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Siu, Kin Lung
Li, Qiang
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Diagnostic and predictive values of circulating tetrahydrobiopterin levels as a novel biomarker in patients with thoracic and abdominal aortic aneurysms

Yixuan Zhang [a], Kin Lung Siu [a], Qiang Li [a], Kimberly Howard-Quijano [b], Jennifer Scovotti [a], Aman Mahajan [b], Hua Cai [a,*]

[a] Division of Molecular Medicine, Department of Anesthesiology and Perioperative Medicine, Division of Cardiology, Department of Medicine, David Geffen School of Medicine at University of California Los Angeles, California, 90095, USA
[b] Department of Anesthesiology and Perioperative Medicine, University of Pittsburgh, Pennsylvania, 15260, USA

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ABSTRACT

We have previously shown that circulating levels of tetrahydrobiopterin (H_4B) function as a robust biomarker for aortic aneurysms in several independent animal models. In the present study, we examined diagnostic and predictive values of circulating H_4B levels in human patients of thoracic aortic aneurysm (TAA) and abdominal aortic aneurysm (AAA) for the first time, while clinically applicable biomarkers for aortic aneurysms have never been previously available. Ninety-five patients scheduled for TAA repair surgeries and 53 control subjects were recruited at University of California Los Angeles (UCLA) Ronald Regan Medical Center, while 44 control subjects and 29 AAA patients were recruited through National Institute of Health (NIH) National Disease Research Interchange (NDRI) program. We had intriguing observations that circulating H_4B levels were substantially lower in TAA and AAA patients, linearly correlated with aortic H_4B levels (blood: $R = 0.8071$, $p < 0.0001$, $n = 75$; plasma: $R = 0.7983$, $p < 0.0001$, $n = 75$), and associated with incidence of TAA (blood: adjusted OR 0.495; 95% CI 0.379–0.647; $p < 0.001$; plasma: adjusted OR 0.501; 95% CI 0.385–0.652; $p < 0.001$) or AAA (blood: adjusted OR 0.329; 95% CI 0.125–0.868; $p = 0.025$) after adjustment for other factors. Blood or plasma H_4B levels below 0.2 pmol/μg serve as an important threshold for prediction of aortic aneurysms independent of age and gender (for TAA risk - blood: adjusted OR 419.67; 95% CI 59.191–2975.540; $p < 0.001$; plasma: adjusted OR 206.11; 95% CI 40.956–1037.279; $p < 0.001$). This threshold was also significantly associated with incidence of AAA ($p < 0.001$ by Chi-square analysis). In addition, we observed previously unrecognized inverse association of Statin use with TAA, and an association of AAA with arrhythmia. Taken together, our data strongly demonstrate for the first time that circulating H_4B levels can serve as a first-in-class, sensitive, robust and independent biomarker for clinical diagnosis and prediction of TAA and AAA in human patients, which can be rapidly translated to bedside to fundamentally improve clinical management of the devastating human disease of aortic aneurysms.

1. Introduction

Aortic aneurysm is the second most prevalent aortic disease next to atherosclerosis [1], which was reported to resulte in 200,000 deaths in 2017 [2]. An aortic aneurysm is defined as a localized dilation that is at least 1.5-fold of the normal diameter of the artery [3,4]. Based on the aortic location affected, aortic aneurysms are classified into abdominal aortic aneurysm (AAA) and thoracic aortic aneurysm (TAA). AAA generally occurs at the infrarenal region [5], whereas most TAA develop at the ascending thoracic aorta/aortic root area (about 60%), and in the descending thoracic aorta area (about 40%) [6]. Clinically, a maximal infrarenal abdominal aortic diameter larger than 3.0 cm is diagnosed as AAA [5,7–9]. Since the normal sizes of thoracic aorta vary based on age, gender, and location (root, ascending, arch, and descending), and that different diagnosis methods (ultrasound, chest x-ray and CT imaging) have different detection characteristics, the diagnosis of TAA covers aortic diameters of 3.6–5.9 cm [4,10,11]. The prevalence of AAA is up to 9% for those older than 65 [12], and a meta-analysis of 56 studies

* Corresponding author. Anesthesiology and Medicine, David Geffen School of Medicine, University of California Los Angeles, USA.
E-mail address: hcai@mednet.ucla.edu (H. Cai).

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indicates that the prevalence of AAA in general population is at 4.8% [13]. The prevalence of TAA is estimated at 4.2% for the general population [14]. Most aortic aneurysms are asymptomatic until a tear (dissection) or rupture occurs, which causes sudden death in more than 90-95% of the AAA cases [5]. The mortality of ruptured TAA is also high at a rate of 97% [15,16]. Nonetheless, effective diagnostic and therapeutic strategies for this devastating human disease of aortic aneurysms have remained lacking. To date, no diagnostic biomarker and oral medicine has been available for better clinical management of aortic aneurysms.

Currently, the aortic aneurysms are incidentally diagnosed by imaging techniques used during a routine health checkup or for the examination of other organs, such as by ultrasonography, computed tomography (CT) and magnetic resonance imaging (MRI), with CT and MRI mostly used as pre-surgery survey of the aneurysms. However, these techniques are not regularly used at clinic for aortic aneurysm diagnosis and/or screening, and cannot detect smaller and growing aneurysms (i.e. less than 3 cm for AAA) or initiating aneurysms with only molecular changes. In view of the silent growth of the aneurysms and the severe consequences of sudden rupture, effective tools for early diagnosis and monitoring are in urgent need to better manage the devastating and in fact, prevalent disease. To date, no diagnostic biomarker, especially circulating biomarker, is available for clinical diagnosis of aortic aneurysms. Previous studies have indicated intermediate roles of inflammation and atherothrombosis in the pathogenesis of AAA [7]. As a result, markers of inflammation (such as IL-6, TNF-α, osteopontin) [17–20] and thrombosis-related proteins (such as fibrinogen) [17,19] have been proposed as potential circulating biomarkers for AAA. However, lack of correlation between elevated levels of IL-6/TNF-α and aneurysm expansion excludes these molecules as biomarkers for AAA [21]. Although circulating levels of osteopontin were shown to be correlated with ultrasound-defined size of AAA, the correlation was not linear [18]. As to fibrinogen, studies have shown that the association between fibrinogen and AAA may be caused by the association of fibrinogen and smoking [22], which represents a strong confounding factor. Meanwhile, elevated fibrillin-1 was proposed to be a possible biomarker for TAA due to its role as a scaffold protein for elastin in the aortic wall [23]. Several other potential biomarkers for TAA such as matrix metalloproteinases (MMPs) and miRNAs (hsa-miR-140-5p, hsa-miR-191-5p and hsa-miR-214-3p) have been reported [24,25]. Nonetheless, none of these proteins/molecules have been shown to have good diagnostic and/or predictive values for aortic aneurysms clinically in patients.

