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Pharmacological Profile of Adenosine A_{2A} Receptors in Patients with Lower Extremity Peripheral Artery Disease and Associated Coronary Artery Disease: a Pilot Study

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Background

Altered blood flow occurs in patients with low extremity peripheral artery disease (LE-PAD). LE-PAD is mostly associated with coronary artery disease (CAD). Adenosine is an endogenous nucleoside that affects both coronary and limb artery blood flow, mostly via the adenosine A_{2A} receptor ($A_{2A}R$). We evaluated $A_{2A}R$ expression and function in peripheral blood mononuclear cells (PBMCs) and the femoral artery tissues of patients with LE-PAD.

Methods

Artery tissues and PBMCs were sampled in 24 patients with intermittent claudication, and compared with PBMCs in 24 healthy subjects. Expression and function of $A_{2A}R$ was studied, using a $A_{2A}R$ antibody with agonist properties, allowing determination of $A_{2A}R$ affinity (K_D) and cAMP production (ie. EC_{50}).

Results

$A_{2A}R$ expression on PBMCs was lower in patients than controls (median 1.3 [range 0.6–1.8] vs 1.75 [1.45–2.1] arbitrary units; $P < 0.01$), and correlated with $A_{2A}R$ expression in artery tissues (Pearson's $r = 0.71$; $P < 0.01$). Basal and maximally stimulated cAMP production of PBMCs was lower in patients vs controls: 172 [90–310] vs 244 [110–380] pg/ 10^6 cells ($P < 0.05$) and 375 [160–659] vs 670 [410–980] pg/ 10^6 cells ($P < 0.05$), respectively. A high K_D/EC_{50} ratio, characteristic of spare receptors, was observed in CAD with inducible-myocardial-ischemia.

Conclusion

$A_{2A}R$ expression in the arteries of patients, correlated with their expression in PBMCs. $A_{2A}R$ expression was lower in patients than in controls. A single blood sample (for measurement of $A_{2A}R$ expression on PBMCs) may help to screen patients with LE-PAD, whereas the presence of spare receptors may help with risk stratification before vascular surgery in CAD patients with high risk of myocardial ischemia.

Introduction

Lower extremity peripheral artery disease (LE-PAD) is the third leading cause of atherosclerotic cardiovascular morbidity, after coronary artery disease (CAD) and stroke [1]. Patients with LE-PAD and patients with CAD share the same risk factors (age, sex, diabetes, tobacco use, hypertension, and hyperlipidemia), with different weights of association compared with CADs [2]. Accordingly, LE-PAD is often underdiagnosed and thus mistreated [3]. Almost two-thirds of patients with LE-PAD are asymptomatic [4], and its diagnosis is challenging, because these patients present a high risk of amputation and premature death [5].

LE-PAD is the result of insufficient blood flow caused by atherosclerosis or arterial thrombosis in the lower extremities. Besides mechanical factors of ischemia, humoral factors may be involved in the maintenance of blood flow in distal arteries. Among the humoral factors, adenosine is an adenosine triphosphate derivative that is released in extracellular spaces during ischemia [6] or inflammation [7]. Adenosine has a strong effect on arterial blood flow via its membrane receptors, namely A_1 , A_{2A} , A_{2B} and A_3 , pending of their pharmacological profile [8]. Adenosine triphosphate and adenosine contribute to the restoration of blood flow during ischemia [9], and play a major role in the control of the associated inflammation process [7]. Adenosine exerts its vasodilatory and anti-inflammatory effects mainly through activation of the A_{2A} receptor ($A_{2A}R$) [10] and the A_{2B} receptor [11,12], which leads to an increase in production of cyclic adenosine monophosphate (cAMP) [10]. The production of cAMP has been correlated with vasodilatation [13].

Thus, the level of expression of these receptors and their functional activity are of great importance for vascular blood flow. The behavior of $A_{2A}Rs$ expressed at the surface of peripheral blood mononuclear cells (PBMCs) mirrors the behavior of $A_{2A}Rs$ found in the heart [14], aorta and coronary artery tissue [15]. However the expression and function of $A_{2A}Rs$ in patients with LE-PAD with intermittent claudication has not been evaluated.

