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Evaluation of Plasma Adenosine as a Marker of Cardiovascular Risk: Analytical and Biological Considerations

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***Background*—**Adenosine is a ubiquitous regulatory molecule known to modulate signaling in many cells and processes vital to vascular homeostasis. While studies of adenosine receptors have dominated research in the field, quantification of adenosine systemically and locally remains limited owing largely to technical restrictions. Given the potential clinical implications of adenosine biology, there is a need for adequately powered studies examining the role of plasma adenosine in vascular health. We sought to describe the analytical and biological factors that affect quantification of adenosine in humans in a large, real-world cohort of patients undergoing evaluation for coronary artery disease.

***Methods and Results*—**Between November 2016 and April 2018, we assessed 1141 patients undergoing angiography for evaluation of coronary artery disease. High-performance liquid chromatography was used for quantification of plasma adenosine concentration, yielding an analytical coefficient of variance (CV_a) of 3.2%, intra-subject variance (CV_i) 35.8% and inter-subject variance (CV_g) 56.7%. Traditional cardiovascular risk factors, medications, and clinical presentation had no significant impact on adenosine levels. Conversely, increasing age (P=0.027) and the presence of obstructive coronary artery disease (P=0.026) were associated with lower adenosine levels. Adjusted multivariable analysis supported only age being inversely associated with adenosine levels (P=0.039).

***Conclusions*—**Plasma adenosine is not significantly impacted by traditional cardiovascular risk factors; however, advancing age and presence of obstructive coronary artery disease may be associated with lower adenosine levels. The degree of intra- and inter-subject variance of adenosine has important implications for biomarker use as a prognosticator of cardiovascular outcomes and as an end point in clinical studies. **(*J Am Heart Assoc*. 2019;8:e012228. DOI: 10.1161/JAHA.119.012228.)**

Key Words: adenosine • biomarker • coronary artery disease • high-performance liquid chromatography • plasma

Adenosine is a purine nucleoside that serves as a crucial intracellular and extracellular regulatory molecule regulating numerous blood and vascular cell types.[1,2] The metabolism of adenosine is regulated by a close balance of production, transport (primarily via equilibrative nucleoside transporters—ENTs) and degradation (primarily via adenosine deaminase—ADA).[3–5] Adenosine circulating in the extracellular space signals mainly via P1 purinergic receptors, G-protein-coupled receptors with differential responses to adenosine depending on which of the 4-subtypes of adenosine receptors (ADOR) are stimulated—ADORA1, ADORA2A, ADORA2B, and ADORA3.[6] Numerous preclinical studies have suggested adenosine regulates vascular homeostasis, with regulatory implications for inflammatory cells, smooth muscle cells, endothelial cells and platelets.[7–12] However, in humans little is known about variance of plasma adenosine concentration (PAC) or factors which influence PAC owing to technical challenges in quantifying levels in large cohorts of patients.

Therapeutically, adenosine's clinical applications have been relatively focused. Intravenous adenosine boluses are predominantly used as a diagnostic and therapeutic agent in

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Clinical Perspective

What Is New?

- Our robust assessment in a large, real-world cohort of patients undergoing evaluation for coronary artery disease demonstrated considerable biologic variability in circulating adenosine levels.
- Advancing age may be associated with reduced circulating adenosine levels, while cardiovascular risk factors and medications did not significantly impact levels.

What Are the Clinical Implications?

- The biologic variability and clinical factors influencing circulating adenosine levels should be considered when using adenosine as an end point in clinical studies or as a predictor of cardiovascular outcomes.

the management of tachyarrhythmias. Secondarily, adenosine and agents that augment PAC have been used for induction of coronary hyperemia for flow-related non-invasive and invasive assessment of myocardial perfusion.[13] Dipyridamole, acting primarily via ENT inhibition to augment adenosine levels, is more broadly employed for its flow-mediated effects and less commonly as an anti-platelet agent.[14] To minimize off target effects, small molecule agents have been developed to target specific adenosine receptors, such as regadenoson (an ADORA2A specific agonist) for maximizing coronary vasodilation.[15] Nonetheless, despite promising preclinical studies, a translational gap exists whereby the therapeutic application of adenosine modulation has been hampered by complex receptor biology and a limited understanding of adenosine levels in human disease pathogenesis.

Clinically, the measurement of circulating adenosine has seen limited use—though some studies have either reported prognostic significance in small cohorts or used PAC as an end point in clinical trials.[16,17] Quantification of PAC by high-performance liquid chromatography (HPLC) is an established methodology with reported analytical variability (CV_a) ranging from 6% to 7%[18] and up to 10%[19] previously, with more contemporary assays yielding CVs of 1% to 3%, in keeping with clinical assay standards.[20] With these protocols, some small studies (n=10) have demonstrated reduced local circulating adenosine levels via coronary sampling immediately following balloon angioplasty for coronary artery disease (CAD).[21] Others report elevated PAC in patients (n=71) with chronic congestive heart failure (CHF), proposing it provides protective effects from rising norepinephrine levels.[22] Interestingly, genetic studies in patients with adenosine monophosphate deaminase locus 1 (AMPD1) mutations (putatively augmenting adenosine levels) demonstrate improved survival in CHF patients.[23,24] AMPD1 carriers have also demonstrated improved cardiovascular survival in those with angiographically documented CAD,[25] though this did not hold true for patients post-revascularization.[26] While all of these studies invoke an adenosine-mediated mechanism, definitive links between atherosclerotic risk factors, disease burden and adenosine levels have yet to be established.[2]

Given the prognostic and therapeutic implications of adenosine levels and the lack of robust human data, we set out to systematically evaluate the analytic characteristics of PAC quantification and determine if traditional cardiac risk factors, cardiac therapies, and/or disease burden are associated with PAC.

Methods

Adenosine Sample Collection and Processing

Blood samples were collected at the time of angiography via a 6-French plastic arterial access sheath (Terumo Medical, Somerset, NJ) placed in the radial artery. Rarely, if this was not possible, then venous samples were collected via peripheral venipuncture. Blood samples (6 mL) were collected in Greiner BioOne Vacuette tubes pre-injected with 2 mL of ice-cold stop solution. Stop solution was composed of 100 µmol/L dipyridamole, 2.5 µmol/L erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), 1 U/mL heparin in 0.9% saline. Tubes were inverted and connected to the access sheath to ensure rapid and direct mixing of blood with stop solution and were maintained on ice before and following draws until processing. Samples were centrifuged at 4°C, 1200*g* for 10 minutes without brakes to limit platelet activation and the supernatant was collected and stored at −80°C until processing. Hemolyzed samples (which result in markedly elevated PAC levels) were excluded on a biological basis in keeping with prior studies.[18] The data that support the findings of this study are available from the corresponding author upon reasonable request.