Our studies have consistently established a novel and important role of endothelial nitric oxide synthase (eNOS) uncoupling in mediating formation of AAA and TAA in mouse models [26–32]. Uncoupling of eNOS, consequent to tetrahydrobiopterin (H_4B) deficiency, leads to increased superoxide production and sustained oxidative stress, which subsequently triggers inflammation and activation of MMPs to result in aneurysm formation. This mediator role of eNOS uncoupling in aneurysm formation is accompanied by deficiencies in aortic and circulating H_4B levels in all of the aneurysm models examined, including Ang II-infused hph-1 and apoE mull mice (as models of AAA), [26,27,29,30,33], and $Fbn1^{C1039G/+}$ Marfan syndrome mice (as model of TAA and AAA) [32]. Furthermore, we have shown that restoration of H_4B by overexpression of the H_4B salvage enzyme dihydrofolate reductase (DHFR), or by upregulation of endogenous DHFR with oral administration of folic acid (FA), robustly attenuates AAA and TAA formation in these novel and classic models of aortic aneurysms, while the aortic and circulating levels of H_4B are also restored [26,27,32]. Intriguingly, we have demonstrated that circulating levels of H_4B linearly correlate with aortic H_4B levels, and that circulating levels of H_4B linearly correlate with, and are indicative of, sizes of abdominal and thoracic aortas, indicating that circulating H_4B levels may be used as a novel diagnostic and predictive biomarker for aortic aneurysms [32,33].

To address the hypothesis that circulating H_4B may serve as a novel diagnostic and predictive biomarker in patients with aortic aneurysms, we compared aortic and circulating levels of H_4B in subjects with and without TAA recruited at the University of California Los Angeles (UCLA) Ronald Regan Medical Center, and in subjects with and without AAA recruited from National Institute of Health (NIH) National Disease Research Interchange (NDRI) program. We found for the first time that aortic and circulating levels of H_4B were substantially decreased in patients with AAA or TAA when compared to that of control subjects. We also demonstrated a linear correlation between circulating H_4B levels and aortic H_4B levels, indicating that circulating H_4B levels can be used as a non-invasive and accurate biomarker to reflect the change in tissue H_4B levels in aortas. Remarkably, our results also revealed significant associations between reduced circulating levels of H_4B, and incidences of TAA and AAA, after adjustment for other factors. Especially, circulating H4B levels below 0.2 pmol/μg is significantly associated with incidence of TAA and AAA, independent of age and gender, establishing a valuable threshold for aortic aneurysm diagnosis and routine screening in general population. Moreover, we found a previously unrecognized association between arrhythmia and AAA, indicating a possible new link of arrhythmia to AAA. We also found an inverse association of diabetes with TAA, especially in males; and a previously unrecognized inverse association between Statin use and TAA, especially in patients who were $\geq$65 years old. Interestingly, increased body weight was significantly associated with incidence of TAA in females, but not in males. Taken together, our data innovatively demonstrate for the first time that circulating H_4B levels can be used as a first-in-class, sensitive and robust diagnostic and predictive biomarker for TAA and AAA in human patients, and that it might be of significant clinical value for early diagnosis of smaller and emerging aortic aneurysms, and for monitoring of recidivation after surgical correction and treatment responses to oral medicines (e.g. folic acid).

2. Methods

2.1. Study populations and collection of clinical information

All protocols involving studies of human tissues and blood samples were approved by Institutional Review Board (IRB) at University of California Los Angeles (UCLA) and National Institute of Health (NIH) National Disease Research Interchange (NDRI) program. For the TAA cohort, blood samples were collected before surgery from 95 patients who were scheduled for TAA repair surgeries between August 2013 and July 2018 at UCLA Ronald Regan Medical Center. Patients were recruited into the study with written consent. Plasma was prepared by centrifugation of blood samples collected using EDTA tubes at 2,000 g for 10 min. Aortic samples of TAA were collected from the aneurismal fragment that was discarded during surgery. For control samples, 53 patients who were scheduled for other cardiovascular surgeries between September 2014 and November 2015 were recruited, from whom only blood samples were collected in EDTA tubes. For the AAA cohort, samples were collected from NIH NDRI program between November 2012 and August 2020. Blood and aortic samples from 29 AAA patients and 44 control subjects were collected from subjects recruited from multi-center locations. Aortic AAA samples were collected from the aneurismal segment. Whole blood samples were also collected in EDTA tubes. Adult subjects aged older than 18 have been included in both AAA and TAA cohorts. Patient characteristics including baseline diseases and medication history have been obtained using a de-identified system.

2.2. HPLC determination of blood/plasma and aortic H_4B levels

Circulating and aortic H_4B levels were measured using high-performance liquid chromatography (HPLC) equipped with a fluorescent detector (Shimadzu America Inc. Carlsbad, CA) as we previously published [26,27,29–40]. For sample preparation from blood or plasma [41,42], equal volume of blood or plasma was incubated with H_4B lysis

buffer (0.1 mol/L phosphoric acid, 1 mmol/L EDTA, 10 mmol/L DL-Dithiothreitol) on ice for 20 min, followed by centrifugation at 12,000 g for 5 min at 4°C. For sample preparation from aortic segments, the aortic tissues were first cleaned of connective and fat tissues and rinsed with ice cold PBS. Then the tissues were lysed in H_4B lysis buffer (~200 μL/100 mg tissue) by mincing with surgical scissors and freezing/thawing using liquid nitrogen, followed by sonication. After incubation on ice for 20 min, the samples were centrifuged at 12,000 g for 5 min at 4°C. Then the supernatant from blood, plasma, or aortic preparations was subjected to differential acidic (0.2 mol/L trichloroacetic acid with 2.5% I_2 and 10% KI) and alkaline (0.1 mol/L NaOH with 0.9% I_2 and 1.5% KI) oxidation as we previously described [26,27,29–40]. After centrifugation, the supernatant was injected into a HPLC system equipped with a fluorescent detector set at 350 nm for excitation and 450 nm for emission. The H_4B concentrations were calculated according to a H_4B (MilliporeSigma, T4425) standard curve and normalized to protein concentration. All of the procedures were carried out in dark.

2.3. ESR determination of eNOS uncoupling activity

Aortic eNOS uncoupling activity was determined using electron spin resonance (ESR, eScan, Bruker) spectrophotometer as we previously published [26,27,29–40]. Aortic samples were minced and homogenized in lysis buffer (20 mmol/L Tris-HCl pH 7.4, 150 mmol/lL NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate, 1% Triton X-100) (~200 μL/100 mg tissue) supplemented with protease inhibitor cocktail (MilliporeSigma, P8340, 1:100). The tissue lysates were sonicated before incubation for 20 min on ice, and then centrifuged at 12,000 g for 10 min. Next, the protein concentrations of the supernatants were determined using a DC protein assay kit (Bio-Rad, CA, USA). To assess eNOS uncoupling activity, aortic tissue lysates were mixed with superoxide-specific spin trap methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, Enzo Life Sciences, #ALX-430-117-M250, 500 μmol/L) and loaded into a glass capillary for immediate detection of superoxide signal using ESR spectrometer. A second measurement was made with the presence of PEG-SOD (polyethylene glycol-superoxide dismutase, MilliporeSigma, #S9549, 20 U/mL). For determination of eNOS uncoupling activity, a third measurement was taken with the addition of NOS inhibitor, L-NAME (Cayman, #80587, 10 μM). When eNOS is coupled, addition of L-NAME increases the measured superoxide due to reduced scavenging of superoxide by nitric oxide produced from coupled eNOS. On the contrary, when eNOS is uncoupled, addition of L-NAME decreases measured superoxide due to the fact that uncoupled eNOS produces superoxide. The ESR settings used were: biofield, 3479; field sweep, 9.00 G; microwave frequency, 9.79 GHz; microwave power, 21.02 mW; modulation amplitude, 2.47 G; 5120 points of resolution; receiver gain, 1000; and kinetic time, 10 min.

