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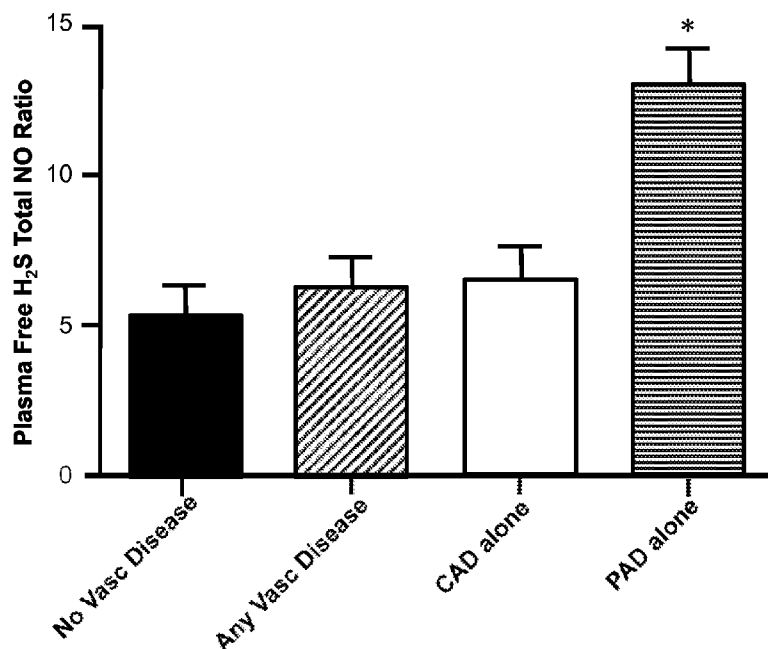
(54) Title: PLASMA H₂S LEVELS AS BIOMARKERS FOR VASCULAR DISEASE

Fig. 2

(57) Abstract: Hydrogen sulfide (H₂S) levels in plasma are useful in diagnosing cardiovascular disease. The ratio of H₂S to NO in plasma is useful in diagnosing peripheral artery disease.



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PLASMA H₂S LEVELS AS BIOMARKERS FOR VASCULAR DISEASE

[0001] The benefit of the October 24, 2012 filing date of United States provisional patent application serial number 61/717,758 is claimed under 35 U.S.C. § 119(e) in the United States, and is claimed under applicable treaties and conventions in all countries. The complete disclosure of the priority application is hereby incorporated by reference in its entirety.

TECHNICAL FIELD

[0002] This invention pertains to biomarkers for vascular disease.

BACKGROUND ART

[0003] Nitric oxide (NO) and hydrogen sulfide (H₂S) are gasotransmitters (gaseous signaling molecules) with many different functions in human biology, including specific roles in cardiovascular physiology. H₂S is a colorless, pungent gas that has multiple functions in physiology, including regulation of neurological function, intestinal inflammation, ischemia reperfusion injury, vasomotor tone regulation, and angiogenesis. NO plays critical roles in regulating vasomotor tone, inflammation, platelet activation, ischemic reperfusion injury, neurological function, and ischemic vascular remodeling. Both of these gaseous mediators are synthesized through enzymatic and non-enzymatic pathways. NO production classically occurs in an enzymatic manner via nitric oxide synthase isoenzymes, with non-enzymatic reduction of nitrite/nitrate serving as an alternative pathway. H₂S is produced by three principal enzymes (cystathionine γ -lyase, cystathionine β -synthase, and 3-mercaptopyruvate transferase), with non-enzymatic generation via glutathione and other sulfane sulfur redox pathways. The biological effects of NO and H₂S occur predominately through small molecule interactions and post-translational protein thiol modifications.

[0004] A clear understanding of H₂S bioavailability and metabolism has been impeded by controversies in its measurement. It is now well-established that non-analytical methods have produced anomalous measurements. The most widely used assay, the methylene blue method, lacks specificity and is subject to various sources of error in the measurement of bioavailable free H₂S. Aside from problematic assay methods, there remains a paucity of information regarding plasma free H₂S levels in normal human physiology and disease states, and regarding the relationship between hydrogen sulfide and nitric oxide bioavailability. The few papers that have explored this area have been hampered by the use of older assay methods and their associated limitations. Recent advances have produced accurate and reliable analytical approaches for measuring free bioavailable H₂S, including the ability to distinguish H₂S bioavailability from different tissues. Information has also been obtained regarding plasma free H₂S levels in young healthy adults; however, there is no comparable information for patients with cardiovascular disease.

