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ORIGINAL RESEARCH

Circulating Ectonucleotidases Signal Impaired Myocardial Perfusion at Rest and Stress

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BACKGROUND: Ectonucleotidases maintain vascular homeostasis by metabolizing extracellular nucleotides, modulating inflammation and thrombosis, and potentially, myocardial flow through adenosine generation. Evidence implicates dysfunction or deficiency of ectonucleotidases CD39 or CD73 in human disease; the utility of measuring levels of circulating ectonucleotidases as plasma biomarkers of coronary artery dysfunction or disease has not been previously reported.

METHODS AND RESULTS: A total of 529 individuals undergoing clinically indicated positron emission tomography stress testing between 2015 and 2019 were enrolled in this single-center retrospective analysis. Baseline demographics, clinical data, nuclear stress test, and coronary artery calcium score variables were collected, as well as a blood sample. CD39 and CD73 levels were assessed as binary (detectable, undetectable) or continuous variables using ELISAs. Plasma CD39 was detectable in 24% of White and 8% of Black study participants (P=0.02). Of the clinical history variables examined, ectonucleotidase levels were most strongly associated with underlying liver disease and not other traditional coronary artery disease risk factors. Intriguingly, detection of circulating ectonucleotidase was inversely associated with stress myocardial blood flow (2.3±0.8 mL/min per g versus 2.7 mL/min per g±1.1 for detectable versus undetectable CD39 levels, P<0.001) and global myocardial flow reserve (Pearson correlation between myocardial flow reserve and log(CD73) −0.19, P<0.001). A subanalysis showed these differences held true independent of liver disease.

CONCLUSIONS: Vasodilatory adenosine is the expected product of local ectonucleotidase activity, yet these data support an inverse relationship between plasma ectonucleotidases, stress myocardial blood flow (CD39), and myocardial flow reserve (CD73). These findings support the conclusion that plasma levels of ectonucleotidases, which may be shed from the endothelial surface, contribute to reduced stress myocardial blood flow and myocardial flow reserve.

Key Words: adenosine ■ coronary artery calcium ■ ectonucleotidase ■ myocardial flow reserve ■ positron emission tomography

Ectonucleotidases are a family of enzymes that are implicated in the suppression of thrombosis and inflammation.[1] As tethered transmembrane proteins on endothelium and leukocytes, ectonucleotidases are expressed on and potentially shed from the plasmalemma, with externally oriented active sites that phosphohydrolyze the terminal phosphate(s) of extracellular nucleotides.[2,3] Ectonucleotidases sequentially degrade nucleotides (ATP, ADP, and AMP), which are released by injured and dying cells, and also act extracellularly as signaling molecules (Figure 1).[4] The end product of the cascade of ectonucleotidase activity is adenosine, which has been shown to have anti-inflammatory and vasorelaxant properties (primarily via the adenosine A_{2A} receptor).[5]

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CLINICAL PERSPECTIVE

What Is New?

- Ectonucleotidases CD39 and CD73 are responsible for extracellular metabolism of ATP and ADP to adenosine, and in this study, circulating ectonucleotidases were inversely correlated with stress myocardial blood flow and myocardial flow reserve.
- Patients with detectible CD39 and elevated CD73 were less likely to have increases in stress myocardial blood flow and myocardial flow reserve in response to regadenoson, an adenosine A_{2A} receptor agonist, implying a relationship between untethered ectonucleotidases and vasodilatory function.

What Are the Clinical Implications?

- There is growing awareness of the impact of microvascular function on patient symptoms, disease progression, and long-term outcomes; the role of ectonucleotidases in microvascular pathophysiology deserves further study.

Nonstandard Abbreviations and Acronyms

ENTPD1	ectonucleoside triphosphate diphosphohydrolase-1
HGPS	Hutchinson-Gilford progeria syndrome
MFR	myocardial flow reserve
NT5E	ecto-5′-nucleotidase

Ectonucleotidases are integral to the maintenance of vascular health, as evidenced by pathology that has been traced to abnormal ectonucleotidase production or function.[6–10] For example, the ectonucleotidase CD39 (also known as ENTPD1 [ectonucleoside triphosphate diphosphohydrolase-1]) catabolizes extracellular ATP and ADP to AMP.[11,12] Genetic variants of CD39 (encoded by *ENTPD1*) have been associated with an increased risk of venous thromboembolism.[13] Individuals with inactivating mutations in the gene encoding CD73 (ecto-5′-nucleotidase [*NT5E*]), which converts AMP to adenosine, develop profound lower extremity vascular calcification. This has been attributed to reduced cAMP production, which enables activation of TNAP (tissue-nonspecific alkaline phosphatase) via alkaline phosphatase, biomineralization associated, an essential enzyme required for ectopic calcification.[8,9,14] Similar to mutations in *NT5E*, inactivating mutations in ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*) cause generalized arterial calcification of infancy.[10]

Hutchinson-Gilford progeria syndrome (HGPS) is a rare disease that results in premature death in humans attributable to stroke or cardiovascular disease, and murine models of HGPS have implicated ectonucleotidases in promoting vascular calcification in HGPS pathogenesis, as well.[15,16] In HGPS, the expression ratio of *CD39* to *ENPP1* is doubled, leading to reduced extracellular pyrophosphate.[15] Specifically inhibiting CD39 (ENTPD1) and TNAP results in preferential metabolism of ATP by ENPP1, reducing vascular calcification and increasing lifespan in an HGPS murine model.[17]

Although ectonucleotidases have been characterized as key regulators of vascular homeostasis, most research has focused on these enzymes in their cell surface–tethered forms. The relationship between tethered and circulating forms of ectonucleotidases and their relative contributions to human pathophysiology remains uncertain. There have been no translational human studies clarifying the relationship of circulating plasma ectonucleotidases with arterial calcification and vasodilation.[18,19] To address this gap in knowledge, we evaluated whether circulating human plasma ectonucleotidases CD39 and CD73 are associated with traditional cardiovascular risk factors, coronary artery calcification, and myocardial flow reserve (MFR), as an indicator of capacity to vasodilate.