2.4. Statistical analysis

All statistical analyses were performed using SPSS Program (Version 26, IBM). For comparison of continuous variables between two groups, the Independent-Samples *t*-test was used. For comparison of categorical variables between two groups, the Chi square and Fisher's exact test were used. Logistic regression analysis was used to evaluate the odds ratios (ORs) and 95% CIs for single or multiple variables in association with incidence of TAA or AAA. Linear regression analyses (r and p values) were performed using GraphPad Prism software. The differences were considered statistically significant with a *P* value less than 0.05.

Table 1
Demographic characteristics of Control subjects and TAA patients.

	Control (n = 52)	TAA (n = 95)	*p* Value
Age (years)	63.15 ± 12.03, n = 52	62.09 ± 12.02, n = 95	p > 0.05
Age ≥ 65 years	n = 28 (52, 53.8%)	n = 45 (95, 43.4%)	p > 0.05
Male gender	n = 38 (52, 73.1%)	n = 67 (95, 70.5%)	p > 0.05
Body weight (kg)	80.04 ± 16.42, n = 52	83.61 ± 20.70, n = 95	p > 0.05
Smoking	n = 24 (52, 46.2%)	n = 47 (92, 51.1%)	p > 0.05
Hypertension	n = 40 (52, 76.9%)	n = 77 (95, 81.1%)	p > 0.05
Diabetes	n = 16 (52, 30.8%)	n = 14 (95, 14.7%)	p = 0.021
Arrhythmia	n = 9 (52, 17.3%)	n = 23 (95, 24.2%)	p > 0.05
ACEI or ARB therapy	n = 21 (52, 40.4%)	n = 50 (93, 53.8%)	p > 0.05
Statins therapy	n = 35 (52, 67.3%)	n = 47 (93, 50.5%)	p = 0.051
Beta blocker therapy	n = 25 (52, 48.1%)	n = 52 (93, 55.9%)	p > 0.05
Blood H_4B ≥ 0.2 pmol/μg	n = 44 (52, 84.6%)	n = 3 (92, 3.3%)	p < 0.001
Plasma H_4B ≥ 0.2 pmol/μg	n = 44 (52, 84.6%)	n = 5 (93, 5.4%)	p < 0.001
Total cholesterol	172.67 ± 38.45, n = 18	164.51 ± 37.42, n = 39	p > 0.05
Triglycerides	160.67 ± 87.61, n = 18	122.21 ± 83.85, n = 39	p > 0.05
LDL cholesterol	93.56 ± 27.63, n = 18	89.61 ± 31.35, n = 38	p > 0.05
HDL cholesterol	50.44 ± 25.06, n = 18	53.68 ± 20.15, n = 40	p > 0.05

Data are shown as means ± SD, n, or n (total n, %). ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker. LDL, low-density lipoprotein. HDL, high-density lipoprotein. Smoking, both former smokers and current smokers were included.

3. Results

3.1. Basic characteristics of TAA patients and control subjects

For the UCLA TAA cohort, 53 control subjects and 95 TAA patients were analyzed (Table 1). The distribution of age, gender and body weight was not different between the two groups. Similarly, no difference was found in history of smoking (including current smokers and ever smokers who quitted later). Specifically, 76.9% of control subjects and 81.1% of TAA patients had hypertension, which was not different between the two groups. The presence of arrhythmia was also not different between control group (17.3%) and TAA group (24.2%). TAA patients had a higher rate of being on ACEI/ARB medication (53.8%) comparing to control subjects (40.4%), although not statistically significant. TAA patients had a higher, but statistically not different, rate of being on beta blockers (55.9% vs. 48.1% in controls). Importantly, we observed a substantial decrease in circulating H_4B levels in both blood and plasma samples in patients with TAA, comparing to the control subjects (Fig. 1, Table 1). Moreover, significantly more TAA patients had blood or plasma H_4B levels below 0.2 pmol/μg, comparing to the control subjects (Table 1).

3.2. Inverse association of diabetes with TAA

Consistent with previous findings [43], our data indicate that presence of diabetes was inversely associated with incidence of TAA (Table 1). To investigate whether the inverse association of diabetes with TAA is age-related, we examined this association in subgroups of those aged below or above 65. We found that comparing to the control subjects, diabetes was less frequent in older TAA patients (older than 65, p = 0.01) (Table 2), whereas no difference in diabetes prevalence observed in TAA patients aged below 65, indicating that the inverse impact of diabetes on TAA development is not present in younger subjects. We also separately examined this relationship in subgroups of different genders. Interestingly, diabetes was present in 39.5% of the

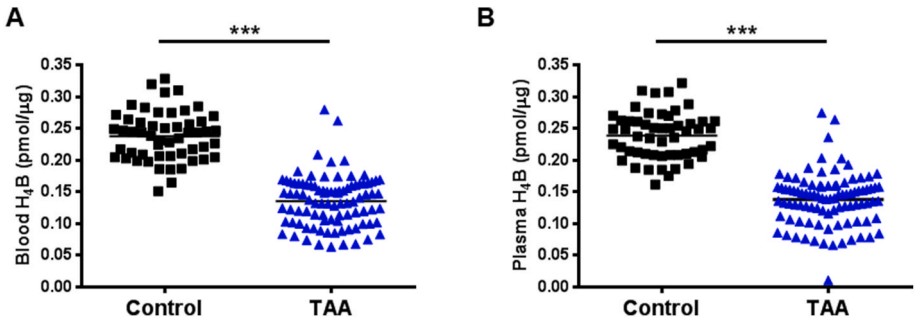

Fig. 1. Decreased circulating H_4B levels in patients with TAA. Circulating levels of H_4B were determined using HPLC as we previously published. Data indicate that circulating H_4B levels were substantially reduced in **(A)** blood (n = 52 for control and n = 92 for TAA) and **(B)** plasma (n = 52 for control and n = 93 for TAA) samples of patients with TAA comparing to those of control subjects. ***p < 0.001.

Table 2
Associations with TAA in age subgroups.