Aliquots were then thawed at room temperature and centrifuged at 1000*g* for 3 minutes and 500 µL of sample was diluted in 500 µL of 4% phosphoric acid. This 1 mL combined solution was then loaded onto a Waters Oasis MCX (Mixed-mode, strong Cation-eXchange) 1 mL cartridge and the sample was eluted with vacuum assistance through the column as per protocol. The sample was then washed with 1 mL of 2% formic acid followed by 500 µL of 100% methanol with vacuum assistance between each wash. The final sample was then eluted using 2 sequential 125 µL elutions with MCX eluting solution (5% NH_4OH in 60/40 acetonitrile/methanol) followed by vacuum assistance to ensure all samples were collected from the vial. Samples were then transferred to vials to undergo HPLC analysis. Adenosine standards were

prepared using pharmaceutical grade adenosine (Sigma PHR11380-1G) diluted in MCX eluting solution.

Samples were analyzed using HPLC on a Waters Alliance E2695 separating module system with sample quantification by Waters 2489 UV/visible detector at 260 nm. The mobile phase was composed of a mixture of Mobile Phase A (10 μmol/L ammonium formate pH3 in 50% acetonitrile:50% water, ranging 1–50%) and Mobile Phase B (10 μmol/L Ammonium Formate pH3 in 95% Acetonitrile:5% Water, ranging 50–99%) with a sample temperature of 4°C and column temperature of 24°C. Samples were then processed through a Waters Xbridge BEH amide SP Vanguard Cartridge pre-column and subsequent Waters Xbridge BEH Amide 2.5 μm, 4.6×150 mm column XP. Data processing was completed using Waters Empower 3 Software.

Assay Validation for Plasma Adenosine Quantification

Quantification of variance is achieved via relative standard deviation (RSD) and the coefficient of variation (CV). Both RSD and CV are percentages representing the standard deviation (SD) divided by the mean value to standardize the variability for a given result. RSD is an absolute value, while CV is not. We report CV in keeping with prior studies.[27] The CV is assessed at multiple stages of our assay and defined accordingly (1) CV_a, CV analytical, the variation of the HPLC assay itself including the processing and analysis of samples (generated via multiple aliquots obtained from a single tube drawn from a single patient), (2) CV_i, CV individual, the intra-subject variation over time generated from serial samples from the same patient collected on different days via $CV_i = (CV_t^2 - CV_a^2)^{1/2}$ and (3) CV_g, CV group, the inter-subject variation within the population of subjects studied (generated from different samples collected from different patients at different times).[28,29] The reference change value (RCV) was calculated via 2.77 $(CV_a^2 + CV_i^2)^{1/2}$, while the index of individuality (II) was calculated by $(CV_a^2 + CV_i^2)^{1/2}/CV_g$, in keeping with prior reports.[28–31] The validation of our HPLC methodology followed good practice guidelines as published previously.[32] Specificity of the assay was maximized by adjusting the gradients and temperatures until adequate separation of the adenosine peak of interest was achieved from the surrounding peaks. The specific identity of the adenosine peak was confirmed by focused degradation of adenosine by ADA followed by quantification to demonstrate loss of the adenosine peak (Figure—Panel A). Repeatability was assessed in both the standards and samples to determine the intra-day assay precision using the same conditions. Standards in eluting solution were injected 10 sequential times from the same vial, while samples prepared with the MCX system were injected 6 sequential times from the same vial. Both the retention times and peak areas were recorded for each run and a mean, SD, and CV reported (Table S1). Linearity and range were assessed by creating 3 individual sample preparations of standards ranging in concentration from 100 to 15 000 nmol/L representing 10% to 1500% of the target concentration of adenosine (1000 nmol/L) (Figure—Panel B, Table S2). These individual samples were run on the same machine on the same day to generate the appropriate curves from which the retention time and peak areas were recorded across each individual preparation with means, standard deviation (SD) and CV then calculated (Table S2). The adenosine standard curve (Figure—Panel B) was then assessed for linearity over a range from 100 to 15 000 nmol/L adenosine concentrations in both elution buffer and matrix (adenosine-depleted plasma generated via ADA degradation of endogenous adenosine).

Ongoing data validation during the sample collection phase was ensured by repetition of a standardized protocol including blank injection before, in the middle of, and following sample injections. Similarly, a blank phosphate-buffered saline sample is processed through the MCX column and quantified. Standards are run with each grouping of samples and the curves are monitored for stability including slope, intercept and R^2. The stability of samples during the HPLC analysis period is ensured by performing 3 injections of a given sample at the start, middle and end of each run to ensure consistent results. We also use 1 sample with which we perform (1) ADA degradation, (2) ADA degradation followed by adenosine spiking post MCX column, and (3) adenosine spiking pre- and post-MCX columns. This process is then repeated in phosphate-buffered saline with both an adenosine spike and an adenosine spike followed by ADA degradation. In this way, constant monitoring of the quality and reliability of results generated is ensured over time.[32]

Biological Sample and Clinical Data Collection

The University of Ottawa Heart Institute is a high volume, tertiary care center providing coronary revascularization services to >1.2 million people.[33] From November 2016 to April 2018, 7252 patients were prospectively enrolled in the CAPITAL (Cardiovascular And Percutaneous Clinical Trials) revascularization registry which indexes clinical data points on patients undergoing coronary angiography and revascularization. In the CAPITAL revascularization registry, coronary artery disease (CAD) was defined as obstructive stenosis ≥50% at the time of angiography in keeping with current clinical standards.[34] Acute coronary syndrome (ACS) was composed of troponin-positive presentations including both non–ST-segment–elevation myocardial infarction and