METHODS

This study included 529 patients presenting to the University of Michigan (Michigan Medicine) nuclear cardiology laboratory for clinically indicated nuclear positron emission tomography stress testing between June 2015 and April 2019. Study participants were prospectively enrolled and provided comprehensive written informed consent for collection of baseline demographic, clinical, and nuclear stress testing data, in addition to a 30-mL blood sample. Blood samples were drawn from existing intravenous lines or via a separate venipuncture if a line was not already in place. The clinical study was reviewed and approved by the University of Michigan institutional review board (HUM00091439). The authors had full access to all of the data in the study and assume responsibility for its integrity and the data analysis. The data that support the findings of this study are available from the corresponding author upon reasonable request. The study inclusion criteria were age ≥18 years, ability to provide consent, recent or planned (within 7 days) cardiovascular stress imaging, and preexisting, functioning intravenous or arterial catheters of sufficient size to allow blood to be drawn, or willingness for a venipuncture. Prisoners, patients

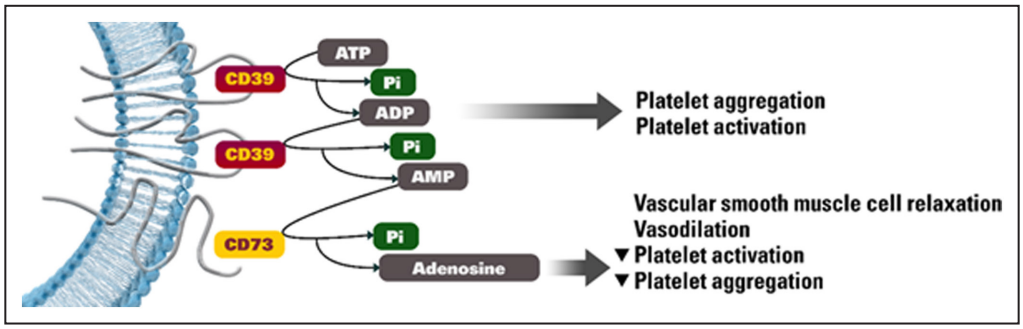

Figure 1. Catabolic activity of CD39 and CD73 produces anti-inflammatory adenosine.
CD39 catabolizes ATP and ADP, generating inorganic phosphate and AMP, the latter of which is then catabolized by CD73 to form adenosine. Adenosine is known to have vasorelaxant and antithrombotic properties. PET indicates positron emission tomography; and P_i, inorganic phosphate.

with severe psychiatric conditions, and those with advanced dementia were excluded from the study.

For patients from whom blood was able to be collected and with a sufficient sample, whole blood was collected in EDTA tubes before stress testing, centrifuged, and stored at −80°C for later analysis. Plasma was serially and numerically coded, and clinical and identifying variables were collected and managed in a secure REDCap electronic database hosted at the University of Michigan.[20]

The primary goal of this study was to determine if circulating plasma ectonucleotidase levels (CD39 or CD73) are associated with baseline demographic or clinical variables, myocardial perfusion and flow reserve, coronary artery calcification, or age given that coronary artery calcification is known to be modulated with age.

Patients were asked to abstain from caffeine and methylxanthines for 24 hours. They were initially injected intravenously with a weight adjusted dose of rubidium-82 under resting conditions with concomitant positron emission tomography imaging (Siemens mCT, Siemens Healthineers, Knoxville, TN) in list mode. After administration of 0.4 mg of regadenoson intravenously, an additional identical dose of rubidium-82 was injected, with imaging again performed in list mode. A low dose computed tomography was obtained for attenuation correction. List mode images were unlisted into dynamic series for quantification of MBF at rest and stress and MFR as the ratio of stress to rest MBF using a 1 tissue compartment model as previously described.[21]

Individuals measuring ectonucleotidase levels and calculating the coronary artery calcium scores were blinded to the baseline demographic and clinical variables. CD73 was measured using an ELISA kit for NT5E (Abcam ab213761) designed for the quantitative measurement of human CD73 in serum and plasma, with plasma samples diluted 1:5 before assaying. CD39 was measured using an ELISA kit for human CD39 serum and plasma (R & D systems DY4397-05), with plasma samples diluted 1:2 before assaying; 100-μL diluted plasma was loaded per well. Samples were measured in duplicate on a VERSAmax microplate reader (Molecular Devices), and means were recorded. A standard curve was used to calibrate readings from each 96-well plate.

Because of our interest in the relationship between plasma ectonucleotidases, age, and coronary calcification, study participants were categorized by age deciles, and the relationship between age and ectonucleotidase expression was determined. Study participants without prior percutaneous coronary interventions underwent coronary artery calcium scoring using the Agatston method.[22] Coronary artery calcium was measured using 4DM (INVIA Solutions). Calcium in the left main, left anterior descending, left circumflex, and right coronary arteries was manually highlighted. The sum of the 4 calcium scores was calculated and reported as the total calcium score for each study participant. Study participants with prior percutaneous coronary intervention were not included in analyses including calcification given the inability to distinguish stent material from coronary calcification.

For analyses, calcium scores were categorized from 0, 1 to 99, 100 to 399, and ≥400 based on the Agatston value generated for each score. The intent was to determine if any relationship existed between age, plasma ectonucleotidase concentration, and coronary artery calcification. To separate aging as a biological process from exposure to disease states over time, we also examined the relationship between ectonucleotidase expression and healthy aging in study participants, excluding those with abnormal stress tests (summed stress score 0), diabetes, smoking, prior myocardial infarction, prior percutaneous coronary intervention, prior coronary artery bypass graft surgery, prior heart transplant, cirrhosis, liver transplant, and those on dialysis.

Baseline characteristics of study participants are presented as mean±SD or median (interquartile range) for continuous variables and percentages for categorical variables. Associations between pairs of categorical variables are based on a chi-squared test of independence or on a Fisher exact test. Because >75% of study participants had undetectable CD39 in the plasma, CD39 is dichotomized as CD39=0 and CD39>0. CD73 and coronary artery calcium are log-transformed because of the variation of these quantities over orders of magnitude. Associations between continuous variables and nonbinary categorical variables, such as race, are determined using 1-way ANOVA. Associations of binary variables with continuous variables are determined using t-tests. Correlations between pairs of continuous variables are based on the Pearson correlation coefficient. All analyses were performed using SPSS 27 or SPSS 28 software (IBM).