	Age <65 years			Age ≥65 years		
	Control (n = 24)	TAA (n = 50)	p value	Control (n = 28)	TAA (n = 45)	p value
Diabetes	n = 4 (24, 16.7%)	n = 7 (50, 14.0%)	p > 0.05	n = 12 (28, 42.9%)	n = 7 (45, 15.6%)	p = 0.01
Statins therapy	n = 11 (24, 45.8%)	n = 20 (49, 40.8%)	p > 0.05	n = 24 (28, 85.7%)	n = 27 (44, 61.4%)	p = 0.027
Blood H_4B (pmol/μg)	0.2400 ± 0.0421, n = 24	0.1340 ± 0.0351, n = 49	p < 0.001	0.2345 ± 0.0355, n = 28	0.1371 ± 0.0446, n = 43	p < 0.001
Plasma H_4B (pmol/μg)	0.2407 ± 0.0415, n = 24	0.1387 ± 0.0388, n = 49	p < 0.001	0.2352 ± 0.0329, n = 28	0.1367 ± 0.0448, n = 44	p < 0.001
Blood H_4B ≥ 0.2 pmol/μg	n = 20 (24, 83.3%)	n = 1 (49, 2.0%)	p < 0.001	n = 24 (28, 85.7%)	n = 2 (43, 4.7%)	p < 0.001
Plasma H_4B ≥ 0.2 pmol/μg	n = 19 (24, 79.2%)	n = 3 (49, 6.1%)	p < 0.001	n = 25 (28, 89.3%)	n = 2 (44, 4.5%)	p < 0.001
Total cholesterol	167.80 ± 51.59, n = 5	172.00 ± 40.86, n = 20	p > 0.05	174.54 ± 34.56, n = 13	156.63 ± 32.64, n = 19	p > 0.05
Triglycerides	161.60 ± 86.26, n = 5	142.00 ± 104.49, n = 20	p > 0.05	160.31 ± 91.62, n = 13	101.37 ± 49.24, n = 19	p = 0.049
LDL cholesterol	89.80 ± 28.83, n = 5	98.50 ± 31.94, n = 20	p > 0.05	95.00 ± 28.22, n = 13	79.72 ± 28.33, n = 18	p > 0.05
HDL cholesterol	58.20 ± 46.06, n = 5	53.38 ± 24.61, n = 21	p > 0.05	47.46 ± 12.14, n = 13	54.00 ± 14.36, n = 19	p > 0.05

Data are shown as means ± SD, n, or n (total n, %). LDL, low-density lipoprotein. HDL, high-density lipoprotein.

Table 3
Associations with TAA in gender subgroups.

	Males			Females		
	Control (n = 38)	TAA (n = 67)	p value	Control (n = 14)	TAA (n = 28)	p value
Body weight (kg)	86.49 ± 13.59, n = 38	88.82 ± 19.52, n = 67	p > 0.05	62.51 ± 8.90, n = 14	71.13 ± 18.21, n = 28	p = 0.046
Diabetes	n = 15 (38, 39.5%)	n = 6 (67, 9.0%)	p < 0.001	n = 1 (14, 7.1%)	n = 8 (28, 28.6%)	p > 0.05
Statins therapy	n = 30 (38, 78.9%)	n = 37 (66, 56.1%)	p = 0.019	n = 5 (14, 35.7%)	n = 10 (27, 37.0%)	p > 0.05
Blood H_4B (pmol/μg)	0.2356 ± 0.0394, n = 38	0.1351 ± 0.0405, n = 66	p < 0.001	0.2411 ± 0.0366, n = 14	0.1364 ± 0.0382, n = 26	p < 0.001
Plasma H_4B (pmol/μg)	0.2354 ± 0.0376, n = 38	0.1411 ± 0.0415, n = 66	p < 0.001	0.2441 ± 0.0353, n = 14	0.1296 ± 0.0412, n = 27	p < 0.001
Blood H_4B ≥ 0.2 pmol/μg	n = 31 (38, 81.6%)	n = 2 (66, 3.0%)	p < 0.001	n = 13 (14, 92.9%)	n = 1 (26, 3.8%)	p < 0.001
Plasma H_4B ≥ 0.2 pmol/μg	n = 31 (38, 81.6%)	n = 5 (66, 7.6%)	p < 0.001	n = 13 (14, 92.9%)	n = 0 (27, 0%)	p < 0.001
Total cholesterol	170.87 ± 36.88, n = 15	163.97 ± 39.74, n = 30	p > 0.05	181.67 ± 53.89, n = 3	166.33 ± 30.34, n = 9	p > 0.05
Triglycerides	170.40 ± 91.03, n = 15	128.63 ± 92.47, n = 30	p > 0.05	112.00 ± 54.62, n = 3	100.78 ± 41.56, n = 9	p > 0.05
LDL cholesterol	94.07 ± 27.71, n = 15	90.93 ± 34.75, n = 29	p > 0.05	91.00 ± 33.18, n = 3	85.33 ± 17.10, n = 9	p > 0.05
HDL cholesterol	43.33 ± 10.98, n = 15	51.61 ± 19.72, n = 31	p > 0.05	86.00 ± 47.09, n = 3	60.78 ± 21.14, n = 9	p > 0.05

Data are shown as means ± SD, n, or n (total n, %). LDL, low-density lipoprotein. HDL, high-density lipoprotein.

control subjects in males, whereas this incidence was only 9.0% (p < 0.001 vs. control subjects) in TAA patients (Table 3). On the contrary, no difference was found in females for prevalence of diabetes between TAA and control groups (Table 3), suggesting a gender difference in the inverse association between diabetes and TAA. Of note, increased body weight in TAA patients was observed in females when compared to control subjects (p = 0.046) (Table 3), implicating a potential relationship between obesity and TAA development in females. Circulating H_4B levels, and the number of patients with H_4B levels ≥0.2 pmol/μg, were significantly reduced in patients with TAA, which were independent of age and gender (p < 0.001 in all subgroup comparisons) (Table 2, Table 3).

3.3. Inverse association of Statin use with TAA

Interestingly, we found that use of Statins was less frequent in TAA patients (p = 0.051) (Table 1), suggesting that lack of lipid-lowering treatment might be associated with incidence of TAA. Although data in Table 1 indicate that lipid levels were not different between control and TAA groups at time of data collection, it does not exclude previous history of dyslipidemia. Further analyses indicate that compared to the control subjects, TAA patients had less use of Statin medication in those older than 65 (p = 0.027) (Table 2), likely implicating that poor dyslipidemia control is associated with development of TAA especially in this

subgroup. Interestingly, no difference in Statin use or triglycerides levels was found between TAA and control groups in those younger than 65 (Table 2). Similar to assessment of diabetes, we also examined possible gender difference in the inverse association of Statin use with TAA. Statin medication was significantly less used in male TAA patients ($p = 0.019$), but not in females (Table 3), indicating that less use of Statins might have contributed more to TAA incidence in males.