The aim of this study was to study $A_{2A}R$ expression and function in situ (femoral tissue) and on PBMCs in patients with LE-PAD compared with in healthy subjects, in order to evaluate the possible systemic regulation of the adenosinergic response.

Methods

Patients

Patients with LE-PAD undergoing vascular surgery for intermittent claudication in La Timone hospital (Marseille, France) were included consecutively. Because the symptoms of myocardial ischemia (MI) are often masked in LE-PAD, and the accuracy of non-invasive

stress tests is usually limited, every patient undergoes invasive coronary angiography. In case of identifiable atheroma plaque >20%, fractional flow reserve and/or one stress imaging test were performed.

Controls were healthy subjects matched for sex and age, with a normal ankle-brachial index ($0.9 < \text{ankle-brachial index} < 1.4$), normal iliac and femoral duplex ultrasound (which includes B-mode echography, pulsed-wave, continuous, color and power Doppler modalities) and no cardiac or vascular history. The protocol was approved by the ethical committee of our institution (CPP Sud Méditerranée). The study conformed to the standards set out in the Declaration of Helsinki. Written informed consent was obtained from all study participants.

Blood Sample Collection

Blood sample collection for measurement of adenosine plasma concentration and PBMC preparation was performed as described previously [15,16]. Briefly, blood was withdrawn, together with a cold stop solution, into vacutainer tubes, to prevent adenosine uptake by red blood cells and deamination into inosine. Samples were centrifuged immediately (4°C , $1500\times g$). The supernatant was pipetted off and stored at -20°C until measurement.

Measurement of Adenosine Plasma Concentration

Measurement of adenosine plasma concentration was performed as described previously [16,17], by liquid chromatography/tandem mass spectrometry, after extraction using a Shimadzu UFLC XR system (Marne-la-Vallée, France). The system was interfaced with an ABSciex 4500 triple quadrupole mass spectrometer (Les Ulis, France), operating with an electrospray ionization source using nitrogen.

Liquid chromatography/mass spectrometry-grade methanol and pure water were purchased from VWR (Fontenay-sous-Bois, France). Formic acid, adenosine, and 2-chloroadenosine were obtained from Sigma Chemical (Saint-Quentin Fallavier, France).

PBMC Preparation

PBMCs were isolated from blood using the Vacutainer CPT system (Becton-Dickinson, Franklin Lakes, NJ) following venous puncture from the brachial vein, according to the manufacturer's instructions.

Artery Tissue Collection

We collected a small artery tissue fragment (mean weight 11 ± 4 mg) from the femoral arteries at the site of the proximal anastomosis of a femoropopliteal or femorofemoral bypass. Fresh tissue samples from arteries were tested extemporaneously for $A_{2A}R$ expression and cAMP (see below).

$A_{2A}R$ Expression Assay

This procedure has been described previously [18,19]. Briefly, $A_{2A}R$ expression in PBMCs or artery tissues was determined by Western blotting, using Adonis, an home made agonist-like monoclonal antibody to human $A_{2A}R$ [18]. PBMCs (0.25×10^6) and tissue samples (3 mg) were solubilized by sonication in lysis buffer containing 5% mercaptoethanol before sodium dodecyl sulfate polyacrylamide (12%) gel electrophoresis using 60x90 mm, 1.5 mm thick minigel (Biorad, Hercules, CA), transfer onto a $0.45 \mu M$ polyvinylidene difluoride filter and saturation with nonfat dried milk. The filter was then incubated with Adonis ($1 \mu g/mL$) and phosphatase alkaline labeled anti-mouse antibodies, successively, and revealed by colored substrate reaction. Bands were scanned and submitted to densitometry analysis using Image J 1.42q software (National Institutes of Health, Bethesda, MD). Results were expressed in arbitrary units defined as pixels for the 45 kDa band ($A_{2A}R$)/pixels of blot background, as described previously [19,20].