RESULTS

Baseline characteristics of the study population are presented in Table 1. Of the 529 study participants enrolled in the study, 329 (62%) were men, and the mean age was 61±12 years. Coronary artery disease risk factors were prevalent in the study population; 384 (73%) had a history of hypertension, 168 (32%) had a history of dyslipidemia, and 199 (38%) had a history of diabetes.

Table 1. Baseline Characteristics of the Study Population

Characteristic	n (%) or mean±SD, n=529
Age, y	61±12
Race	
White	435 (82.2%)
Black	62 (11.7%)
Asian	6 (1.1%)
Hispanic	1 (0.2%)
Other*	19 (3.6%)
Sex (% men)	329 (62%)
Body mass index	34±9
Hypertension	384 (73%)
Dyslipidemia	168 (32%)
Diabetes	199 (38%)
Smoking (current or within 5 y)	122 (23%)
Family history of coronary artery disease	117 (22%)
Prior myocardial infarction	140 (26%)
Prior percutaneous coronary intervention	100 (19%)
Prior heart transplant	81 (15%)
Liver disease	101 (19%)
Cirrhosis	55 (10%)

*Other indicates race other than White, Black, or Asian. Other races could be American Indian or Alaska Native, Native Hawaiian or Other Pacific Islander.

Detectible CD39 ranged between 0.2 and 20 377 pg/mL. Associations of CD39 (as a dichotomous variable: CD39=0 or CD39>0) with baseline demographic, clinical, and laboratory values are presented in Table 2.[23] At baseline, CD39 was more likely to be detected in White (24%) compared with Black (8%) study participants (P=0.02). Study participants with liver disease (35% versus 20% with no liver disease, P<0.01), cirrhosis (55% versus 19% with no cirrhosis, P<0.001), and prior liver transplant (53% versus 19% with no prior transplant, P<0.001) were more likely to have detectable plasma CD39. Correspondingly, detectable CD39 was associated with elevated aspartate aminotransferase (35.1±22.1 versus 29.9±18.4 IU/L, P=0.04), elevated bilirubin (1.1±1.5 versus 0.7±0.7 mg/dL, P<0.01), and lower albumin (3.9±0.6 versus 4.1±0.4 g/dL, P<0.01), when compared with study participants without detectable CD39.

Given the differences in detectable CD39 by race and the presence of liver disease, we further explored the relationship between race, liver disease, and detectable CD39. More White (22%) than Black (8%) study participants had liver disease. In a subanalysis excluding those with liver disease or cirrhosis, White patients were still more likely (21%) than Black patients (3%) to have detectable circulating CD39 (Table S1).

Detectable plasma CD73 ranged between 759 and 82 931 pg/mL. Several baseline variables showed significant associations with log-transformed CD73 (Table 3). Log-transformed CD73 was higher in study participants with liver disease compared with study participants without liver disease (8.2±1.1 versus 7.7±0.8, P<0.001), study participants with cirrhosis compared with those without cirrhosis (8.8±1.2 versus 7.7±0.8, P<0.001), and study participants with prior liver transplant compared with those without prior liver transplant (8.8±1.2 versus 7.7±0.8, P<0.001). Correspondingly, log-transformed CD73 was positively associated with elevated aspartate aminotransferase (correlation coefficient=0.40, P<0.001), alanine transaminase (correlation coefficient=0.26, P<0.001), alkaline phosphatase (correlation coefficient=0.40, P<0.001), bilirubin (correlation coefficient=0.2, P<0.001), and was negatively associated with albumin (correlation coefficient=−0.28, P<0.001).

Because of the interest in understanding the association between detectable plasma ectonucleotidase and age, study participants were stratified by decades of age (Figure 2 and Figure 3). CD39 was undetectable in the majority of participants all age deciles, with the peak (50–59 year age category) having >40% of study participants with detectable CD39 (P<0.001 based on a Fisher's exact test for the overall analysis). There was no significant association between log(CD73) and age decile.

We were interested in understanding if comorbidities influenced the relationship between age and

Table 2. Associations of CD39 (As a Dichotomous Variable: CD39=0 or CD39>0) With Baseline Demographic, Clinical, and Laboratory Values*

Variable	CD39=0 n=363	CD39>0 n=105	*P* value
Age, y	61.3±12.2	60.1±10.7	0.34
Sex			0.09
Men	217 (75%)	73 (25%)	
Women	146 (82%)	32 (18%)	
Race			0.02
White	298 (76%)	96 (24%)	
Black	49 (93%)	4 (8%)	
Asian	3 (60%)	2 (40%)	
Other†	10 (77%)	3 (23%)	
Hypertension			0.06
Yes	274 (80%)	69 (20%)	
No	89 (71%)	36 (29%)	
Dyslipidemia			0.81
Yes	247 (78%)	69 (22%)	
No	115 (77%)	35 (23%)	
Diabetes			>0.99
Yes	138 (78%)	40 (22%)	
No	225 (78%)	64 (22%)	
Smoking (current or quit within 5 y)			0.19
Yes	90 (83%)	19 (17%)	
No	272 (76%)	86 (24%)	
Family history of coronary artery disease			>0.99
Yes	81 (78%)	23 (22%)	
No	274 (78%)	79 (22%)	
Prior myocardial infarction			0.51
Yes	90 (75%)	30 (25%)	
No	273 (78%)	75 (22%)	
Prior PCI			0.83
Yes	67 (76%)	21 (24%)	
No	296 (78%)	84 (22%)	
Prior CABG			>0.99
Yes	32 (78%)	9 (22%)	
No	331 (78%)	96 (23%)	
Heart transplant			0.10
Yes	47 (69%)	21 (31%)	
No	316 (79%)	84 (21%)	
Liver disease			<0.01
Yes	62 (65%)	33 (35%)	
No	292 (80%)	71 (20%)	
Cirrhosis			<0.001
Yes	23 (45%)	28 (55%)	
No	331 (81%)	76 (19%)	
Liver transplant			<0.001
Yes	22 (47%)	25 (53%)	
No	332 (81%)	79 (19%)	
On dialysis			0.50
Yes	9 (69%)	4 (31%)	
No	345 (78%)	100 (23%)	
Body mass index	34.8±9.7	33.1±7.5	0.052
AST	29.9±18.4	35.1±22.1	0.04
ALT	30.8±21.7	30.3±17.8	0.84
Alk Phos	97.0±66.1	101.1±51.8	0.59
Phosphate	3.52±0.83	3.47±0.68	0.71
Total protein	7.04±3.36	6.84±0.67	0.56
Albumin	4.1±0.4	3.9±0.6	<0.01
Bilirubin	0.7±0.7	1.1±1.5	<0.01
Creatinine	1.21±0.84	1.34±0.91	0.16
Glomerular filtration rate‡	53.8±12.2	51.4±13.4	0.11
INR	1.18±0.53	1.29±0.44	0.16

Alk Phos indicates alkaline phosphatase (IU/L); ALT, alanine aminotransferase (IU/L); AST, aspartate aminotransferase (IU/L); CABG, coronary artery bypass graft; INR, international normalized ratio; and PCI, percutaneous coronary intervention.