3.4. Circulating H_4B as a diagnostic and predictive biomarker for TAA in human patients

Our latest work has demonstrated a linear correlation between plasma H_4B levels and aortic H_4B levels in $Fbn1^{C1039G/+}$ mice of TAA/AAA model when H_4B is either deficient or restored with oral folic acid administration, indicating that circulating H_4B levels are accurately reflective of tissue H_4B levels during the development and treatment of TAA/AAA [32]. We have also shown that reduced aortic and plasma H_4B levels were significantly and linearly correlated with increased size of aortic root in $Fbn1^{C1039G/+}$ mice [32]. To examine whether circulating levels of H_4B were reduced in patients with TAA and corrected with aortic levels of H_4B in TAA patients, we measured both circulating and aortic levels of H_4B, and analyzed their interrelationship, and the indicative value of circulating H_4B for the incidence of TAA. As shown in Fig. 2A and Fig. 2B, both blood ($R = 0.8071$, $p < 0.0001$, $n = 75$) and plasma ($R = 0.7983$, $p < 0.0001$, $n = 75$) H_4B levels were linearly correlated with aortic H_4B levels in patients with TAA. Since the control subjects did not go through surgeries to allow collection of aortas, which was different from surgical removal of TAA segment in TAA patients, we did not collect aortic H_4B data from control subjects. Of note, there was a clear correlation between blood and plasma H_4B levels in subjects with or without TAA (Fig. 2C) ($R = 0.9729$, $p < 0.0001$, $n = 145$). These data indicate that circulating H_4B levels, either measured using blood or plasma samples, can be used as an accurate indicator of aortic tissue levels of H_4B in TAA patients.

To examine whether reduced circulating H_4B levels are associated with incidence of TAA in patients, logistic regression was carried out using SPSS version 26. The results indicate that decreased blood and plasma H_4B levels were significantly associated with incidence of TAA. Specifically, for every 0.01 pmol/μg decrease in blood H_4B levels, the risk of having TAA increases by 1.88 fold (OR 1.88; 95% CI 1.529–2.387; $p < 0.001$). For every 0.01 pmol/μg decrease in plasma H_4B levels, the odds of having TAA increase by 1.85 fold (OR 1.85; 95% CI 1.508–2.278; $p < 0.001$). For every 0.01 pmol/μg increase of blood H_4B level, the odds of having TAA decrease by 47.7% (OR 0.523; 95% CI 0.419–0.654; $p < 0.001$). Likewise, for every 0.01 pmol/μg increase of plasma H_4B level, the odds of having TAA decrease by 46% (OR 0.54; 95% CI 0.439–0.663; $p < 0.001$). To further evaluate the role of circulating H_4B as a potential biomarker of TAA, we conducted multivariable analysis using logistic regression. Our analyses showed that reduced blood (adjusted OR 0.495; 95% CI 0.379–0.647; $p < 0.001$) and plasma (adjusted OR 0.501; 95% 0.385–0.652; $p < 0.001$) H_4B levels were significantly associated with incidence of TAA after adjustment for age, gender, body weight, smoking history (current and ever smokers), hypertension, diabetes, arrhythmia, and use of ACEI/ARB, Statins, or beta blocker. Based on the values of adjusted OR, these data indicate that for every 0.01 pmol/μg increase in blood/plasma H_4B levels, the odds of having TAA decrease by 50.5% and 49.9% respectively. Furthermore, blood (OR 419.67; 95% CI 59.19–2975.54; $p < 0.001$; OR defines the ratios for below 0.2 pmol/μg compared with above 0.2 pmol/μg) and plasma (OR 206.11; 95% CI 40.96–1037.28; $p < 0.001$; OR defines the ratios for <0.2 pmol/μg compared with above ≥0.2 pmol/μg) levels of H_4B below 0.2 pmol/μg were significantly associated with incidence of TAA after adjustment for age, gender, body weight, smoking, hypertension, diabetes, arrhythmia, and use of ACEI/ARB, statins, or beta blocker. To be specific, one's risk of TAA increases by 419.67-fold if the blood H_4B levels drop below 0.2 pmol/μg, or 206.11-fold if the plasma H_4B levels drop below 0.2 pmol/μg, when compared to circulating H_4B levels ≥0.2 pmol/μg after normalization to other factors. Taken together, these data indicate that circulating H_4B levels can be used as a first-in-class, sensitive, robust and independent diagnostic and predictive biomarker for TAA in patients.

3.5. Basic characteristics of AAA patients and control subjects of NIH NDRI cohorts

For the NDRI AAA cohort, 44 control subjects without AAA and 29 subjects with AAA were recruited. Due to the fact that surgical repairs of AAA nowadays mostly involve endovascular procedures, discarded AAA tissues are no longer available from these surgeries. The aortic tissue samples collected from the NDRI cohorts were obtained by post-mortem isolation from patients with ruptured aneurysms, or other causes of

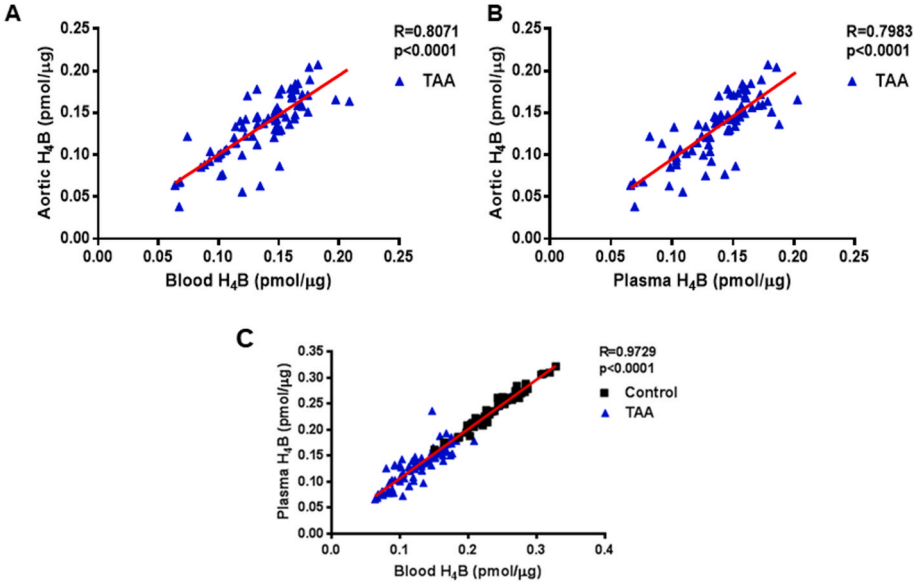

Fig. 2. Circulating H_4B levels correlated linearly with aortic H_4B levels in patients with TAA. Circulating levels of H_4B were determined using HPLC as we previously published. Data indicate that **(A)** Blood ($n = 75$) and **(B)** plasma ($n = 75$) levels of H_4B were linearly correlated with aortic H_4B levels in patients with TAA. **(C)** Linear correlation between blood and plasma levels of H_4B in both control and TAA patients ($n = 145$).

Table 4
Demographic characteristics of Control subjects and AAA patients.