cAMP Measurement

The assay was performed on freshly prepared PBMCs and artery tissues collected during surgery, as described previously [15]. cAMP measurement using Adonis as agonist has been described previously [19,20]. Briefly, samples were immediately placed into Roswell Park Memorial Institute (RPMI) culture medium containing an inhibitor of phosphodiesterase ($100 \mu M$ 3-isobutyl-1-methylxanthine) with and without the presence of $1 \mu M$ Adonis. After 2 hours of incubation at $37^\circ C$, samples were centrifuged ($1500 \times g$, 10 min) and supernatants were frozen ($-80^\circ C$) until cAMP quantitation. Frozen tissues were then homogenized in 6% trichloroacetic acid at $4^\circ C$ to obtain a 10% (w/v) homogenate. Samples were centrifuged ($2000 \times g$, 15 min, $4^\circ C$), supernatants were recovered, and the pellets discarded. The supernatants were washed four times with 5 volumes of water-saturated diethyl ether, and the upper organic layer from each wash was discarded. The aqueous extract was lyophilized and stored ($-80^\circ C$) until analysis. The cAMP concentration was determined using the Amersham Biotrak Kit (GE Healthcare Bio-Sciences, Uppsala, Sweden). Dodecyl-trimethyl-ammonium

bromide acetate buffer was used to stop the incubation step. The competitive enzyme immunoassay was carried out in microplates according to the manufacturer's instructions, and optical density (450 nm) was measured. Wells without cells were used to determine nonspecific binding. Results were expressed as a percentage of (standard or sample optical density – nonspecific binding optical density) vs (zero standard optical density – nonspecific binding optical density). A standard curve from 0 to 6400 pg/well was generated to quantify cAMP production.

Adonis K_D /EC₅₀ Determination on PBMCs

Adonis binding to the PBMC surface triggers cAMP production, and allows determination of both binding capacity (dissociation constant K_D) and functional response (cAMP production, ie, half-maximal cAMP production [EC₅₀]) variables [19,20]. PBMCs (0.75×10^6 cells) were incubated with seven increasing concentrations of Adonis (0, 0.028, 0.56, 0.112, 0.225, 0.45, and 0.9 μ M) in 0.5 mL culture medium for 90 min at room temperature, with shaking). PBMCs were then either washed once with phosphate buffered saline (pH 7.3) to eliminate unbound Adonis or centrifuged without washing for K_D or EC₅₀ determination, respectively.

K_D was defined as the concentration of Adonis at which 50% of the binding sites were occupied. For K_D determination, we used Western blotting to establish the binding curve of Adonis to A_{2A}R on PBMCs. PBMCs (0.25×10^6) previously incubated with increasing concentrations of Adonis were solubilized in reducing conditions and submitted to electrophoresis and blotting as described above for A_{2A}R expression assay. The reducing conditions led to the dissociation of Adonis into its heavy and light chains. Only the blotted kappa light chain (25 kDa) was visualized (see Figure 1) using phosphatase alkaline labeled anti-mouse kappa light chain antibodies and colored substrate reaction. The bands were then scanned and submitted to densitometry analysis (Image J software) and values expressed as arbitrary units (ie, pixels generated by the light chain band versus pixels generated by the background signal, as described previously [19,20]). K_D was evaluated from the data analysis obtained with each of the six increasing concentrations of Adonis, as described below.

EC₅₀ was defined as the concentration of Adonis that led to half-maximal cAMP production when incubated with PBMCs. Briefly, we addressed the cAMP production level of PBMCs (0.75×10^6 cells) induced by incubation of increasing doses of the agonist-like Adonis using the cAMP Biotrak Kit, as described above.

K_D and EC₅₀ values were calculated using non-linear regression analysis (Prism® software; GraphPad Software, San Diego, CA). The presence of spare A_{2A}Rs was evaluated

using the K_D/EC_{50} ratio, as reported previously [19,20].

Statistical Analysis

According to numerous previous reports of the group on clinical classification of patients based on purinergic profiling, a difference $>15\%$ in A_{2A} R expression and $>100\%$ in K_D/EC_{50} ratio was considered to be with pathophysiological consequences. Thus groups of at least 20 subjects were found to be necessary for statistical analysis. Patients data were expressed as median [range], or mean \pm standard deviation. Correlations between biological variables were quantified and tested using Pearson's rank correlation coefficient. Comparisons of biological variables between patients and controls were performed using two-way analysis of variance. A multivariate analysis to address the correlation between artery A_{2A} R and PBMC A_{2A} R was performed. Receiver operating characteristic curve was established to define the best threshold value for expression and for A_{2A} R expression to discriminate patients from controls. The areas under the curve and their 95% confidence intervals were estimated. All statistical tests were two-sided, and P values <0.05 were considered statistically significant. Analysis was performed with SPSS software, version 13.0, 2004 (SPSS Inc, Chicago, IL).