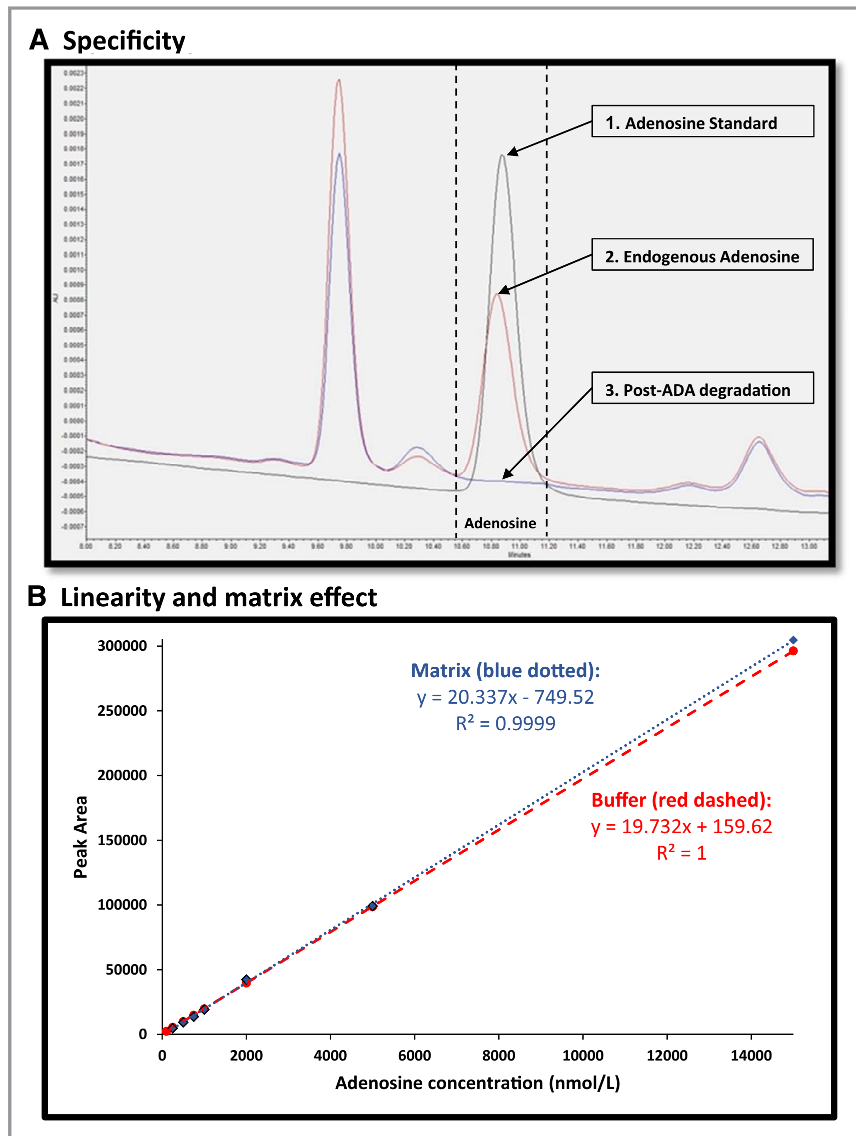

Figure. Validation of adenosine high-performance liquid chromatography methodology. **A**, Superimposed chromatograms of 3 separate samples demonstrating a distinct adenosine peak free of interfering peaks. Multiple superimposed peaks including (1) adenosine standard in elution buffer, (2) endogenous adenosine in plasma (matrix) sample, and (3) endogenous plasma sample following degradation of adenosine with adenosine deaminase resulting in no detectable adenosine peak confirming peak specificity. **B**, Curve generated by plotting peak areas by adenosine concentration for both standards in buffer (dashed red line) and standards in plasma sample matrix (dotted blue line) demonstrating excellent linearity and minimal matrix effect. ADA indicates adenosine deaminase.

ST-segment–elevation myocardial infarction cases. Diabetes mellitus (DM) was based on either a hemoglobin A1c (HbA1c) ≥6.5% on presentation or a prior DM diagnosis or presence of medical therapy for DM. Tobacco use was dichotomized into smokers (active smoking at the time of sample collection) or non-smokers (not smoking at the time of sample collection). Positive family history was defined as CAD in a first-degree relative aged <55 years for men and <65 years for women. Dyslipidemia and hypertension were defined as either a prior diagnosis of either condition or the presence of the appropriate medical therapy for either diagnosis on presentation. This study received approval from the University of Ottawa Heart Institute ethics review board (Protocols #20180562-01H, #20160516-01H and #20170126-01H) and informed consent was completed. Of this cohort of patients undergoing evaluation for coronary artery disease, 1174 patients had blood samples collected for analysis.

Statistical Methods

Data are reported as mean±SD, median±interquartile range or number and percentage (%) where appropriate. Statistical testing was completed using GraphPad Prism 7.04, SigmaStat and SAS v9.4. Biological data were assessed for normality using D'Agostino and Pearson or Shapiro-Wilk normality tests. Following log transformation of the data set, no statistical outliers were identified. Comparisons of 2 groups of non-parametric data were performed using Mann–Whitney test. All analyses defined significance as a 2-tailed $P<0.05$, unless otherwise specified. Log-transformation of all data was completed before regression analysis. Linear regression was performed for age with demonstrated 95% confidence intervals. Univariable linear regression was similarly performed for all factors with a predetermined $P<0.2$ used to identify factors for inclusion in subsequent multivariable linear regression analysis with significance defined as $P<0.05$ in keeping with prior studies.[35]

Results

HPLC methodology validation and analytical variability

Robust assay specificity was demonstrated generating a discernible adenosine peak free of interference from surrounding peaks. Focused degradation of adenosine by ADA demonstrated complete abrogation of the adenosine peak (Figure—Panel A). Repeatability of the HPLC assay itself was assessed by sequential injections from the same preparation of (1) standard (10 sequential injections on the same day of 1000 nmol/L adenosine, representing 100% target concentration) and (2) sample (6 sequential injections on the same day of serum from a single subject). This approach demonstrated a CV for retention time, peak area, and peak height of 0.12%, 2.19%, and 0.65% for standards and 0.16%, 1.52%, and 1.96% for samples, respectively (Table S1). Our assessment of linearity and range included reporting the retention time and peak area for all adenosine values ranging from 100 to 15 000 nmol/L (10–1500% of target)—producing CV ranging from 0.02% to 0.22% for retention time, while the CV for peak areas ranged from 0.78% to 7.03% at 250 nmol/L (Table S2). The lowest quantified value, 100 nmol/L of adenosine, demonstrated a variance of 12.87% identifying a lower limit for reliable quantification by this assay. Plotting the peak areas generated as a function of the adenosine concentrations generates a line of best fit with equation $y=19.732x+159.62$, $R^2=1$ (Figure—Panel B, dashed line). The impact of matrix (plasma) was assessed with the same range of standard concentrations and compared with the curve generated with standards in elution buffer on the same plot (Figure—Panel B, dotted line), resulting in a trendline of $y=20.337x-749.52$, $R^2=0.9999$.

Intra and inter-subject variability

The variability of our assay and methodology was assessed at each level of quantification in an unselected cohort of patients undergoing assessment for coronary artery disease (Table 1). First, intra-tube variability was assessed using multiple aliquots from the same blood tube drawn from 1 subject demonstrating a CV of 3.2%—establishing the analytical CV (CV_a) for our assay. Next, intra-subject variability was assessed, first for inter-tube variability using separate draws at the same time point yielding a CV of 23.0%. Intra-subject variation was then assessed within the same day and on separate days. Adenosine levels drawn throughout a single day incrementally increased the CV to 30.1%, while serial collections on the same patient across multiple days (mean 35.8±33.1 days) further increased the CV to 35.8%—establishing the CV_i for our assay. Lastly, inter-subject variation (CV_g) was assessed using all adenosine levels in the entire cohort, noting a CV_g of 56.7% (Table S3). Overall, this resulted in a RCV of 98.9% and an II of 0.63.

Patient and procedural characteristics

From an initially recruited 1174 patients, we excluded duplicate samples of the same patients (33 in total) leaving 1141 patients included in the final analysis. The cohort's baseline demographics are summarized in Table 2. The

Table 1. Intra- and Inter-Subject Variation

	No. of Subjects	No. of Samples	Adenosine (nmol/L)	SD (nmol/L)	CV (%)
Intra-subject					
Intra-tube, same time (CV_a)	18	92	1042.0	33.5	3.2
Inter-tube, same time	29	95	1240.0	256.1	23.0
Different time, same day	17	39	1090.8	349.3	30.1
Different time, different day (CV_i)	31	64	1216.6	457.3	35.8
Inter-subject					
Different time, different day (CV_g)	1141	···	1067.2	605.3	56.7

CV indicates coefficient of variation; CV_a, analytical CV; CV_g, inter-subject CV; CV_i, intra-subject CV; SD, standard deviation.