[0005] It has recently been found that H₂S can be used as an alternative to NO in promoting vasodilation. H₂S also plays a role in regulating atherogenesis at the cellular and molecular levels. In addition, H₂S can affect endothelial nitric oxide synthase (eNOS) expression and function while increasing nitrite anion reduction to NO, thereby providing an alternate pathway to regulate NO bioavailability. However, no information currently exists regarding biochemical relationships between NO and H₂S in clinical settings.

[0006] Coronary Artery Disease (CAD) and Peripheral Artery Disease (PAD) currently afflict millions of adults in the U.S. and worldwide. However, early, asymptomatic CAD and PAD individuals often remain undiagnosed. Only about a third of U.S. PAD patients receive recommended therapies for secondary prevention. There exists an unfilled need for easy-to-implement diagnostic tests for PAD and CAD.

[0007] Several studies suggest that H₂S plays important roles in atherosclerosis pathogenesis, ischemic vascular remodeling, and tissue ischemia reperfusion injury. H₂S has been shown to decrease oxidation of low-density lipoprotein (LDL) and to decrease the uptake of oxidized LDL by macrophages in antioxidant responses. H₂S impairs the migration of monocytes into the subendothelial layer via reduced

expression of intercellular adhesion molecule-1 and monocyte chemoattractant protein-1. H₂S has also been found to inhibit foam cell formation and to inhibit vascular smooth muscle cell proliferation. H₂S has been reported to reduce vascular calcification in a rat model via down regulation of alkaline phosphatase activity and osteopontin gene down regulation.

[0008] Kleinbongard P, et al., Plasma nitrite concentrations reflect the degree of endothelial dysfunction in humans. *Free radical biology & medicine*. 2006;40:295-302 reported that plasma nitrite levels are inversely related to the number of cardiovascular risk factors, such that the greater the number of risk factors, the lower the plasma nitrite levels.

[0009] In experimental systems it has been reported that plasma free H₂S and NO have an influence upon one another. C. Coletta et al., Hydrogen sulfide and nitric oxide are mutually dependent in the regulation of angiogenesis and endothelium-dependent vasorelaxation. *Proceedings of the National Academy of Sciences of the United States of America*. 2012;109:9161-9166.

[0010] Differences in total hydrogen sulfide or nitric oxide bioavailability have been reported in studies using various detection methods that cannot detect specific biochemical forms of hydrogen sulfide or nitric oxide metabolites (e.g., the methylene blue method or sulfide-sensitive electrodes). Differences in hydrogen sulfide levels may occur in congestive heart failure, Kawasaki disease, Alzheimer's disease, vascular dementia, hypertension, and coronary heart disease. No prior study has suggested that plasma hydrogen sulfide or nitric oxide metabolites are indicative of peripheral artery disease or coronary artery disease. See, e.g.: Chen L, Ingrid S, Ding YG, Liu Y, Qi JG, Tang CS, Du JB: Imbalance of endogenous homocysteine and hydrogen sulfide metabolic pathway in essential hypertensive children. *Chinese medical journal* 2007;120:389-393; Heiss C, Lauer T, Dejam A, Kleinbongard P, Hamada S, Rassaf T, Matern S, Feelisch M, Kelm M: Plasma nitroso compounds are decreased in patients with endothelial dysfunction. *Journal of the American College of Cardiology* 2006;47:573-579; Jain SK, Bull R, Rains JL, Bass PF, Levine SN, Reddy S, McVie R, Bocchini JA: Low levels of hydrogen sulfide in the blood of diabetes patients and streptozotocin-treated rats causes vascular inflammation? *Antioxidants & redox signaling* 2010;12:1333-1337; Jain SK, Manna P, Micinski D,