Results

Twenty-four patients with LE-PAD (20 men and four women; mean age 70 ± 7.5 years) undergoing vascular surgery for intermittent claudication were included consecutively. The main clinical features and surgical treatment data are presented in Table 1, and concomitant coronary artery status is presented in Table 2. The coronary arteries of seven patients (29.1%) were without identifiable atheroma plaque or with minimal disease (stenosis $<20\%$). Only nine patients (37.5%) had significant coronary stenosis ($>70\%$) documented by invasive coronary angiography with inducible MI (Table 2). The remaining eight patients were considered to present nonhemodynamically significant coronary artery stenosis, despite one patient having visually-perceived diameter stenosis $>70\%$, because noninducible MI and fractional flow reserve >0.8 were noted. None of the patients had cardiovascular events during hospitalization.

The control group comprised 19 men and five women (mean age 67 ± 5 years and see methods). Adenosine plasma concentration was significantly higher in basal conditions in patients compared with controls: 0.82 [0.5–1] vs 0.6 [0.4–0.78]; $P<0.001$.

A_{2A}R Expression

A_{2A}R expression measured on PBMCs in arbitrary units was lower in patients compared with in controls: 1.3 [0.6–1.8] vs 1.75 [1.45–2.1] arbitrary units; $P < 0.01$ (Figure 1). The lower A_{2A}R expression was found in patients with inducible MI.

Significant correlation ($P < 0.001$) was observed between A_{2A}R expression measured on PBMCs and in patients' artery tissues (Figure 2). A multivariate analysis was performed to address the correlation between A_{2A}R present in artery and A_{2A}R in PBMC. Only BMI was associated with A_{2A}R ($p = 0.047$). We then performed a multivariable analysis including these 2 variables and the association between artery A_{2A}R and PBMC A_{2A}R remained significant ($p = 0.009$).

When we added to the model the cardiovascular risk factors and the coronary artery disease status, we observed an independent association between artery A_{2A}R and PBMC A_{2A}R ($p = 0.014$). Receiver operating characteristics (ROC) curve was performed to establish the best sensitivity and/or specificity of A_{2A}R expression (Figure 1, supplementary data). A cut-off value of 0.73 AU is associated with 73% sensitivity and 88% specificity. A cut-off value of 0.82 or 0.69 is associated with a 100% sensitivity or specificity, respectively.

A_{2A}R function

Basal cAMP production levels were addressed in PBMC samples, and were found to be lower in patients versus controls (172 [90–310] vs 244 [110–380] pg/10⁶ cells; $P < 0.05$). PBMCs from patients and controls were submitted to a saturating dose (1 μM) of Adonis, and the resulting maximal cAMP production was lower in patients than in controls: 375 [160–659] vs 670 [410–980] pg/10⁶; $P < 0.05$ (Figure 3A). A_{2A}Rs in arteries remain functional as incubation of artery tissues in the presence of Adonis leads to a significant increase in cAMP, close to that measured on PBMCs in term of percentage increase (Figure 3B).

K_D and EC₅₀

While no significant difference was found in the affinity of Adonis for A_{2A}Rs expressed on PBMCs between patients and controls (Figure 4A), EC₅₀ was significantly lower in patients (Figure 4B) compared with controls. However, this difference was mostly the result of the low EC₅₀ values found in the nine patients with inducible MI-documented CAD. For the same reason, the K_D/EC₅₀ ratio was higher in this patient group (Figure 4C). Figure 5 shows the affinity and cAMP production curves in patients with LE-PAD, with or without inducible MI.