average age was 66.3±11.8 years (Figure S1A) with 70.8% being male. The cohort underwent angiography for indications that included ACS (39.5%) and stable CAD (39.9%). Risk factors included 30.4% with diabetes mellitus, 18.5% active smokers, 61.3% with dyslipidemia, 16.5% with positive family history, and 64.7% with hypertension. Coronary artery disease was known before angiography in 38.1% patients, with 24.7% reporting a prior myocardial infarction and 34.6% having had prior revascularization with either percutaneous coronary intervention or coronary artery bypass grafting. Medical therapy in the cohort included 53.5% on angiotensin-converting enzyme inhibitors/ARB (Angiotensin II receptor blocker), 59.4% on beta blockers, 79.7% on statins, 88.4% on aspirin and 87.7% on P2Y12 inhibitors. A total of 25 recruited patients did not undergo angiography, but had samples collected via venous access, leaving 1116 patients who underwent angiography for which procedural details were indexed (Table 3). After excluding those with prior revascularization, CAD, or myocardial infarction, 633 cases remained that underwent angiography, of which 431 cases remained that had de novo obstructive CAD at the time of sample collection—ranging from 1 vessel (40.1%), 2 vessel (28.5%) to 3 vessel (31.3%) disease. Of the entire cohort, 23.5% underwent percutaneous coronary intervention with placement of 1 stent (46.2%), 2 stents (32.4%) or ≥3 stents (21.4%).

Impact of Cardiovascular Risk Factors on PAC

Established cardiovascular risk factors were assessed for impact on circulating adenosine levels (Table 4). Smokers did not show a statistical difference in adenosine levels compared with non-smokers (917 [607–1325] nmol/L versus 932 [635–1357] nmol/L, P=0.858). As well, there was no statistical difference in adenosine levels between those that did and did not have a history of dyslipidemia (909 [626–1350] versus 953 [645–1390] nmol/L, P=0.292), hypertension (936 [634–1363] versus 917 [621–1353] nmol/L, P=0.701), or family history of CAD (953 [609–1376] versus 926 [635–1350] nmol/L, P=0.896). Sex did not impact adenosine levels with males (925 [630–1345] nmol/L) (demonstrating similar levels to females (949 [645–1398] nmol/L, P=0.293). Diabetes mellitus as a dichotomized variable did not significantly impact adenosine levels with diabetics and non-diabetics (974 [604–1438] versus 913 [639–1313] nmol/L, P=0.238). Moreover, in the 294 diabetic patients with HbA1c values available, there was no significant relationship between HbA1c and adenosine levels (r=0.03, R^2=0.001, P=0.59, Figure S2A). The impact of age on adenosine levels was also assessed (Figure S2B) showing a statistically significant inverse association between age and PAC (R^2=0.005, r=−0.07, P=0.02). Next, we performed additional analysis following division of the cohort into those aged ≤65 (n=533)

Table 2. Baseline Demographics

	Number or Mean	Proportion (%) or Standard Deviation
Number of patients	1141	...
Age	66.3	11.8
Male	806	70.8
Indication for angiography		
Acute coronary syndrome	451	39.5
STEMI	24	2.1
NSTEMI	292	25.6
Unstable angina	135	11.8
Stable coronary artery disease	455	39.9
Staged PCI	124	10.9
Shock	2	0.2
Arrhythmia	19	1.7
Heart failure/LV dysfunction	90	7.9
Past medical history		
Diabetes mellitus	347	30.4
Type I	7	2.0
Type II—diet controlled	13	3.7
Type II—non-insulin therapy	233	67.1
Type II—insulin therapy	94	27.1
Smoking	211	18.5
Dyslipidemia	699	61.3
Family history	188	16.5
Hypertension	738	64.7
Prior cerebrovascular accident	80	7.0
Peripheral arterial disease	84	7.4
Atrial fibrillation	119	10.4
Prior coronary artery disease	435	38.1
Prior myocardial infarction	282	24.7
Prior angiogram	462	40.5
Prior PCI	317	27.8
Prior coronary artery bypass grafting	78	6.8
Medications		
ACE inhibitor/ARB	610	53.5
Beta blocker	678	59.4
Calcium channel blocker	158	13.8
Statin	909	79.7
Oral anticoagulation	61	5.3
Intravenous unfractionated heparin	132	11.6
Subcutaneous LMWH	123	10.8
Acetylsalicylic acid	1009	88.4

Continued

Table 2. Continued

	Number or Mean	Proportion (%) or Standard Deviation
P2Y12	1001	87.7
Clopidogrel	685	68.4
Ticagrelor	315	31.5
Prasugrel	1	0.1

ACE indicates angiotensin-converting enzyme; ARB, angiotensin receptor blocker; LMWH, low-molecular weight heparin; LV, left ventricular; PCI,percutaneous coronary intervention; SD, standard deviation; STEMI, ST-segment–elevation myocardial infarction; NSTEMI, Non–ST-segment–elevation myocardial infarction.

and those aged >65 (n=608) years, demonstrating reduced adenosine in the >65-year cohort (895 [610–1315] nmol/L) than the ≤65-year cohort (971 [649–1397] nmol/L, P=0.027).

Impact of Medical Therapy on Plasma Adenosine Levels

Medical therapy for cardiovascular risk reduction was assessed for impact on PAC (Table 4). No difference in adenosine levels was noted comparing patients taking to those not taking angiotensin-converting enzyme inhibitors/ARBs (943 [645–1353] nmol/L versus 909 [620–1363] nmol/L, P=0.419) beta blockers (958 [644–1356] nmol/L versus 887 [615–1357] nmol/L, P=0.317) calcium channel blockers (974 [618–1398] nmol/L versus 919 [634–1346] nmol/L, P=0.433) and statins (919 [618–1341] nmol/L versus 937 [690–1419] nmol/L, P=0.141). Similarly, anticoagulants used preceding angiography did not impact adenosine levels with unfractionated heparin (985 [619–1397] versus 925 [634–1350] nmol/L, P=0.549) or subcutaneous low-molecular weight heparin (907 [654–1400] versus 932 [630–1350] nmol/L, P=0.568). Finally, we evaluated the impact of antiplatelet therapy on PAC as previous data suggesting ticagrelor may impact adenosine metabolism (Table 4).[16,17] No difference in adenosine was seen with acetylsalicylic acid (ASA) (919 [626–1361] versus 958 [683–1285] nmol/L, P=0.65). Similarly, in the class of P2Y12 inhibitors, clopidogrel did not affect adenosine levels (953 [637–1400] versus 904 [609–1289] nmol/L, P=0.158). Interestingly, ticagrelor therapy was associated with a reduction in adenosine levels compared with those not on ticagrelor (875 [595–1254] versus 955 [650–1408] nmol/L, P=0.012).

Impact of Coronary Artery Disease on Plasma Adenosine Levels

Finally, we assessed the impact of CAD presence and burden on plasma adenosine levels (Table 4). In the total cohort, absence of obstructive CAD was associated with higher adenosine levels than patients with obstructive CAD (909 [618–1325] versus 995 [686–1460] nmol/L, P=0.026). No differences between patients presenting as ACS (non–ST-segment–elevation myocardial infarction /ST-segment–elevation myocardial infarction) versus non-ACS and were observed (n=311, 932 [637–1346] nmol/L versus n=830, 928 [630–1363] nmol/L, P=0.971). Disease burden, as assessed by presence of de novo multivessel (>1 vessel) disease, failed to show any association with PAC levels compared with those with single-vessel disease (902 [616–1306] versus 926 [604–1356] nmol/L, P=0.676).