Lieblong BJ, Kahlon G, Morehead L, Hoeldtke R, Bass PF, 3rd, Levine SN: In African American type 2 diabetic patients, is vitamin D deficiency associated with lower blood levels of hydrogen sulfide and cyclic adenosine monophosphate, and elevated oxidative stress? *Antioxidants & redox signaling* 2013;18:1154-1158; Jiang HL, Wu HC, Li ZL, Geng B, Tang CS: [Changes of the new gaseous transmitter H₂S in patients with coronary heart disease]. *Di 1 jun yi da xue xue bao = Academic journal of the first medical college of PLA* 2005;25:951-954; Kleinbongard P, Dejam A, Lauer T, Jax T, Kerber S, Gharini P, Balzer J, Zotz RB, Scharf RE, Willers R, Schechter AN, Feelisch M, Kelm M: Plasma nitrite concentrations reflect the degree of endothelial dysfunction in humans. *Free radical biology & medicine* 2006;40:295-302; Kovacic D, Glavnik N, Marinsek M, Zagozen P, Rovani K, Goslar T, Mars T, Podbregar M: Total plasma sulfide in congestive heart failure. *Journal of cardiac failure* 2012;18:541-548; Li XH, Zhang CY, Wu JX, Zhang T: Changes in plasma hydrogen sulfide and nitric oxide levels and their clinical significance in children with Kawasaki disease. *Chinese medical journal* 2011;124:3445-3449; Liu XQ, Liu XQ, Jiang P, Huang H, Yan Y: [Plasma levels of endogenous hydrogen sulfide and homocysteine in patients with Alzheimer's disease and vascular dementia and the significance thereof]. *Zhonghua yi xue za zhi* 2008;88:2246-2249; and Sun NL, Xi Y, Yang SN, Ma Z, Tang CS: [Plasma hydrogen sulfide and homocysteine levels in hypertensive patients with different blood pressure levels and complications]. *Zhonghua xin xue guan bing za zhi* 2007;35:1145-1148.

[0011] Numerous studies have tried to identify useful biomarkers for vascular diseases including PAD. Multiple indicators have been examined, including soluble adhesion molecules, inflammatory mediators, cytokines and other plasma proteins. However, the clinical utility of these markers as an indicator of vascular disease is still not clear and under active investigation. There is an unfilled need for improved biomarkers for cardiovascular disease.

DISCLOSURE OF THE INVENTION

[0012] We have discovered that plasma free hydrogen sulfide (H₂S) and nitric oxide (NO) levels are biomarkers associated with cardiovascular disease. Measuring the level of plasma free H₂S, either alone or in conjunction with measurement of the NO level, provides an indicator of cardiovascular disease due to Coronary Artery

Disease (CAD), Peripheral Artery Disease (PAD), or both. Several known markers of vascular inflammation were also measured: plasma thrombospondin-1 (TSP-1), Interleukin-6 (IL-6), and soluble intercellular adhesion molecule-1 (sICAM-1). Multiple regression analyses showed that elevated H₂S levels were associated with cardiovascular disease, independent of diabetes mellitus, hypertension, dyslipidemia, smoking status, race, gender, or age. Patients with PAD alone can be identified by the ratio of plasma H₂S to plasma NO. Patients thus identified as having PAD, CAD, or both, or as being at risk for those diseases may then be given appropriate therapies, including therapies that are otherwise previously known in the art.

[0013] To explore the relationship between bioavailable free H₂S and NO in patients with and without vascular disease, we undertook an unbiased observational clinical study to measure these molecules in plasma from patients presenting for coronary or peripheral angiography. We quantified nanomolar amounts of plasma free H₂S, using the monobromobimane (MBB) method of H₂S measurement. Plasma total NO levels were measured using tri-iodide chemiluminescent analysis.

[0014] We found that the plasma free H₂S levels were significantly elevated in patient populations with any early-stage vascular disease, including PAD and CAD. Increased H₂S levels were an independent risk factor, independent of previously known cardiovascular risk factors, as shown by multiple regression analyses. The plasma levels of H₂S thus are useful as biomarkers for vascular disease, and patients thus identified can be given preventive therapies.

[0015] We examined possible alternative interpretations for the observed elevated H₂S levels, e.g., that elevated plasma free H₂S levels might be a compensatory response to endothelial dysfunction and dysregulation of NO bioavailability. Interestingly, we found that plasma NO levels were significantly reduced in all patients with cardiovascular disease as compared to reported normal levels of NO in healthy persons, with subjects having PAD alone showing the greatest deficit in NO levels. We observed that patients with PAD also had higher levels of plasma free H₂S as compared to patients with CAD. These findings suggest that vascular disease mechanisms are different between patients with CAD and patients with PAD.