*Categorical variables are presented as number (%), and continuous variables are presented as mean±SD. Differences between numbers reported for a given variable and the total number of study participants because of missing data for these variables.

†Other indicates race other than White, Black, or Asian. Other races could be American Indian or Alaska Native, Native Hawaiian or Other Pacific Islander.

‡Glomerular filtration rates (mL/min per 1.73 m^2) reported in this study were calculated using the Modification of Diet in Renal Disease study equation (estimated glomerular filtration rate $=175\times(SCr)^{-1.154}\times(age)^{-0.203}\times0.742$ [if women]×1.212 [if Black race]), where SCr=standardized serum cystatin C in mg/L and age is in years.[23]

circulating ectonucleotidases. We performed a subanalysis with 73 healthy participants. These participants did not have any stress test abnormalities, did not have diabetes, did not smoke, did not have cirrhosis, were not evaluated for liver transplant, were not on dialysis, and had no prior myocardial infarction, percutaneous coronary intervention, coronary artery bypass graft surgery, or heart transplant. Age was not significantly correlated with log-transformed CD73 (r=−0.1, P=0.38) in these participants, and there was not a significant difference in age between participants with CD39=0 (60.4) and participants with CD39>0 (60.5, P=0.97).

There was not a significant difference between categories of coronary artery calcium, for CD39=0 versus CD39>0 (P=0.31) (Table S2). The correlation between log-transformed CD73 and log-transformed coronary artery calcium was also not significant (P=0.77) (Table S3).

Table 3. Associations of log(CD73) With Baseline Demographic, Clinical, and Laboratory Values*

Variable	log(CD73) if variable present	log(CD73) if variable not present	*P* value
Sex (men)	7.8±1.0	7.7±0.7	0.38
Race			0.53
White	7.8±0.9		
Black	7.6±0.9		
Asian	7.5±0.5		
Other†	8.0±0.7		
Hypertension	7.7±0.9	7.9±0.9	0.1
Dyslipidemia	7.7±0.9	7.8±0.9	0.18
Diabetes	7.9±1.1	7.7±0.7	0.055
Smoking (current or quit within 5 y)	7.9±1.0	7.8±0.9	0.22
Family history of coronary artery disease	7.9±0.9	7.7±0.9	0.1
Prior myocardial infarction	7.7±0.7	7.8±0.9	0.22
Prior PCI	7.8±0.8	7.8±0.9	0.96
Prior CABG	7.9±0.8	7.8±0.9	0.42
Heart transplant	7.8±0.9	7.8±0.9	0.99
Liver disease	8.2±1.1	7.7±0.8	<0.001
Cirrhosis	8.8±1.2	7.7±0.8	<0.001
Liver transplant	8.8±1.2	7.7±0.8	<0.001
On dialysis	7.8±1.0	7.8±0.9	0.88
Age	−0.08		0.09
Body mass index, kg/m^2	−0.02		0.74
AST	0.4		<0.001
ALT	0.26		<0.001
Alk Phos	0.4		<0.001
Phosphate	−0.07		0.34
Total protein	−0.01		0.90
Albumin	−0.28		<0.001
Bilirubin	0.2		<0.001
Creatinine	0.01		0.84
Glomerular filtration rate‡	−0.05		0.34
INR	0.08		0.21

Alk Phos indicates alkaline phosphatase (IU/L); ALT, alanine aminotransferase (IU/L); AST, aspartate aminotransferase (IU/L); CABG, coronary artery bypass graft; INR, international normalized ratio; and PCI, percutaneous coronary intervention.

*The table summarizes log(CD73) with the mean±SD for categorical variables and the Pearson correlation coefficient for log(CD73) with continuous variables.

†Other indicates race other than White, Black, or Asian. Other races could be American Indian or Alaska Native, Native Hawaiian or Other Pacific Islander.

‡Glomerular filtration rates (mL/min per 1.73 m^2) reported in this study were calculated using the Modification of Diet in Renal Disease study equation (estimated glomerular filtration rate $=175\times(\text{SCr})^{-1.154}\times(\text{age})^{-0.203}\times0.742$ [if women]×1.212 [if Black race]), where SCr indicates standardized serum cystatin C in mg/L and age is in years.[23]

The relationship between plasma ectonucleotidase expression and positron emission tomography stress testing variables was determined for dichotomized CD39 (Table 4) and log(CD73) (Table 5). Stress MBF was higher in study participants without detectable plasma CD39 compared with those with detectable CD39 (median and interquartile range, 2.49 (1.88–3.44) versus 2.33 (1.79–2.88) mL/min per g, $P<0.001$). Log(CD73) was negatively associated with MFR (correlation coefficient −0.19, $P<0.001$), suggesting that increasing log(CD73) values were associated with decreasing MFR. Decreased log(CD73) was noted in study participants with normal MFR compared with those without normal MFR (7.6±0.8 versus 8.0±1.0 pg/mL, $P=0.002$).

In a separate sensitivity analysis (data not shown), we separately excluded study participants with underlying liver disease. The major conclusions on the relationships between ectonucleotidases, age, coronary calcification, comorbidities, and stress myocardial flow parameters did not differ when study participants with underlying liver disease were excluded from the overall population.