Table 3. Procedural Details

	Number or Mean	Proportion (%) or Standard Deviation
Number of patients undergoing angiography	1116	...
Access		
Radial	1015	90.9
Femoral	97	8.7
Brachial	4	0.4
Access site medications		
Calcium channel blocker	585	52.4
Nitroglycerin	420	37.6
Procedural medications		
Heparin	966	88.1
Mean dose (U)	6678	2378
Bivalirudin	53	4.7
Glycoprotein IIb/IIIa inhibitors	2	0.2
Adenosine	68	6.1
Intravenous	30	2.7
Intracoronary	36	3.2
Nitroglycerin	457	40.9
Number of cases with de novo obstructive (≥50%) CAD	431	38.6
Lesion-burden		
1 lesion	121	28.1
2 lesions	97	22.5
3 lesions	68	15.8
4 lesions	59	13.7
≥5 lesions	88	20.4
Vessel-burden		
1 vessel	173	40.1
2 vessel	123	28.5
3 vessel	135	31.3
Number of cases with a stent deployed	262	23.5
1 stent	121	46.2
2 stents	85	32.4
≥3 stents	56	21.4

Table 4. Impact of Risk Factors, Medications, and Coronary Artery Disease on Adenosine

	Present		Absent		
	n	Median (IQR) (nmol/L)	n	Median (IQR) (nmol/L)	*P* Value
Cardiovascular risk factors					
Age >65 y	608	895 (610–1315)	533	971 (649–1397)	0.027*
Diabetes mellitus	347	974 (604–1438)	793	913 (639–1313)	0.238
Smoking	211	917 (607–1325)	930	932 (635–1357)	0.858
Dyslipidemia	699	909 (626–1350)	442	953 (645–1390)	0.292
Family history	188	953 (609–1376)	953	926 (635–1350)	0.896
Hypertension	738	936 (634–1363)	403	917 (621–1353	0.701
Male	806	925 (630–1345)	335	949 (645–1398)	0.293
Medications					
Acetylsalicylic acid	1023	919 (626–1361)	117	958 (683–1285)	0.650
Clopidogrel	685	953 (637–1400)	456	904 (609–1289)	0.158
Ticagrelor	315	875 (595–1254)	826	955 (650–1408)	0.012*
ACE inhibitor/ARB	610	943 (645–1353)	531	909 (620–1363)	0.419
Beta blocker	678	958 (644–1356)	463	887 (615–1357)	0.317
Calcium channel blocker	158	974 (618–1398)	983	919 (634–1346)	0.433
Statin	909	919 (618–1341)	232	937 (690–1419)	0.141
Unfractionated heparin	132	985 (619–1397)	1009	925 (634–1350)	0.549
Low-molecular weight heparin	123	907 (654–1400)	1018	932 (630–1350)	0.568
Coronary artery disease					
CAD	941	909 (618–1325)	200	995 (686–1460)	0.026*
De novo CAD burden >1 vessel	548	902 (616–1306)	315	926 (604–1356)	0.676
Acute coronary syndrome	311	932 (637–1346)	830	928 (630–1363)	0.971

ACE indicates angiotensin-converting enzyme; ARB, Angiotensin II receptor blocker; CAD, coronary artery disease; IQR, interquartile range.
*$P<0.05$.

Multivariable Linear Regression Analysis

To assess the association of variables with PAC, we first performed a log-transformation of adenosine values (Figure S1B and S1C) followed by a univariable linear regression to identify potential associated variables (Table S4). Individual variables associated with a $P<0.2$ were identified including total number of vessels (P=0.104), age (P=0.009), hemoglobin A1c (P=0.062), sex (P=0.193), statin (P=0.176), P2Y12 (clopidogrel and ticagrelor) (P=0.195). After multivariable analysis (Table S5), only age (P=0.039) remained inversely associated with PAC.

Discussion

Despite abundant preclinical research linking adenosine to vascular disease, the current study is the first to evaluate the relationship of plasma adenosine levels with known cardiovascular risk factors, medical therapy and disease presence in humans. Herein, we report the performance of a high-throughput protocol for rapid HPLC-based adenosine quantification with performance parameters in congruence with good practice guidelines.[32] In the current cohort, our assay produces intra-subject and inter-subject variability consistent with other biomarkers of cardiovascular disease. Notably, in the current data set traditional cardiovascular risk factors and medical therapies were not associated with significant changes in plasma adenosine levels. In contrast, age and CAD presence were inversely associated with plasma adenosine levels—a finding for which age alone remained statistically significant after multivariable adjustment.

In our study of >1100 patients, traditional cardiovascular risk factors including hypertension, diabetes mellitus, family history, smoking, dyslipidemia, and sex did not associate with adenosine levels, while age was inversely correlated. Age is known to impact other established markers of cardiovascular disease. For example, low-density lipoprotein is known to diminish with advancing age at a rate of only 0.8% annually, though this still translates to important clinical implications.[36]

Similarly, NT-proBNP (N-terminal pro-brain natriuretic peptide), an established marker for diagnosis, monitoring and outcomes in heart failure, is known to have a biological variance closely mirroring that of adenosine.[37,38] Moreover, it is also impacted by age, necessitating age-specific reference intervals and having diminished predictive abilities at more advanced ages.[39,40] Hence, while the annual incremental impact of advancing age on adenosine may be small, the cumulative impact of age over time remains an important consideration when establishing adenosine's performance as a diagnostic, prognostic and monitoring clinical test. Smokers have lower adenosine levels in their sputum, with increased adenosine levels and ADORA3 and 1 noted post cessation.[41] However, there has been no definitive link between smoking and circulating adenosine levels in keeping with our data. Similarly, extensive literature links diabetes mellitus to adenosine levels—however, these associations typically focus on augmented ADA levels, postulating that this leads to reduced circulating adenosine.[42] Our data do not demonstrate any overt differences in PAC between those with and without diabetes mellitus, while not evaluating an impact on receptor activity *nor* in specific vascular beds. Nonetheless, our study provides adequate power across subgroups to evaluate the impact of risk factors in humans and suggests that age may incrementally contribute to a decline in PAC—a finding which confounds smaller observational studies.

The use of medications for cardiovascular risk reduction did not demonstrate any significant associations with adenosine levels, with none of the angiotensin-converting enzyme inhibitors/ARBs, beta blockers, calcium channel blockers, statins, or heparins demonstrating any significant differences. In contrast, antiplatelet medications have been studied extensively for their putative impact on PAC. Specifically, ticagrelor has garnered significant attention with postulations that observed pleiotropic effects may stem from modulation of adenosine biology. In one study, 60 ACS patients were randomized to ticagrelor or clopidogrel with ticagrelor increasing plasma adenosine levels compared with those receiving clopidogrel, ostensibly via inhibition of red blood cell uptake.[17] However, a recent randomized crossover study in 54 ACS patients compared ticagrelor, prasugrel and clopidogrel—failed to demonstrate any significant augmentation in adenosine levels with ticagrelor compared with clopidogrel or prasugrel.[16] In our all-comers cohort with over 300 patients on ticagrelor therapy, reduced PAC was noted in those on ticagrelor compared with those not receiving ticagrelor. However, any non-randomized data set is innately confounded by the fact that ticagrelor is differentially employed in clinical practice, with ACS patients preferentially receiving ticagrelor given its superior clinical outcomes in ACS patients.[43] Indeed, this was observed in our data set with 68.8% of patients on ticagrelor presenting as an ACS versus only 27.9% of those not on ticagrelor. The differential use of ticagrelor in our cohort leads to innate differences between the populations which limit further analysis. Hence, our study was not intended to specifically address the impact of P2Y12 agents on adenosine levels but adds to the growing debate of the impact of ticagrelor on circulating adenosine levels.