Discussion

The main results of this study are: (1) A_{2A}R expression evaluated on PBMCs correlates with A_{2A}R expression evaluated on artery tissues in patients with LE-PAD; (2) A_{2A}R expression on PBMCs was lower in patients than in controls; and (3) patients with CAD with inducible MI had the lowest A_{2A}R expression, associated with the highest K_D/EC₅₀ ratio. Our study shows that the behavior of A_{2A}R expressed at the surface of PBMCs mirrors the behavior of A_{2A}R found in femoral tissues.

We found low expression of A_{2A}R in PBMCs from patients with LE-PAD, and confirmed the decreased expression of A_{2A}R previously observed in the coronary artery and aortic tissues of patients with CAD [15,19,20]. Low A_{2A}R expression is involved in reduced blood flow. Diagnosing symptomatic LE-PAD is difficult because symptoms of LE-PAD are protean, tools for detection (ankle-brachial index, questionnaires) lack sensitivity, and imaging is limited, by availability (duplex ultrasound) or the need to use intravenous contrast and/or ionizing radiation (computed tomography angiography, magnetic resonance angiography). A_{2A}R expression seems to be a simple screening test that can be used, in addition to clinical assessment, to identify patients with a less severe form of LE-PAD (obstructive LE-PAD with intermittent claudication), thereby avoiding unnecessary invasive and noninvasive tests.

The prevalence of significant obstructive CAD is high (>50%) in patients who have LE-PAD [21]. The presence of CAD in patients with LE-PAD may necessitate coronary revascularization, depending on the severity and urgency of LE-PAD symptoms [22]. Screening for CAD in patients with LE-PAD may be useful for risk stratification, as morbidity and mortality are mainly cardiac [22]. As myocardial infarction is a major cause of perioperative death in vascular surgery [21], substantial efforts, such as risk stratification and routine or selective coronary angiography/revascularization before vascular surgery [23], have been made to improve cardiovascular outcome during the operation. Nevertheless, clinical guidelines only recommend coronary angiography/revascularization before vascular surgery in some clinical situations [24], especially inducible MI. Here we found that only 9 of 24 patients had inducible MI; in these nine patients, EC₅₀ was lower than K_D, suggesting the presence of spare A_{2A}R [25]. Interestingly, in patients without CAD or without inducible MI, EC₅₀ was higher than K_D. The presence of spare receptors is suspected when maximal cAMP production occurs despite a weak fraction of receptors being occupied by the agonist (here Adonis). It is possible that spare A_{2A}R are multimerised and, in this activated state, can

induce maximal cAMP when crosslinked by only few molecules of Adonis. The presence of spare A_{2A} Rs has been shown to be associated with positive exercise stress testing [19] or reduced flow fraction reserve (<0.80) [20], suggesting that the presence of spare receptors is associated with MI. We found the same results in our patients with LE-PAD.

It is well established that the inflammatory process plays a major role in the pathogenesis of atherosclerosis [26,27]. Activation of A_{2A} R leads to anti-inflammatory effects [28,29] and, conversely, a decrease in A_{2A} R activation promotes inflammation, which in turn favors atherosclerosis. Thus, low levels of A_{2A} R expression and function may participate in the inflammatory process in patients with CAD. The increase in adenosine plasma concentration observed in patients with LE-PAD may be the consequence of adenosine release by endothelial and muscle cells, in order to attenuate hypoxic inflammation and provide metabolic tissue adaptation to ischemia [30-34].

Clinical implications: the biological criteria reported here cannot currently substitute LE-PAD investigations but in spite of the weak population size, our findings have two consequences in clinical practice: i) the A_{2A} R expression measurement, that only require a usual blood collection, may help to highlight in a large population patients with a lower extremity precarious arterial blood flow; ii) the K_D/EC_{50} ratio that detects the presence of spare receptors (19,20), may help to screen among LE-PAD patients those who need stress echocardiography, exercise stress testing or coronary angiography before surgery.

Study limitations: It should be interesting to include LE-PAD with acute ischemia and conversely those with less severe LE-PAD.

Conclusions

This work reflects the relative importance of the adenosinergic system in diagnosing cardiovascular processes, such as LE- PAD. We found that A_{2A} R expression in the femoral arteries of patients with LE-PAD correlated with A_{2A} R expression in PBMCs. The low level of expression of A_{2A} Rs on PBMCs may help to detect patients with LE-PAD, whereas A_{2A} R function (presence of spare A_{2A} Rs) may help to screen for associated CAD (inducible MI) in high-risk patients using a single blood sample test. These results serve as preliminary evidence that requires confirmation in larger and ad hoc cohorts.