DISCUSSION

Previously, deficient functional ectonucleotidase activity has been shown to be culpable for the development of heritable peripheral vascular disease and responsible for tuning the inflammatory response to myocardial ischemia and infarction.[7–9,24–26] As these modulators of the extracellular purinergic cascade could potentially be shed from endothelial cells or circulating leukocytes, we queried whether ectonucleotidases CD39 and CD73 may represent circulating biomarkers. These could be assessed with traditional cardiovascular risk factors, coronary artery calcification, and potentially even predict myocardial flow and vasodilatory reserve. Here, we show that detectable CD39 was increased in White compared with Black study participants. Of the clinical history variables examined, ectonucleotidase expression was most strongly associated with underlying liver disease and not other traditional coronary artery disease risk factors. Intriguingly, detection of circulating ectonucleotidase was inversely associated with stress MBF (CD39) and MFR (CD73) (Figure 4). It will be interesting in future studies to clarify how the density or function of ectonucleotidases and A_{2A} receptors might regulate myocardial microvascular flow and resistance.

We had hypothesized that declining age-related arterial endothelial ectonucleotidase expression, possibly associated with higher detectable circulating ectonucleotidase expression, would be associated with increasing age, coronary artery calcification, comorbidities associated with coronary artery disease, and indicators of microvascular function. Our hypotheses were based on prior observations of increased

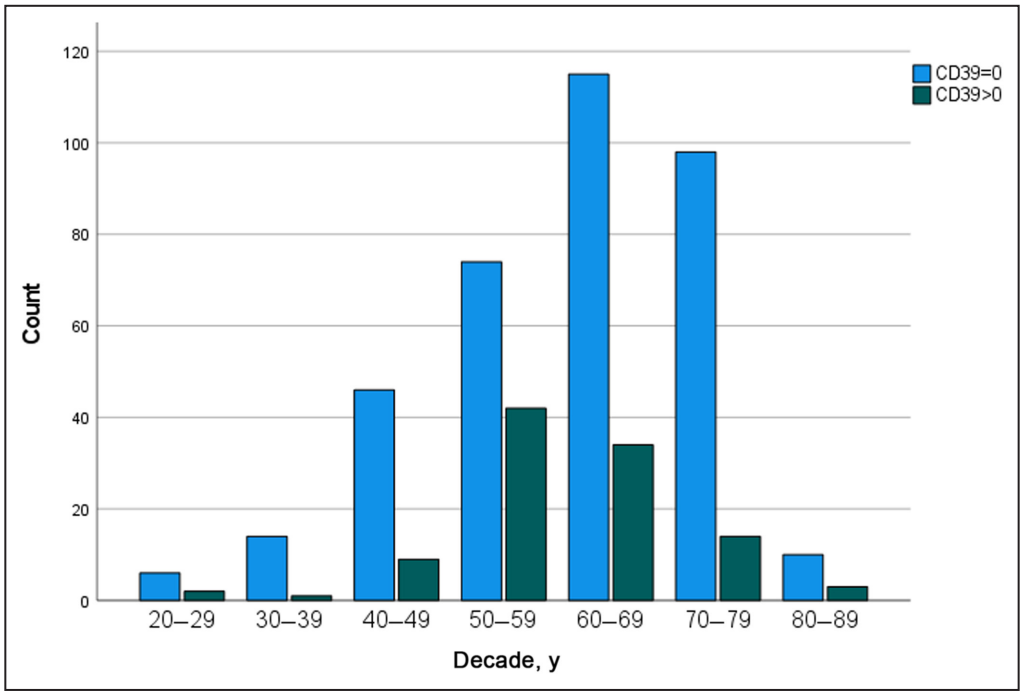

Figure 2. Decade of age and the presence of detectable plasma CD39.
*P<0.001 based on Fisher exact test.

expression of CD39 in study participants aged 65 to 85 years and decreased CD39 and CD73 mRNA in centenarians (Table S4).[27,28] The authors concluded that CD39 and CD73 gene expression may increase in middle age, but healthy aging was associated with lower levels.[27] In this study, we found some evidence that circulating CD39 was more likely to be detectable in the 50- to 59-year age group compared with other adult age deciles, though no such pattern was observed for circulating CD73. We conclude that while

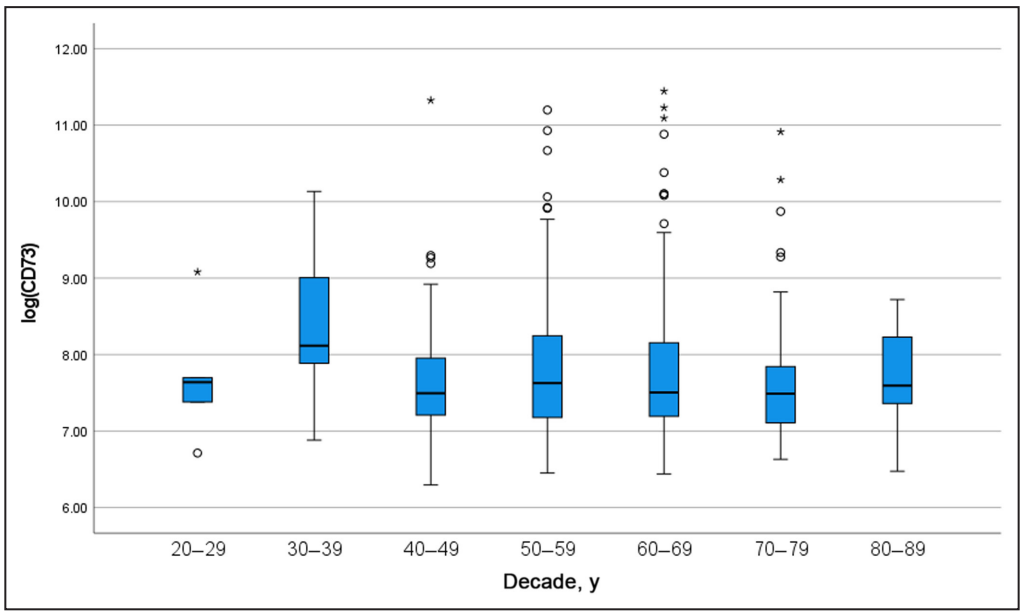

Figure 3. Decade of age and plasma log(CD73).
Box plots represent median and 25th and 75th percentiles. Circles represent data points that are >1.5 and <3 times below or above the box limits. Stars represent points that are >3 times below the lower or above the upper box limits. P=0.08 based on ANOVA; n per age category: 20 to 29 (n=6), 30 to 39: (n=15), 40 to 49 (n=55), 50 to 59: (n=113), 60 to 69: (n=147), 70 to 79 (n=114), 80 to 89 (n=13).

