Clinical

Outcomes Utilizing Revascularization and Aggressive Drug Evaluation) trial, 32% of patients presented an unstable angina.[25] These 2 studies showed clinical benefit of an FFR-guided strategy in ACS patients. Furthermore, it was evidenced that FFR-guided strategy for complete revascularization during ACS has the potential to decrease unnecessary interventions during primary PCI.[26] Little doubt exists about the value of the cut-off value in ACS patients, ie, 0.80. For Hakeem and colleagues,[27] ACS patients appeared to have a cut-off of 0.84, on the basis of receiver-operating characteristic analysis. FFR <0.84 seems to present the best predictive accuracy for events in another work.[28] In our small trial, FFR study was higher than 0.90 in every patient in group 2 without any event rate at 1 year.

Adenosine is released in the plasma by endothelial cells and myocytes during ischemia, particularly in the coronary artery.[29] The adaptive response to the decrease in coronary blood flow consists of vasodilation of the coronary arteries via activation of the A$_{2A}$R, which are coupled to the cAMP pathway—cAMP production and coronary vasodilation being correlated.[7] Thus, A$_{2A}$R regulates the coronary blood flow, particularly in ischemic conditions. Although A$_{2A}$R are strongly expressed in the coronary system, it is difficult to investigate the adenosinergic pathway in the coronary artery. The fact that the expression and function of A$_{2A}$R in heart tissues[30] and coronary arteries[31] correlate with expression and function of A$_{2A}$R in PBMC provides a unique window to link the adenosinergic system to ischemia in the coronary arteries.

When Adonis binds to A$_{2A}$R, the resulting quantity of activated receptors will depend on the receptor density, Adonis affinity, and intrinsic efficacy. Once activated, the A$_{2A}$R stimulates various signal transduction pathways (Figure 6), leading to measurable physiological responses, here the coronary blood flow measured by the FFR test. The pharmacological response of an agonist relative to its maximal effect is not always proportional to the fraction of bound receptors.[32] A high K_D/EC_{50} ratio means that the K_D value is greater than the corresponding EC_{50} and, consequently, that surface receptor sites are partially unoccupied, constituting a "reserve" when a given low agonist concentration maximally stimulates a pharmacological response: this situation has been theorized as the spare receptors.[33] In an animal model, activation of only 5% of A$_{2A}$R leads to maximal coronary conductance and, thus, vasodilation.[6] Here, the positive threshold for the K_D/EC_{50} ratio was set to 1.8, meaning that 55% of sites bound by Adonis gave half-maximal cAMP production and that higher ratio values were associated with a significant FFR test. This reasoning means, in turn, that above a reserve of 45% of unoccupied sites, there is significant ischemia, as documented by FFR. Paradoxically, the presence of spare receptors was associated here with low A$_{2A}$R expression and, consequently, low efficacy in CAD patients with significant FFR. Like Adonis for A$_{2A}$R, some ligands for the 5-hydroxytryptamine 2A receptor have been
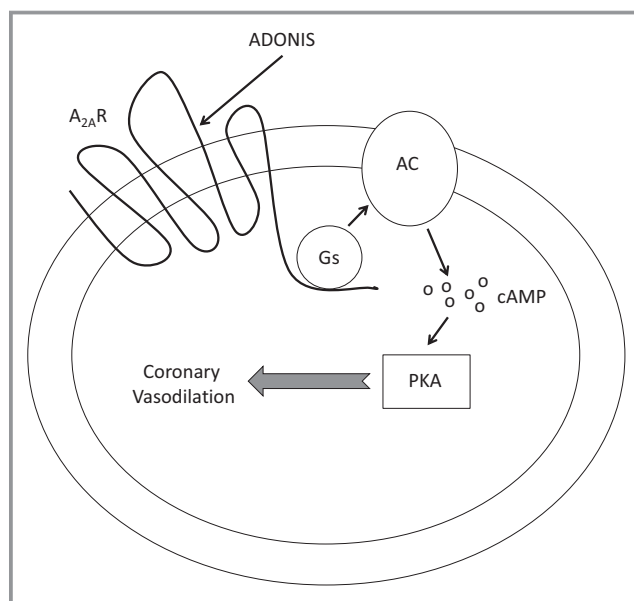
Figure 6. Mechanism of action of A$_{2A}$R on coronary vascular wall. Adonis binds to a linear epitope on the second extracellular loop of A$_{2A}$R. Upon stimulation by Adonis, the third intracellular loop of A$_{2A}$R couples with the stimulatory subunit of G-proteins (Gs) and triggers adenylate cyclase (AC) activity. Production of cAMP activates protein kinase-mediated signaling pathways and acts on Ca^{++}/K^+ channels of vascular smooth muscle leading eventually to coronary dilation. PKA indicates protein kinase A.

shown to have a lower efficacy with a greater receptor reserve according to the activated signal transduction pathway.[34] Here, nearly one-third of the patients with suspected CAD and non-contributive electrocardiography and troponin had FFR <0.8. Albeit the existence of an FFR "gray zone" from 0.75 to 0.80 was previously reported for decision-making,[35–37] where the chosen FFR threshold value of 0.80 was clearly associated with the presence of spare A$_{2A}$R displaying a high K_D/EC_{50} ratio. Importantly, this test may help distinguish patients who require hospitalization in a cardiac department or monitoring only.

Study Limitations

Firstly, this is a single-center study. However, as patient demographics were comparable to several recent studies including consecutive patients with symptoms suggestive of CAD, we consider our results to be representative for unselected patient cohorts presenting to the emergency or cardiology department. Secondly, 60 patients with CAD represent too small a cohort to correctly assess ischemia, and confirmation in larger studies is warranted before the spare A$_{2A}$R assay can be adopted into clinical practice. Thirdly, because of the emergency context, exercise stress testing, myocardial perfusion scintigraphy, stress echocardiography, and MSCTA were only performed in some patients. It might be interesting to perform all of these non-invasive

diagnostic tests for each patient. Finally, despite initial concerns of impaired microvascular function during ACS that might yield a false negative FFR result, we didn't measure CFR and microresistance. The microvascular function does not matter for the accuracy of the FFR measurement.[38] Only ACS statut constitutes an issue for FFR measurement (duration and intensity of ischemia, embolization spasm) while the clinical impact of these changes is minimal. Earlier data comparing FFR measurements at the acute phase or a week later would have led the same clinical decision about the need for revascularization.[35] At least, our study included patients on average 4 days after the index period (delay for performing non-invasive tests), which suggested a majority of stabilized "unstable syndromes" while we have excluded high-risk patients.

Conclusions

Optimization of cardiac risk stratification of patients suspected to have CAD and non-contributive electrocardiography and troponin (ie, suspected CAD) is a key priority in emergency medicine. Risk stratification based on prognostic scoring systems such as GRACE improves the selection of higher-risk patients for invasive management. The accuracy of diagnostic strategies is key to preventing inappropriate hospital admissions and minimizing morbidity and costs. In low- or intermediate-risk acute coronary syndromes without ST-segment elevation or stable angina, functional parameters (exercise stress testing, stress echocardiography, MSCTA, myocardial perfusion scintigraphy, FFR) have become more important for management. In our study, the detection of spare A$_{2A}$R appears to be a promising diagnostic tool for screening patients with CAD and a hemodynamically significant coronary lesion.

Acknowledgments

Sources of Funding

Disclosures

None.

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