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Disclosures

None

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Legends

Figure 1. (A) $A_{2A}R$ expression level evaluated on PBMCs from 24 patients with LE-PAD and 24 healthy subjects (controls). Among patients with LE-PAD, nine had associated CAD with documented inducible MI (see Methods). Expression was measured by western blot and expressed in arbitrary units (AU; see Methods). All patients with CAD with inducible myocardial ischemia (MI) are detailed in Table 2. (B) Examples of western blots in patients and controls. a: $P < 0.01$ compared with controls. b: $p < 0.01$ compared with patients without inducible MI; MW indicates molecular weight.

Figure 2. Correlation (Pearson's r) between $A_{2A}R$ expression evaluated on PBMCs and in femoral or iliac artery tissues. Expression was measured by western blot, and expressed in arbitrary units (see Methods).

Figure 3. Production of cAMP in (A) PBMCs and (B) artery tissues from 24 patients with LE-PAD and controls, evaluated in basal conditions and after incubation with Adonis, a monoclonal antibody against the $A_{2A}R$, with agonist properties. a: $P < 0.01$ compared with basal conditions. b: $P < 0.05$ compared with patients' basal conditions.

Figure 4. (A) Affinity (K_D) and (B) half-maximal cAMP production (EC_{50}) in PBMCs from 24 patients with LE-PAD and 24 healthy subjects (controls), evaluated using Adonis, an antibody with agonist properties for the $A_{2A}R$. (C) K_D/EC_{50} ratio. NS indicates not significant. Numbers refer to patients with associated coronary artery disease mentioned in table 2.