Table 4. Dichotomized Plasma CD39 and Positron Emission Tomography Stress Test Variables.

Variable	Median (IQR) for CD39=0 n=363	Median (IQR) for CD39>0 n=105	*P* value
Summed stress score	0 (0–6)	0 (0–7)	0.6
Summed difference score	0 (0–2)	0 (0–2)	0.45
Rest global defect	2 (0–8)	2 (0–6)	0.62
Stress global defect	1 (0–11)	2 (0–11)	0.58
Reversibility	0 (0–3)	0 (0–3)	0.65
TID ratio	1.02 (0.98–1.05)	1.00 (0.97–1.05)	0.28
Rest myocardial blood flow (mL/min·g)	1.17 (0.88–1.55)	1.12 (0.84–1.44)	0.14
Stress myocardial blood flow (mL/min·g)	2.49 (1.88–3.44)	2.33 (1.79–2.88)	0.02
Myocardial flow reserve*	2.19 (1.68–2.63)	2.03 (1.63–2.66)	0.37

Variable	Variable present	Variable not present	
	n (%)	n (%)	*P* value
Normal myocardial flow reserve			0.13
Yes	173 (80%)	43 (20%)	
No	115 (73%)	43 (27%)	

Mann–Whitney *U* test used for median and interquartile ranges. IQR indicates interquartile range; and TID ratio, transient ischemic dilation ratio.
*Myocardial flow reserve (where <2.0 abnormal and ≥2.0 normal).

there may be a hyperbolic relationship between circulating CD39 and CD73 and age, the relationship is likely subtle and possibly more influenced by cellular ectonucleotidase expression, rather than circulating plasma ectonucleotidases.

In this study, we found no significant association between circulating plasma CD39 or CD73 and any traditional cardiovascular risk factors such as hypertension, hyperlipidemia, diabetes, or smoking. We did observe that White study participants were more likely to have detectable circulating CD39 than Black study participants. Differential ectonucleotidase expression by race has not been previously reported to our knowledge; whether this represents a haplotype association or other demographic, environmental, or structural interactions remains to be determined. Counter to our hypotheses, an association between circulating ectonucleotidases and traditional cardiovascular risk factors and stress testing parameters such as summed stress score or stress global defect was not present in this study.

Table 5. Log-Transformed CD73 and Positron Emission Tomography Stress Test Variables

Variable	Correlation coefficient	*P* value
Summed stress score	−0.02	0.67
Summed difference score	−0.02	0.69
Rest global defect	−0.02	0.66
Stress global defect	−0.03	0.54
Reversibility	−0.03	0.60
TID ratio	0.04	0.39
Rest MBF	0.1	0.056
Stress MBF (mL/(min·per g)	−0.05	0.38
MFR* (mL/min·per g)	−0.19	<0.001

Variable	Variable is present (mean±SD for log(CD73))	Variable is not present (mean±SD log(CD73))	*P* value
Normal MFR*	7.6±0.8	8.0±1.0	0.002

MBF indicates myocardial blood flow; MFR, myocardial flow reserve; and TID ratio, transient ischemic dilation ratio.
*Myocardial flow reserve (using <2.0 abnormal and ≥2.0 normal).

Our study findings may be compared with those of Jalkanen et al.[2,29] In prior studies, investigators evaluated plasma ATP and ADP levels and serum CD39 and CD73 activity in 226 study participants with stable peripheral artery disease admitted for nonurgent imaging.[29] Serum ATP, ADP, and CD73 values were higher in study participants with atherosclerosis of the peripheral vessels than in controls without clinically evident peripheral artery disease. Subsequently, Jalkanen et al went on to describe that CD73 expression was elevated in the vasa vasorum of developing plaques, but expression was lost in mature occlusive plaques removed at the time of peripheral vascular endarterectomy, thus implicating that elevated serum CD73 activity was elevated because of shedding of CD73 from diseased arterial walls.[2]

The regulation and shedding of ectonucleotidases remains incompletely explored. Human *ENTPD1* (which encodes the ectonucleotidase CD39) promoter polymorphisms have been described that increase transcriptional activity by 8-fold,[13] while interleukin-4