[0016] Plasma free H₂S levels alone did not distinguish the particular form of vascular disease; i.e., whether the patient had CAD, PAD, or both. In addition, plasma TSP-1, IL-6, and sICAM-1 levels did not correlate with H₂S or NO bioavailability in either vascular disease condition. However, we identified a novel inverse relationship with NO bioavailability in patients with PAD. The ratio of plasma free H₂S to NO was significantly greater in patients with PAD alone as compared to patients with CAD alone, or patients with both CAD and PAD, or control patients with no vascular disease. Thus, the ratio of plasma H₂S to plasma NO can be used to identify patients with PAD alone.

[0017] The novel method can be used in diagnosing, screening, and determining efficacy of therapeutic treatments for vascular disease. Because it uses standard blood specimens, there is minimal risk of harm to the patient. It is highly sensitive, accurate, and clinically validated.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] **Figures 1A and 1B** depict the effect of different types of blood collection tubes on plasma H₂S levels. **Figure 1A** depicts plasma free H₂S in the different types of collection tubes versus lysed red blood cells. **Figure 1B** illustrates the amount of cell-free hemoglobin in the collection tubes versus lysed red blood cells.

[0019] **Figure 2** depicts the ratio of plasma free H₂S to total NO, calculated for different vascular disease cohorts.

MODES FOR CARRYING OUT THE INVENTION

[0020] Embodiments of this invention include a method for diagnosing vascular disease, including early vascular disease (peripheral artery disease, coronary artery disease, or both) in a subject, comprising obtaining a blood sample from the subject, determining the level of free hydrogen sulfide in plasma from the blood sample; and comparing the level of free hydrogen sulfide in the sample to the level of free hydrogen sulfide in a normal control population, wherein a level of hydrogen sulfide in a sample that is significantly greater than the level in the normal control population is indicative of vascular disease.

[0021] A second embodiment includes a method for diagnosing peripheral artery disease, including early peripheral artery disease in a subject, comprising obtaining a blood sample from the subject, determining the level of free hydrogen sulfide and the level of nitric oxide in plasma from the blood sample; and comparing the ratio of free hydrogen sulfide to nitric oxide in the sample to the same ratio in a normal control population, wherein a ratio of hydrogen sulfide to nitric oxide in the sample that is significantly above the ratio found in the normal control population is indicative of peripheral artery disease.

METHODS

[0022] Abbreviations

CAD	Coronary artery disease
H ₂ S	Hydrogen sulfide
IL-6	Interleukin-6
LDL	Low-density lipoprotein
NO	Nitric oxide
PAD	Peripheral arterial disease
sICAM-1	Soluble Intercellular adhesion molecule-1
TSP-1	Thrombospondin-1

[0023] Materials and Tools

	5-sulfosalicylic acid
ABI	Ankle brachial index
ANCOVA	Analysis of covariance
	Drabkin assay
DTPA	Diethylenetriaminepentaacetic acid
	Eclipse XDB-C18 column
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
	Lithium heparin vacutainer tubes
MBB	Monobromobimane
Nitric oxide preservation solution	800 mM potassium ferricyanide, 17.6 mM N-ethylmaleimide, and 6% nonidet P40
RP-HPLC	Reversed phase high-performance liquid chromatography
	Shimadzu Prominence HPLC
Tris-HCl	Tris-hydrochloride

Example 1. Study design

[0024] The clinical study complied with the Declaration of Helsinki and was approved by the Institutional Review Board of the Louisiana State University Health Sciences Center at Shreveport (LSUHSC-S). It was registered at www.clinicaltrials.gov, NCT 01407172.