	Control (n = 44)	AAA (n = 29)	*p* Value
Age (years)	70.77 ± 15.23, n = 44	75.90 ± 10.12, n = 29	p > 0.05
Age ≥ 65 years	n = 30 (44, 68.2%)	n = 25 (29, 86.2%)	p > 0.05
Male gender	n = 26 (44, 59.1%)	n = 19 (29, 65.5%)	p > 0.05
Body weight (kg)	80.48 ± 30.03, n = 43	82.55 ± 24.40, n = 28	p > 0.05
Smoking	n = 28 (44, 63.6%)	n = 22 (28, 78.6%)	p > 0.05
Hypertension	n = 27 (44, 61.4%)	n = 25 (29, 86.2%)	p = 0.022
Diabetes	n = 10 (34, 29.4%)	n = 5 (28, 17.9%)	p > 0.05
Arrhythmia	n = 7 (44, 15.9%)	n = 13 (29, 44.8%)	p = 0.007
ACEI or ARB therapy Therapy	n = 14 (36, 38.9%)	n = 19 (28, 67.9%)	p = 0.021
Statins Therapy	n = 7 (36, 19.4%)	n = 12 (28, 42.9%)	p = 0.042
Beta blocker Therapy	n = 5 (36, 13.9%)	n = 12 (25, 48.0%)	p = 0.003
Blood H_4B ≥ 0.2 pmol/μg	n = 30 (44, 68.2%)	n = 0 (24, 0%)	p < 0.001

Data are shown as means ± SD, n, or n (total n, %).

death (e.g. cardiac arrest, respiratory arrest, heart failure) but with co-existing aneurysms, or subjects died of sudden incidents for the control group. As shown in Table 4, no difference was found between control and AAA groups in age, gender, body weight, and smoking history. Similar to the TAA cohort, AAA patients had less co-existing diabetes compared to the control group (Table 4). Of note, higher percentage of hypertension (p = 0.021), current hypertension therapy (p = 0.021), current Statins therapy (p = 0.042), and use of beta blocker (p = 0.003) were observed among subjects with AAA when compared to non-AAA controls (Table 4). In addition to associations with various medications, a previously unrecognized higher co-existence of arrhythmia (p = 0.007) was observed in subjects with AAA (Table 4). Importantly, we found that circulating levels of H_4B in blood and aortic tissues were substantially decreased in the AAA group (Fig. 3A and B). Furthermore, using 0.2 pmol/μg as a threshold similar to analyses in TAA patients as described above, we found that more control subjects had blood H_4B levels above 0.2 pmol/μg comparing to AAA patients (Table 4). Of note, the AAA subjects had even lower levels of H_4B for most to around 0.12–0.14 pmol/μg, indicating that AAA patients might be more sensitive to the diagnostic threshold than the TAA subjects. We also found a significant increase in L-NAME-dependent superoxide production in the aortic tissues from the AAA group (Fig. 3C), confirming accompanying eNOS uncoupling activity in subjects with AAA.

3.6. *Circulating H_4B as a diagnostic and predictive biomarker for AAA*

We analyzed H_4B levels using blood samples and aortic tissues of both control subjects and AAA patients. Importantly, we found that blood H_4B levels were clearly correlated with aortic H_4B levels (R = 0.9363, p < 0.0001, n = 69) (Fig. 4A). We also examined the correlation between aortic eNOS uncoupling activity, and aortic and circulating (blood) H_4B levels. As shown in Fig. 4B and C, both aortic (R = −0.3330, p = 0.0063, n = 66) and blood (R = −0.3805, p = 0.0023, n = 62) H_4B levels were negatively correlated with aortic eNOS uncoupling activity, reflected by L-NAME-sensitive superoxide production, indicating that circulating H_4B levels serve as an accurate indicator of aortic eNOS uncoupling activity in patients with AAA, which represents the mechanistic changes mediating AAA development at molecular levels.

Using logistic regression analyses, we identified previously unrecognized, significant association between incidence of AAA with blood H_4B levels (p = 0.014), aortic eNOS uncoupling activity (p = 0.012), hypertension (p = 0.027), arrhythmia (p = 0.009), current hypertension medication (p = 0.024), current lipid-lowering medication/use of Statins (p = 0.046), and current use of beta blocker (p = 0.005). Especially, for every 0.1 pmol/μg decrease in blood H_4B levels, the odds of having AAA increase by 2.02-fold (OR 3.021; 95% CI 1.247–7.353; p = 0.014). For every 0.1 pmol/μg increase in blood H_4B level, the odds of having AAA decrease by 66.9% (OR 0.331; 95% CI 0.136–0.802; p = 0.014). The association between blood H_4B levels and AAA incidence remained significant (adjusted OR 0.329; 95% CI 0.125–0.868; p = 0.025) after adjustment for gender, diabetes, arrhythmia, hypertension therapy, lipid-lowering medication, and beta blocker therapy. Hence, for every 0.1 pmol/μg increase in blood H_4B level, the odds of having AAA decrease by 67.1%, and that circulating H_4B levels can function as a first-in-class, sensitive, robust and independent diagnostic and predictive biomarker for AAA.

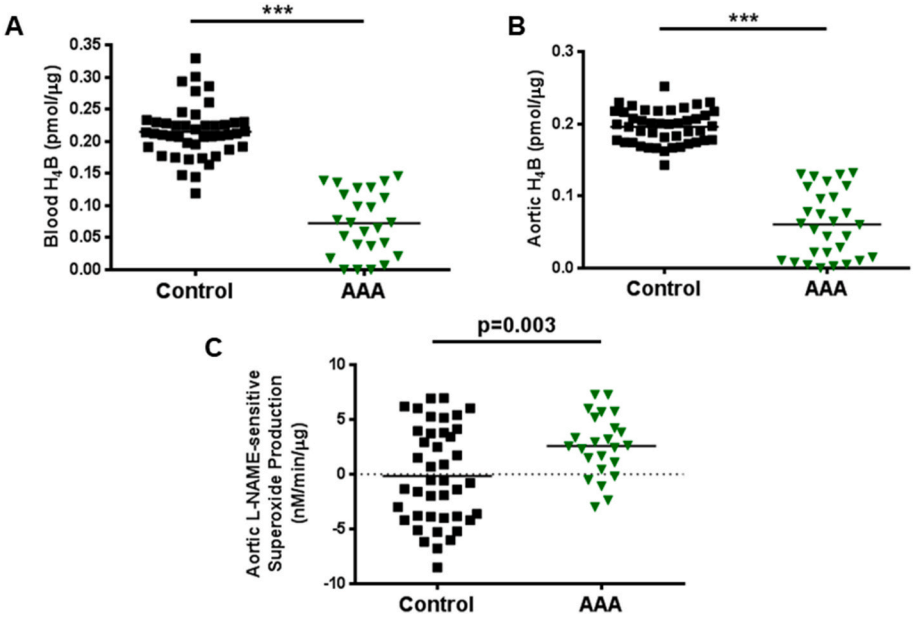

Fig. 3. Patients with AAA had reduced circulating and aortic H_4B levels, and elevated aortic eNOS uncoupling activity. Significantly reduced **(A)** blood (n = 44 for control and n = 25 for AAA) and **(B)** aortic (n = 44 for control and n = 29 for AAA) levels of H_4B in patients with AAA comparing to control subjects. ***p < 0.001. **(C)** Activity of eNOS uncoupling, represented by L-NAME-sensitive superoxide production, was significantly elevated in patients with AAA (n = 42 for control and n = 24 for AAA). p = 0.003.