Preclinical research has suggested adenosine plays an important role in modulating the pathogenesis of atherosclerosis particularly with modulation of systemic inflammation.[44–46] Indeed, our data suggest an inverse association between obstructive CAD and PAC. However, subgroup analysis failed to show any significant differences in adenosine levels across a spectrum of disease burden (ie, multivessel disease) or presentation (ie, acute coronary syndrome). Animal studies have noted increased activity of vascular ADA (resulting in reduced circulating adenosine levels) as a mediator of atherosclerosis—proposing ADA inhibition (augmenting circulating adenosine) as a possible therapeutic approach.[47] Similarly, genetic studies in humans lend support to the hypothesis that adenosine is a vascular protective molecule.[23–25] In humans, patients with CAD and genetic variations that purportedly augment circulating adenosine levels have reduced adverse cardiovascular events.[25] Our data now lend credence to this hypothesis—establishing a potential relationship between the presence and absence of disease. Whether adenosine acts as a prognosticator of events needs to be established in larger cohorts.

In spite of intensive research in the field of adenosine biology, the systematic development and evaluation of adenosine as a potential biomarker has not been previously performed owing largely to the technical limitations of sampling and existing quantification methods.[29] The currently reported assay yields technical performance that meets and exceeds good practice guidelines.[32] With a CV_a 3.2%, CV_i 35.8%, CV_g 56.7%, a RCV of 98.9%, and an II of 0.63, our assay performed in line with many known markers of coronary artery disease—such as C-reactive protein (CRP).[48–50] Indeed, from an assay perspective, a CV_a of 3.2% is markedly improved over early assays reporting CV_a ranging from 6% to 7%[18] and up to 10%,[19] while falling closely in line with contemporary assays yielding CV_a's of 1% to 3%, keeping with clinical assay standards.[20] The balance of these variances is crucial to assessing the clinical utility of a test. A test with high index of individuality (>1.4) will perform well as a diagnostic test based on population-level reference intervals, while a low index (<0.6) will not, as significant changes for a given subject may still fall within a population-based reference range.[29,31] Comparatively, CRP has a CV_a 5.2%, CV_i 42.2%, and CV_g 92.5%.[28] Having a large CV_g coupled with a relatively smaller CV_i means that individuals could have early disease-related changes without rising above a given reference interval, requiring relatively large changes in value before confidence in

its significance is noted (Table S3).[27,28] Indeed, the RCV (smallest percentage change not likely because of CV_a or CV_i at significance of $P \leq 0.05$) is 118% for CRP and index of individuality was 0.46—a substantial change in value.[28] Comparatively, the RCV for adenosine in our assay is 98.9% with an II of 0.63, translating to similar considerations when determining its clinical utility and optimal interpretation. In fact, the variance of CRP leads to up to 46% of patients alternating between low- and high-risk categories despite a stable clinical status, translating to a 10% to 20% probability of making an erroneous risk assignment based on a single CRP value.[51] Despite this, CRP remains an established predictor of cardiovascular outcomes in those with[48] and without CAD[49] and predicts reduction of cardiovascular events in response to medical therapy[50]—supporting its role in current guidelines.[52,53] We demonstrate a similar variance profile to CRP for PAC in humans—meaning the variability in humans will require large sample sizes to adequately detect disease associations *or* to evaluate the impact of therapies on PAC.[54] Thus, clinical tests with this variance profile, such as adenosine, will have little utility in identifying early disease-related changes in the context of a healthy reference interval, favoring serial monitoring for significant changes in individual patients instead[28]—important implications for interpreting previous studies in humans and powering future evaluations of PAC as a marker or end point.

Certainly, our study is not without its limitations. The data are observational in nature and subject to all the limitations of this design. However, clinical and procedural data were prospectively collected in a nested registry design, limiting potential biases a solely retrospective approach may introduce. Second, the relatively large variability does open the possibility of regression dilution bias whereby significant differences may not be seen on account of inherent measurement errors.[55] Hence, despite being substantially larger than any previous human study, we are at risk of not detecting a modest association where one exists. Third, differences in absolute PAC values exist across varying collection methodologies reported. However, the uniform processing procedures and robust analytical variation of this study lends itself to unidirectional variance—whereby any potential errors would exist uniformly throughout the cohort and not impact the ability to detect biological differences present. Finally, our protocol was designed to evaluate adenosine levels assayed by peripheral collection - the primary method performed in humans. Hence, these values may not reflect levels in local tissues or specific vascular beds and thus does not preclude organ/tissue specific changes in adenosine. However, as demonstrated by our analysis, adequately powered studies to assess local adenosine levels may be difficult owing to technical factors and variability, with future studies requiring robust protocols and statistical methodology.

Conclusions

In humans, plasma adenosine levels are not significantly impacted by traditional cardiovascular risk factors or medical therapy for cardiovascular disease; however, advancing age and the presence of coronary artery disease may be associated with diminishing adenosine levels. Large prospective studies of basal levels and variation of adenosine for prediction of future cardiovascular events are warranted.

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Disclosures

None.

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Supplemental Material

Table S1. Repeatability assessment of adenosine assay.

	Injection	Retention Time	Peak Area	Peak Height
Standards	1	10.677	19635	1494
	2	10.665	19584	1460
	3	10.665	19210	1477
	4	10.656	19369	1473
	5	10.651	19194	1483
	6	10.65	18989	1477
	7	10.65	19029	1476
	8	10.643	18751	1469
	9	10.637	18403	1464
	10	10.638	18521	1471
	Mean	10.65	19068.50	1474.40
	SD	0.01	416.88	9.62
	CV (%)	0.12	2.19	0.65
	Injection	**Retention Time**	**Peak Area**	**Peak Height**
Samples	1	10.887	5906	384
	2	10.934	6088	374
	3	10.932	6024	391
	4	10.928	5861	384
	5	10.931	5906	389
	6	10.93	6036	396
	Mean	10.92	5970.17	386.33
	SD	0.02	90.85	7.55
	CV (%)	0.16	1.52	1.96

Generated via 10 sequential injections of 1000nM adenosine standard for standards assessment and 6 sequential injections of a single patient sample for samples assessment. Retention time in minutes. SD – standard deviation, CV – coefficient of variation

Table S2. Linearity and range assessment of standards.