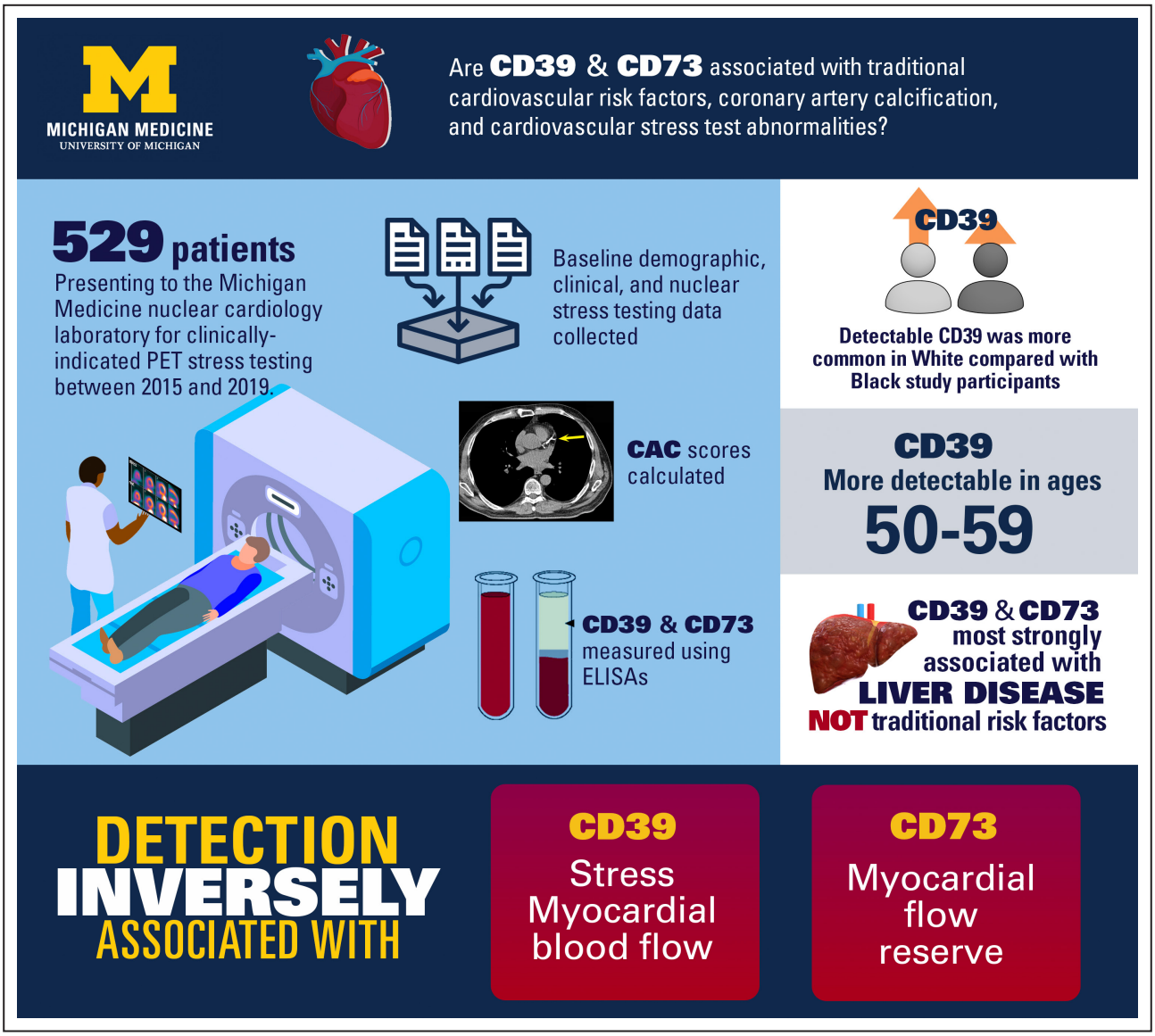

Figure 4. Summary of the key study findings.
Study findings support an inverse relationship between plasma ectonucleotidases, stress myocardial blood flow (CD39), and myocardial flow reserve (CD73). CAC indicates coronary artery calcium; and PET, positron emission tomography.

inhibits *ENTPD1* transcription through the phosphorylation of STAT6 (signal transducer and activator of transcription 6) and the upregulation of GATA3 (GATA binding protein 3), GFI1 (growth factor independent 1 transcriptional repressor), and YY1 (Yin Yang 1).[30] Soluble, circulating CD39 has been described,[31,32] with contribution from circulating microparticles.[33–35] Prior studies suggest increased extracellular CD39 release in response to increased intracellular cyclic AMP.[19,36] CD73 is a GPI (glycosylphosphatidylinositol)-anchored ectonucleotidase that becomes soluble when the GPI anchor is shed by the action of phosphatidylinositol-specific phospholipase; CD73 sheds variably in response to stimuli between lymphocytes and endothelial cells.[3] Ectonucleotidase shedding from the cell surface also occurs in response to shear stress.[37] Kanthi et al previously described reduced CD39 expression (in an animal model) in atheroprone disturbed flow vascular regions.[38] Soluble CD39[35] and CD73[29,39] maintain ectonucleotidase enzymatic activity. Taken together, we hypothesize that higher detectable circulating ectonucleotidase expression may reflect endothelial dysfunction and pathological shedding. Whether this is an actively mediated or passive process requires further investigation.

Striking evidence links genetic defects in CD73 to profound peripheral arterial calcification.[8] In the absence of functional CD73, reduced local adenosine

disinhibits tissue nonspecific alkaline phosphatase activity, resulting in inorganic phosphate production, breakdown of pyrophosphate (an endogenous inhibitor of calcification), and ultimately results in increased vascular calcification.[8,9,40] Based on these data, which are, to date and to our knowledge the largest and only biomarker study attempting to characterize the relationship between circulating ectonucleotidases and cardiovascular disease, we do not believe that circulating plasma ectonucleotidases play any appreciable role on the development or inhibition of coronary artery calcification.

In this study, we noted an association between detectable circulating ectonucleotidase and study participants with underlying liver disease. This was a single-center study with an active liver transplantation program; select patients undergoing liver transplant evaluations undergo positron emission tomography stress testing as part of their pre-liver transplantation evaluation. This population may have been overrepresented in our study population compared with the general population.

Prior studies have demonstrated that adenosine (via ectonucleotidase activity) modulates the liver's response to injury and is involved in the regulation of liver fibrosis.[41–45] The findings described in this manuscript are consistent with prior reports of increased ectonucleotidase expression in the setting of liver disease and fibrosis.[46–49] Increased CD39 transcript and protein expression in response to liver injury is dependent upon Sp1 transcription factor activation.[50] During the pathologic process of liver fibrosis, hepatic stellate cells and portal fibroblasts differentiate into hepatic myofibroblasts.[51] This process is associated with CD73 transcriptional upregulation, dependent upon SP1 (specificity protein 1) and small mothers against decapentaplegic promoter elements.[51] In addition, Snider et al detected higher levels of the NT5E-2 splice variant in study participants with cirrhosis and hepatocellular carcinoma, implying a link between the encoded CD73 isoform and subsequent diseases characterized by abnormal tissue growth, including fibrosis-induced cirrhosis and hepatic cancer.[42,52,53] Mice with a hepatocyte-specific deletion of *NT5E* develop spontaneous age-dependent liver disease.[53] In addition to supporting liver homeostasis, CD73 is a critical regulator of hepatocyte responses to different forms of stress, following alcoholic, chemically induced, and ischemic injury.[42,43,54,55] Therefore, it is not surprising that we observed the highest serum levels of CD73 (and its catalytic partner enzyme CD39) in patients with liver disease.