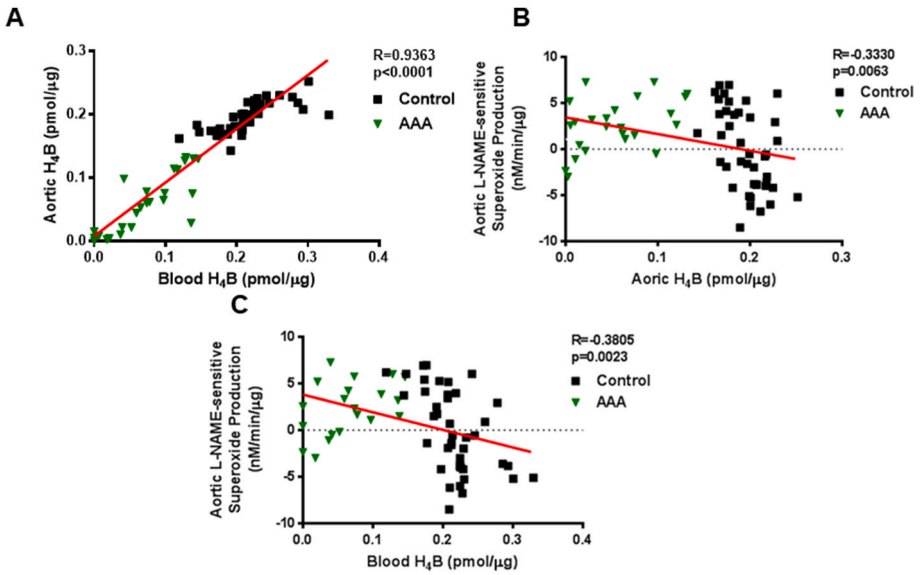

Fig. 4. Both aortic and blood levels of H_4B negatively correlated with aortic eNOS uncoupling activity in control subjects and AAA patients. (A) Blood H_4B levels correlated linearly with aortic H_4B levels in both control subjects and AAA patients (n = 69 total). Aortic eNOS uncoupling activity was negatively correlated with **(B)** aortic (n = 66) and **(C)** blood (n = 62) H_4B levels.

4. Discussion

The most significant and innovative finding of the present study is that circulating H_4B levels can serve as a robust, diagnostic and predictive biomarker for aortic aneurysms in human patients. Reduced blood and plasma H_4B levels were significantly associated with incidence of TAA independent of other risk/compounding factors. Reduced blood H_4B levels were also significantly associated with incidence of AAA independent of other risk/compounding factors. Of note, decreased circulating H_4B levels correlated well with decreased aortic H_4B levels in both TAA and AAA patients, indicating an accurate predicting value of circulating H_4B for tissue H_4B bioavailability in aneurysm patients. Importantly, decreased H_4B levels in blood and aortas correlated with increased eNOS uncoupling activity in patients with AAA, implicating that circulating H_4B levels, reflective of eNOS uncoupling activity at tissue levels, can be used as an indicator of aneurysm formation at molecular levels (more below). We also found that blood or plasma levels of H_4B below 0.2 pmol/μg were markedly associated with incidence and predicted risk of TAA and AAA, demonstrating that circulating H_4B levels below 0.2 pmol/μg can be used as a highly sensitive, valuable and clinically applicable diagnostic and profiling threshold for TAA and AAA. In addition, we observed an inverse relationship between TAA and diabetes as shown previously [44], and a boarder line negative association between TAA and use of Statins indicating potential contribution to TAA formation of poor lipid management. In the cohort of AAA patients, we found a novel association of AAA with arrhythmia, as well as previously unrecognized association of AAA with medication of hypertension beyond hypertension itself, association of AAA with lipid-lowering medication/use of Stains or use of beta blocker, and an inverse association of AAA with diabetes. Collectively, these data strongly demonstrate that circulating H_4B levels can be used as a robust and accurate diagnostic/predictive biomarker for aortic aneurysms in human patients. Since we have repetitively shown that in animal models of TAA and AAA, namely Ang II infused hph-1 mice, Ang II infused apoE mice, and Fbn1 Marfan syndrome mice, circulating levels of H_4B are time-dependently predictive of aneurysm growth and progression for being reduced at weeks 1–2 proceeding aneurysms being formed/detected at weeks 2–4 [26, 32, 33], we anticipate that circulating H_4B levels also have predictive values in diagnosing progressing aneurysms in human patients, although further studies are warranted to investigate this further in a perspective study. In addition, based on the predictive values of circulating H_4B for tissue level eNOS uncoupling activity both in animal models and in the current study of human patients [26–32], we anticipate that circulating H_4B are also of early diagnostic value in reflecting tissue eNOS uncoupling activity that drives aneurysm formation at molecular levels before aneurysms can be detected/diagnosed using ultrasound and/or other imaging techniques.

Currently, the diagnosis and monitoring strategies of aortic aneurysms have been lacking, with aneurysms incidentally diagnosed only through imaging of other organs during a routine health checkup. No pharmaceutical therapy or medicine is available to restrain the growth or rupture of aortic aneurysms [7]. Establishing a sensitive and accurate biomarker to screen and monitor the development and progression of aortic aneurysms can no doubt improve the care of patients with known and unknown aortic aneurysms. In this study, we examined the hypothesis of using circulating H_4B levels as a biomarker for both TAA and AAA in patients, based on our consistent observations of its biomarker role/value in both TAA and AAA formation in various animal models [26–33]. In Ang II-infused hph-1 and apoE null mice, as well as in DHFR knockout mice, we have demonstrated that AAA formation is attributed to H_4B deficiency and eNOS uncoupling activity [26–30,33]. Upregulation of H_4B levels by oral administration of folic acid, restoration of DHFR (H_4B salvage enzyme) function by miRNA192-5p inhibitor, or inactivation of NADPH oxidase (NOX) isoforms 1, 2, 4 using knockout approach, is able to recouple eNOS to attenuate AAA and TAA formation [26–29,31–33]. Consistent to these findings in animal models, data in the present study from human patients also indicate that mechanistic regulation of tissue H_4B bioavailability and eNOS uncoupling activity, which can be accurately detected through measurements of circulating H_4B levels, is positively associated with presence of aneurysms.