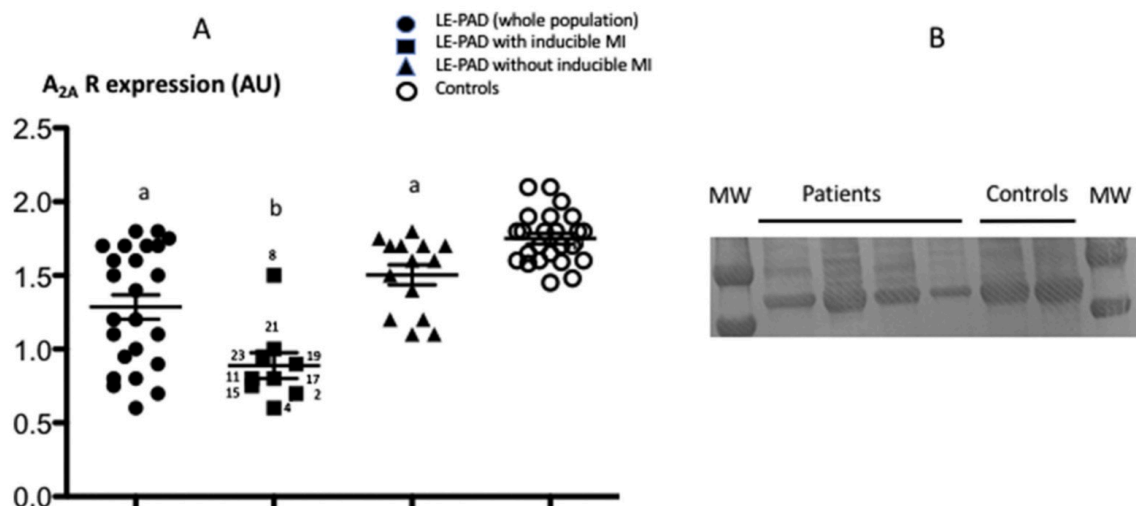


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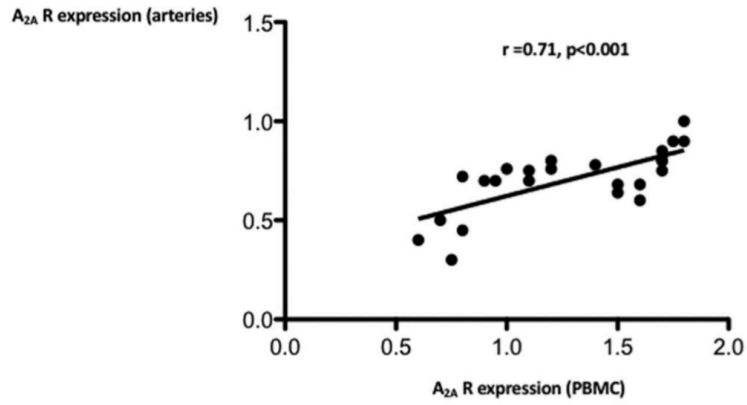


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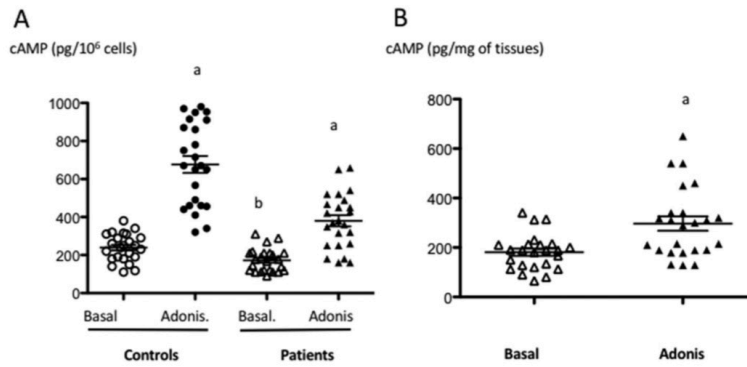


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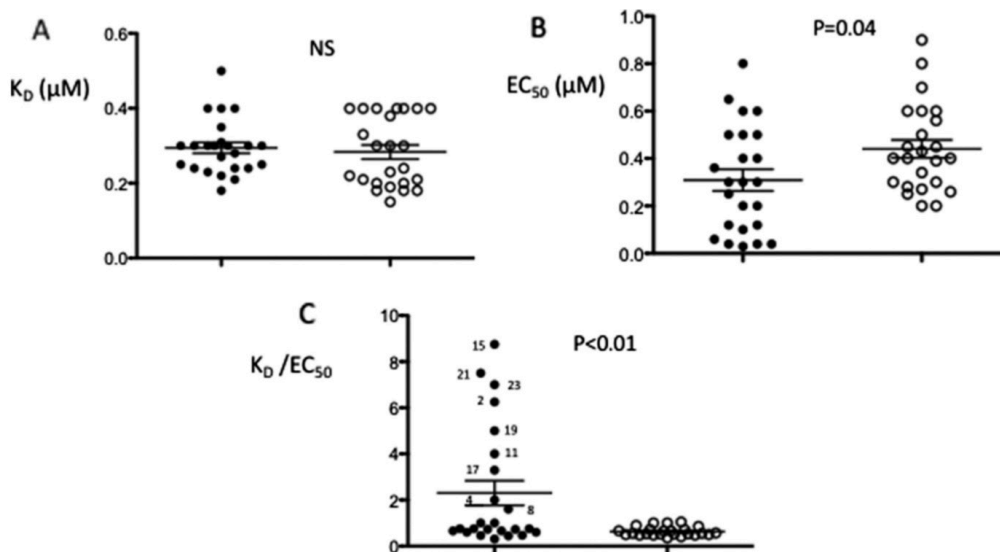


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Supplement file

Figure 5. Receiver operating characteristics curves for A₂ adenosine receptor expression evaluated on peripheral blood mononuclear cells in 24 LE-PAD patients vs 20 controls.

Figure 6. Affinity and cAMP production curves in peripheral blood mononuclear cells of patients with LE-PAD (n=24). The reducing conditions led to the dissociation of Adonis (a home-made antibody against the adenosine A_{2A} receptor, with agonist properties) into its heavy and light chains. Only the blotted kappa light chain (25 kDa) was visualized (see Methods). **(A)** Patients with LE-PAD (n=15) without inducible myocardial ischemia; and **(B)** patients with LE-PAD with associated coronary artery disease and inducible myocardial ischemia (n=9).