HPLC Parameters (N=3/level)		Retention Time (min)			Peak Area		
Adenosine (nM)	**% Target**	**Mean**	**SD**	**CV (%)**	**Mean**	**SD**	**CV (%)**
15000	**1500**	10.63	0.01	0.11	296199.00	2312.07	0.78
5000	**500**	10.65	0.01	0.05	98654.67	480.60	0.49
2000	**200**	10.68	0.01	0.09	39632.33	569.87	1.44
1000	**100**	10.66	0.02	0.16	19773.00	261.86	1.32
750	**75**	10.66	0.02	0.19	14807.67	371.25	2.51
500	**50**	10.68	0.00	0.02	9886.00	334.39	3.38
250	**25**	10.73	0.02	0.22	5397.33	379.43	7.03
100	**10**	10.75	0.01	0.07	2322.33	298.89	12.87

Generated via 3 separate injections of each concentration of adenosine standard ranging from 10-1500% of the target adenosine ceoncentration. HPLC – high performance liquid chromatography, SD – standard deviation, CV – coefficient of variation

Table S3. Comparison of adenosine variation to established markers.

	CVi (%)	CVg (%)
Adenosine	**35.8**	**56.7**
C-reactive protein (CRP)	42.2-52.6	84.4-92.5
N-terminal (NT)-proBNP	30-50	99-130
Insulin	21.1	58.3
Vanillylmandelic acid (24 hour urine)	22.2	47
Cortisol	20.9	45.6
Creatinine kinase (CK)	22.8	40
Calcium (24 hour urine)	27.5	36.6
Haptoglobin	20.4	36.4
Lipase	23	33.1
Thyroid stimulating hormone (TSH)	19.7	27.2
Low-density lipoprotein (LDL)	8.3	25.7

CV – coefficient of variation, CVi – intra-subject CV, CVg – inter-subject CV[1-3]

Table S4. Univariable linear regression.

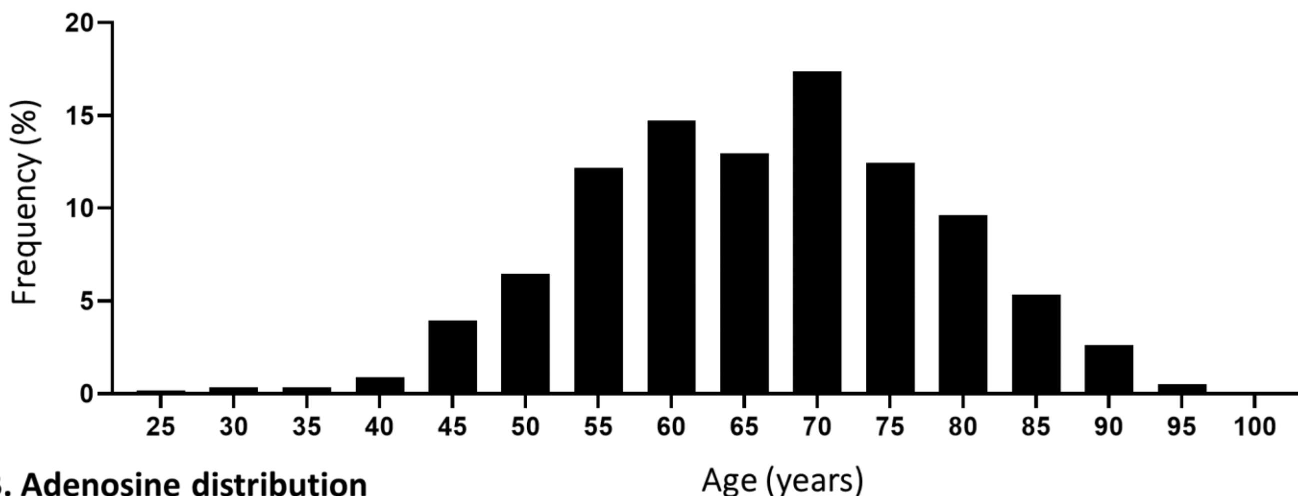
	p	P<0.2
Age	0.009	*
Sex	0.193	*
Coronary artery disease - number of vessels	0.104	*
Acute coronary syndrome	0.950	
ST-elevation myocardial infarction	0.395	
Non-ST-elevation myocardial infarction	0.609	
Diabetes	0.219	
Smoking	0.998	
Dyslipidemia	0.298	
Family History	0.993	
Hypertension	0.553	
Prior coronary artery disease	0.536	
Prior myocardial infarction	0.243	
Prior percutaneous coronary intervention	0.387	
Prior coronary artery bypass grafting	0.734	
ACE inhibitor/Angiotensin receptor blocker	0.483	
Acetylsalicylic acid	0.864	
Beta Blocker	0.422	
Calcium Channel Blocker	0.379	
Statin	0.176	*
Intravenous unfractionated heparin	0.558	
Subcutaneous low molecular weight heparin	0.516	
P2Y12	0.195	*
Hemoglobin A1c	0.062	*
Creatinine	0.816	
Hemoglobin	0.921	

Table S5. Multivariable linear regression.

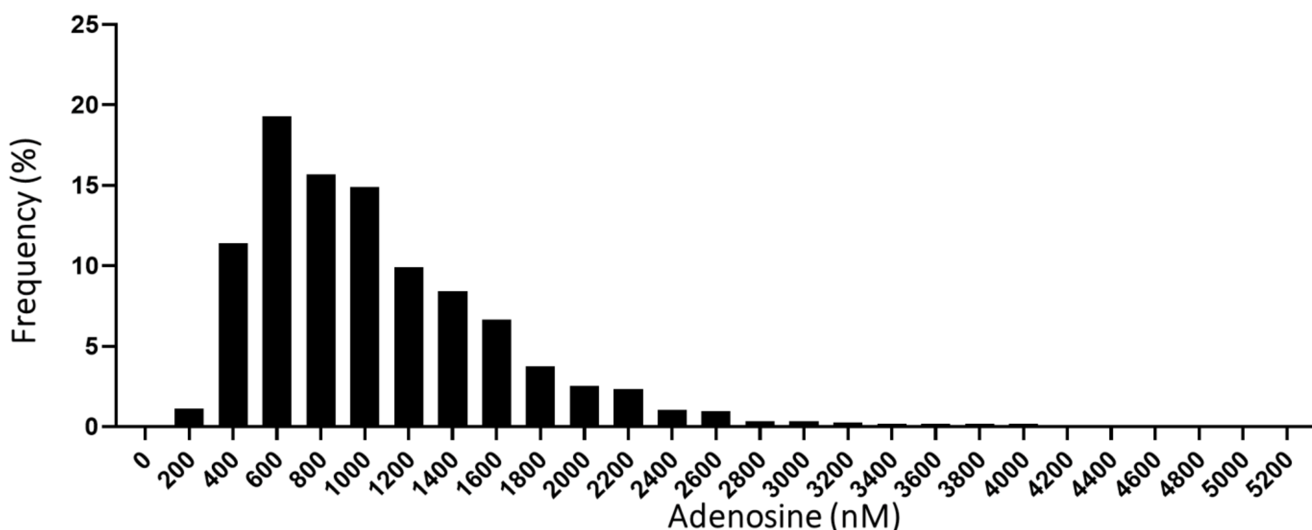
	p	p<0.05
Age	0.039	*
Sex	0.237	
Clopidogrel	0.297	
Ticagrelor	0.537	
Statin	0.504	
Coronary artery disease - number of vessels	0.332	
Hemoglobin A1c	0.088	

Figure S1. Distribution of adenosine and age. Histograms depicting the relative frequency in percentage (%) for each designated bin of term. Specifically, (A) demonstrates age distribution throughout the entire cohort (N=1,141) of patients undergoing workup for coronary artery disease, while (B) provides adenosine distribution across the entire cohort. **(C)** Logarithmic transformation of adenosine values across the entire cohort.