The essential role of H_4B deficiency in the formation of AAA is supported by our findings in the DHFR knockout mice, where aortic H_4B levels were endogenously diminished [30]. Ang II infused DHFR knockout mice had increased incidence of AAA and exaggerated hypertension [30]. Likewise, in Fbn1 mice as a classical model for TAA formation, formation of TAA and AAA is accompanied by decreased aortic and circulating levels of H_4B [32]. Whereas, restoration of H_4B by oral administration of folic acid also diminished TAA and AAA formation in this model [32]. Importantly, levels of circulating H_4B were linearly correlated with sizes of aortas in both AAA and TAA, under both

diseased and treatment conditions, implicating a remarkable diagnostic and predictive value of circulating H_4B levels for both development of the disease and responses to treatment [26,27,29–33], which is consistent and validated in our current study with data from human TAA and AAA patients. Unlike the non-linear correlation with size of AAA of osteopontin [18], linear correlation of H_4B has great advantage in predicting and monitoring the progression of aortic aneurysms in patients and to evaluate treatment efficacies by oral medications (e.g. folic acid per our previous publications) [26,27,29,30,32,33], and can be used to prevent unpredictiable lethal rupture by arrangement of elective surgeries in those suitable. Although correlation between circulating or aortic tissue H_4B and the sizes of TAA was not identified in this study, it is due to the limitation that only large aneurysms for surgical corrections were included in the analysis, missing data points from patients with smaller TAA. Importantly, monitoring circulating H_4B levels are considered beneficial not just for evaluation of disease development and progression, but also for assessment of treatment efficacies and post-surgery re-occurrence of the aneurysms.

Furthermore, based on the distribution of the data of circulating H_4B levels in our cohort (Figs. 1 and 3A), we proposed that circulating H_4B level below 0.2 pmol/μg can be considered as the threshold for diagnoses of both TAA and AAA. Indeed, compared to control groups, significantly more patients with circulating H_4B levels < 0.2 pmol/μg were found in the groups of TAA and AAA patients, or all of the subgroups (Tables 1–4) of aged <65 years, ≥ 65 years, males, or females. We also found that circulating H_4B levels <0.2 pmol/μg (blood OR 419.672; 95% CI 59.191–2975.540, $p < 0.001$; plasma OR 206.113; 95% CI 40.956–1037.279, $p < 0.001$; OR defines the ratios for <0.2 pmol/μg compared with ≥0.2 pmol/μg) were significantly associated with incidence of TAA after adjustment for other factors. Compared to the subjects whose circulating H_4B levels ≥0.2 pmol/μg, one's risk of TAA increases by 419.67-fold or 206.11-fold when the blood or plasma H_4B levels drop to <0.2 pmol/μg. These data suggest that our proposed aortic aneurysm biomarker of reduced H_4B levels, especially at <0.2 pmol/μg, is applicable to general population independent of age and gender. Of note, our data clearly show that H_4B levels of either whole blood or plasma are accurately and sensitively indicative of tissue H_4B levels that are reflective of aortic aneurysm formation at molecular levels. Therefore, we anticipate that similar to findings in animal models [26,27,29–33], circulating H_4B as a novel biomarker can be applied to diagnosis of smaller and growing aneurysms that cannot be detected by imaging techniques such as ultrasound due to detection limit. The limitation of the study is relatively small sample size for subgroup analyses. However, this is largely attributed to the fact that AAA surgeries now mostly involve endovascular repair. It is therefore challenging to collect enough tissue samples for the study. That is why we recruited through NIH NDRI program to procure samples from all of the participating national medical centers.

Of note, our data reveal that arrhythmia (types undifferentied) is associated with incidence of AAA (Table 4). As shown in Table 4, 7 out of 44 (15.9%) control subjects have arrhythmia, whereas 13 out of 29 (44.8%) AAA subjects have co-existing arrhythmia (Table 4) ($p = 0.007$). This seems to share some similarities with previous reports that patients with atrial fibrillation, the most common form of arrhythmia, have increased risk of developing aortic aneurysms (adjusted hazard ratio 1.243, $p < 0.001$) [45]. In addition, others have also reported presence of dilated aortic root or ascending thoracic aorta in patients with atrial fibrillation [46]. Therefore, our results suggested that performing echocardiography surveillance in patients with arrhythmia might help to identify silent AAA. On the other hand, diabetes has been reported to be inversely associated with TAA and AAA [43]. Consistent to previous reports, we also observed negative association between presence of TAA/AAA and diabetes in our study. Indeed, a meta-analysis by D'cruz RT et al. reported inverse association between diabetes and TAA, although whether the protection is due to hyperglycemic condition or anti-diabetic drugs remain to be investigated further [47].

One of the interesting findings of our study is the association between less use of Statins and incidence of TAA. We observed less incidence of TAA in subjects with Statins medication ($p = 0.051$). In addition, this association became more significant when we examined subgroups of aged ≥65 ($p = 0.027$) or male gender ($p = 0.019$). No more association was found in subgroups of age <65 or female gender, suggesting age- and gender-dependent correlation of Statin use and TAA. These data seem to indicate that better control of dyslipidemia maybe of protection against TAA especially in older population and males. Of note, it was reported that Statin use is associated with decreased risk of adverse events (death, dissection, or rupture) in patients with TAA [48], and that dyslipidemia has been previously recognized as a risk factor for aneurysm formation [49]. These previous literatures support our proposal to evaluate the relationship between aneurysm formation and dyslipidemia medication further. On the other hand, no association with Statin use was found in AAA cohort, possibly due to smaller sample size. Mixed results were reported regarding the association of Statin use and AAA. No association between Statin use and AAA growth was reported in a cohort of 652 AAA patients [50]. However, a systematic review and meta-analysis indicate that Statin therapy is associated with reduced growth, lower rupture risk, and reduced post-repair mortality [51].

Importantly, although human data are not readily available, findings of our own and others have shown that in animal models, tissue H_4B levels (data on circulating levels not available from most of the models) never reduce to the same low levels in aneurysm conditions (0.8–2.0 pmol/mg protein) [26,27,29,30,32,33] in conditions such as hyperlipidemia (10 pmol/mg protein, apoE null mice) [52], diabetes mellitus (6.0 pmol/mg protein, STZ-induced diabetic mice) [53], hypertension (4.0 pmol/mg protein, Ang II-infused hypertensive mice) [26] or ischemia-reperfusion injury of the heart (8.0 pmol/mg protein, ischemia reperfusion/high glucose injured mice) [54]. Therefore, combined with previous observations, our findings innovatively establish a selective and reliable biomarker role of circulating H_4B levels, accurately reflective of tissue H_4B levels, for the incidence of aortic aneurysms differentially from other conditions. Taken together, our data demonstrate for the first time that circulating H_4B levels can serve as a first-in-class, sensitive and robust biomarker for aortic aneurysms in human patients. We believe that implementation of routine checkup of circulating H_4B levels can help to identify aneurysms at early stage of molecular changes only, especially for the diagnosis and management of silent, small but growing aneurysms with high risk of unpredicted rupture. It is anticipated to effectively predict or monitor progression of aneurysms, treatment responses (e.g. to oral medicines and RNA drugs), and re-occurrence post surgeries (and preventive efficacies by treatments). These diagnostic and predictive values of circulating H_4B levels, establishing circulating H_4B levels as a first-in-class, sensitive, robust and independent biomarker for aortic aneurysms that is readily translatable to bedside, would no doubt fundamentally improve clinical management of the devastating human disease of aortic aneurysms for which clinically applicable biomarkers have never previously been available.

Data availability

Data will be made available on request.

Acknowledgement

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