A notable observation from this study was the inverse relationship between circulating ectonucleotidases and MFR and stress MBF. Adenosine is known to be vasodilatory, and the extracellular concentration depends on cellular release, cellular reuptake, and purinergic metabolism by ectonucleotidases.[56,57] Specifically, adenosine is generated by sequential phosphohydrolysis of ATP and ADP to AMP to adenosine by ectonucleotidases.[58] Adenosine binds to specific adenosine receptor types (and subtypes) and is known to regulate myocardial and coronary function.[59] While coronary flow is regulated by multiple adenosine receptors, the A_{2A} adenosine receptor is thought to be the predominant receptor subtype responsible for coronary blood flow and dilates in both endothelial-dependent and -independent manners.[59,60]

One may expect that higher cell surface–tethered ectonucleotidases would result in adenosine production and higher MFR. Here, we found that circulating ectonucleotidases were associated with lower MFR and stress MBF. Based on prior studies, we attribute the detection of plasma ectonucleotidases in study participants with these flow characteristics to shedding of ectonucleotidases from endothelium, possibly attributable to endothelial dysfunction.[2,29,38,61] While this study does not prove causation (eg, that reduced local endothelial ectonucleotidase expression causes impaired MBF—possibly microvascular), it does raise a plausible hypothesis that local impaired adenosine production could contribute to impaired MBF, even in the absence of atherosclerotic, obstructive coronary artery disease.[62,63] The vasodilator used in this study was regadenoson, a selective adenosine A_{2A} receptor agonist that mimics the effects of adenosine.[64,65] We do not believe the administration of regadenoson would have impacted the findings of this study, and this does not rule out a possible role for the adenosine A_{2A} receptor in the link between detection of circulating ectonucleotidase and MBF and MFR.

Limitations of the current study are that study participants were recruited from the stress testing laboratory at the University of Michigan (a single center) and may not be generalizable to other populations or geographical locations. The majority of the study population were men and White, a reflection of the demographic characteristics of the population referred for stress testing and recruited in a consecutive fashion. Ectonucleotidase expression was measured from plasma samples and may not be reflective of circulating cell-bound leukocyte ectonucleotidase expression or of endothelial ectonucleotidase expression. Ectonucleotidase expression rather than activity was determined, though this is likely more reflective of what would be feasible if applied to clinical practice. This study remains subject to the limitations of a human biomarker study, providing hypotheses-generating observations and does not prove causality.

Much remains to be determined to elucidate the relationship between ectonucleotidases, adenosine receptors, and myocardial microvascular function.

Understanding the molecular underpinnings of impaired MBF (with or without obstructive coronary artery disease) is critical given that the risk of death for those with myocardial infarction with nonobstructive coronary artery disease is >10% within 5 years.[66] Adenosine can provoke vasodilation in a nonendothelial-dependent fashion, though in the vessel wall, endogenous adenosine production stems from the enzymatic activity of membrane-bound ectonucleotidases, primarily expressed on the endothelium.[67,68] Our findings, in concert with prior studies, strongly imply that pathological shedding of endothelial ectonucleotidases and the potential resultant reduction in local adenosine are linked with MBF.

ARTICLE INFORMATION

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Supplemental Material

Tables S1–S4
Reference[69]

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Supplemental Material

Table S1. Counts and percentages of White and Black study participants with detectable CD39, only including participants without liver disease or cirrhosis*

Race of study participant	**CD39 = 0** **n = 279**	**CD39 > 0** **n = 67**
White	235 (79%)	64 (21%)
Black	44 (94%)	3 (6%)

*p = 0.026

Table S2. Dichotomized CD39 and Coronary Artery Calcium Score*

CAC score category	CD39 = 0 n = 298	CD39 > 0 n = 80
0 (n = 182)	150 (82%)	32 (18%)
1-99 (n = 50)	36 (72%)	14 (28%)
100-399 (n = 58)	46 (79%)	12 (21%)
400+ (n = 88)	66 (75%)	22 (25%)

*p = 0.31 based on Chi-squared test comparing CAC categories

CAC = coronary artery calcium

Table S3. Log-transformed CD73 and Coronary Artery Calcium Score*

CAC score category	Log-transformed CD73
0 (n = 178)	7.7 ± 0.8
1-99 (n = 48)	7.8 ± 1.1
100-399 (n = 59)	7.8 ± 1.0
400+ (n = 87)	7.8 ± 1.0
Overall (n = 372)	7.8 ± 0.9

*p = 0.77 based on ANOVA

CAC = coronary artery calcium

Table S4. Major findings of previous studies linking CD39/CD73 levels to age or disease state

Author(s) & Year published	Ectonucleotidase & directional change with age/disease	Methods of Quantification	Age categories (in years)	Major comorbidities in study population
Fang et al., 2016 [20]	CD39; increase with age	CD4+ T-cell CD39 mRNA transcript levels evaluated by qPCR	older: $65 \leq x \leq 85$ younger: $20 \leq x \leq 35$	None
Crooke et al., 2017 [21]	CD39 and CD73; higher levels in older adults compared to middle and younger adults and centenarians	CD39 and CD73 mRNA transcript levels evaluated via qPCR	centenarian: $x \geq 100$ older: x = 79* middle-aged: x = 45* young: x = 23*	None
Jalkanen et al., 2015 [22]	CD39 and CD73; lower CD39 activity associated with higher incidence disease; higher CD73 activity associated	nucleotidase serum activity levels were assessed using TLC and quantified via scintillation β-	older: $x \geq 60$	Stable peripheral artery disease of the lower extremities

	with increased atherosclerosis	counting		
Friedman et al., 2009 [40]	CD39; lower levels associated with higher incidence of disease	CD39 mRNA transcript levels of SNP variations evaluated via qPCR, both in the presence and absence of Crohn's disease	N/A	Crohn's disease
Guzman-Flores et al., 2015 [41]	CD39 and CD73; CD39 expression increased with age and CD73 expression decreased with age and disease (T2D & obesity), in lymphocytes	CD39 and CD73 expression levels in total lymphocytes measured via flow cytometry	N/A	Type II diabetes, obesity
Chen et al., 2021[69]	CD73; higher CD73 expression associated with worse diseased-state	CD73 expression levels were reported via an immunoreactive score calculated based on immunohistochemical staining	older: $x \geq 60$	Esophageal squamous cell carcinoma, esophagectomy

		results		

*median ages of participants in this age bracket

SNP = single nucleotide polymorphism; TLC = thin-layer chromatography; T2D = type II diabetes