A. Age distribution



B. Adenosine distribution



C. Log-transformed adenosine distribution

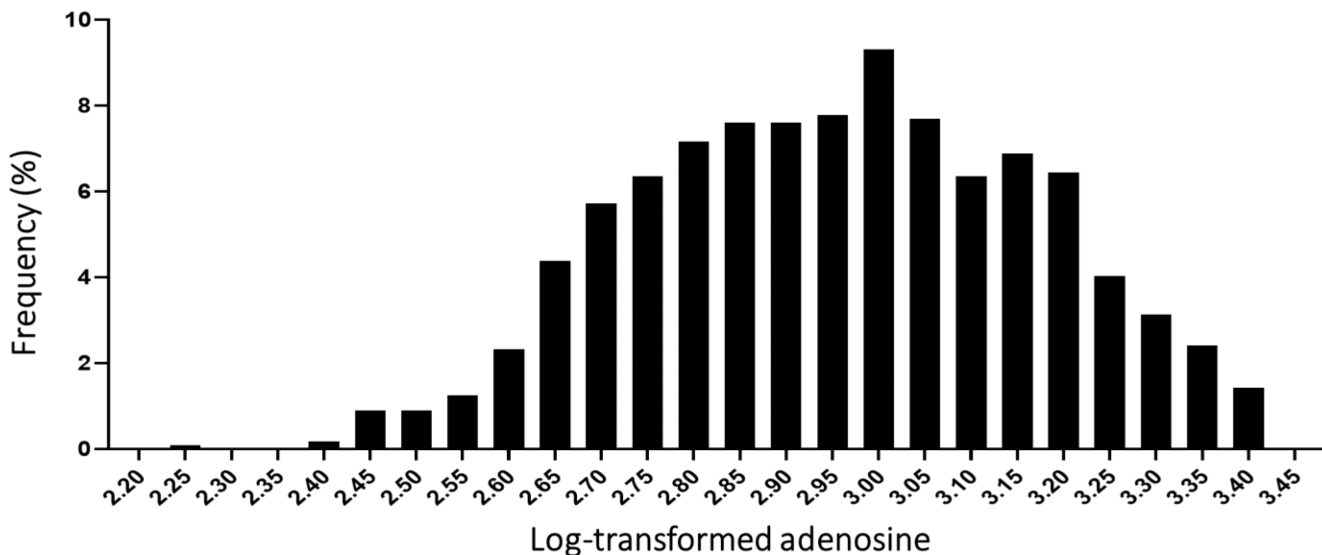
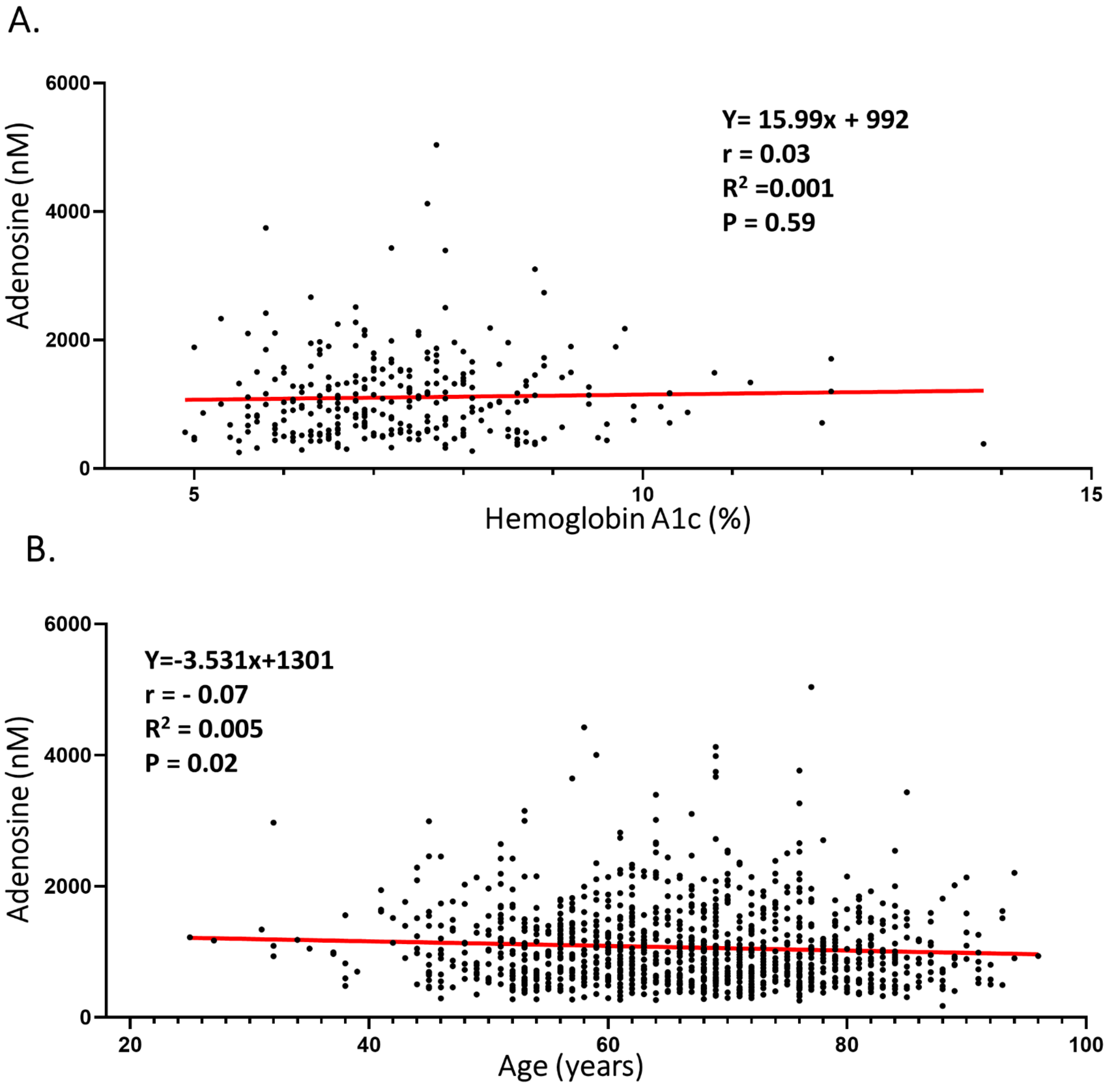


Figure S2. Association of hemoglobin A1c and age with adenosine. (A) Univariable analysis completed for all diabetic patients with a hemoglobin A1c indexed (N =294), demonstrating no significant relationship between HbA1c and adenosine (p=0.59). **(B)** Univariable linear regression of adenosine levels and age (years) in the entire cohort (N= 1,141) demonstrating a negative correlation (p=0.02).



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