[0025] Patients over the age of 40 undergoing cardiac catheterization or peripheral angiogram via a major arterial approach at the LSUHSC-S cardiac catheterization laboratory or vascular surgery suite were enrolled in the study. The protocol broadly enrolled patients with suspected cardiovascular disease, which was then either confirmed or ruled out by angiography. Patients presented for angiography for the usual clinically accepted indications, including coronary angiography, unstable angina, non-ST elevation myocardial infarction, and stable angina. Peripheral angiography was performed for claudication and critical limb ischemia. A single physician operator performed the ankle brachial index (ABI) measurement using the straight wrapping method with an 8 MHz handheld Doppler as recommended by Aboyans V, et al., Measurement and interpretation of the ankle-brachial index: A scientific statement from the American Heart Association. *Circulation*. 2012;126:2890-2909. ABI was measured prior to angiography in all patients. In this way, patients were recruited and enrolled in an unbiased manner before they were separated into distinct disease cohorts as described in **Example 3**.

Example 2. Exclusion criteria

[0026] Seventy-eight patients were excluded from the study for various reasons. Patients were excluded if they were unable to provide informed consent, were enrolled in another clinical trial requiring the use of experimental therapeutic agents, were pregnant/nursing, or for other issues (e.g. cancellation of the procedure). Patients with non-atherosclerotic PAD (e.g. Buerger's disease) were excluded, as were those with an ABI>1.3 (indicative of a non-compressible vessel) unless they had already been diagnosed with PAD. To avoid delays in care, patients presenting with ST elevation myocardial infarction or cardiogenic shock were excluded.

Samples were also excluded for quality control failures (e.g. exceeding collection tube incubation time or specimen hemolysis).

Example 3. Patient cohorts and historical data

[0027] One-hundred ninety-three patients were studied, including 74 PAD patients and 119 non-PAD patients who were subsequently diagnosed with or without CAD after cardiac catheterization. Initial groups were based on the ABI or known PAD status: (1) patients without PAD as defined by $0.9 < \text{ABI} < 1.3$; or (2) patients with PAD as defined by an $\text{ABI} < 0.9$, or documented PAD on a peripheral angiogram, or with prior peripheral arterial interventions.

[0028] The two groups were then divided according to the presence or absence of CAD on the basis of their coronary angiogram using currently accepted definitions, i.e. the presence of stenosis greater than 50% in at least one major epicardial coronary artery (diameter > 2 mm). In a few cases, coronary angiography could not be performed, and CAD diagnosis was based on medical history or history of prior myocardial infarction.

[0029] Medical records were studied and patient interviews were conducted to assess traditional vascular disease risk factors: hypertension, dyslipidemia, diabetes, obesity, smoking, race, and gender.

Example 4. Blood collection

[0030] Under routine aseptic conditions, a vascular access sheath was placed in the femoral or radial artery. The location was at the operator's discretion. Then 12 ml of blood was collected into a sterile 20 ml syringe after bleed back (to ensure freedom from contamination with the saline used to flush the catheters). The blood sample was directly injected into two plastic 6 ml lithium heparin vacutainer tubes. Lithium heparin collection tubes were preferred, after initial experiments using healthy blood donors had demonstrated decreased hemolysis as compared to EDTA collection tubes, particularly when the EDTA tube was incorrectly filled. After inversion 4-5 times to ensure adequate mixing with anticoagulant, the tube was placed on ice and transported to the research laboratory. To ensure consistency

across samples, specimens were centrifuged at 9 minutes after collection, for 4 minutes at 1500 x g.

[0031] **Figures 1A and 1B** depict the effect of different types of blood collection tubes had on measured plasma H₂S levels. Venous blood was collected from healthy volunteers in either lithium heparin or EDTA vacutainer collection tubes. **Figure 1A** depicts plasma free H₂S for the different collection tube types versus lysed red blood cells (**p<0.01 versus lithium heparin collection tubes). **Figure 1B** depicts the amount of cell-free hemoglobin measured using the Drabkin assay in plasma from blood collected in various tube types versus lysed red blood cells (**p<0.01 versus lithium heparin collection tubes).

Example 5. MEASUREMENT OF PLASMA FREE H₂S AND TOTAL NO

[0032] Plasma samples were analyzed for H₂S as otherwise described in X. Shen et al Measurement of plasma hydrogen sulfide in vivo and in vitro. *Free radical biology & medicine*. 2011;50:1021-1031. Briefly, plasma free H₂S was measured by reversed-phase high-performance liquid chromatography (RP-HPLC) after derivatization with excess MBB to form the stable sulfide-dibimane derivative. Then 30 µl of plasma was mixed with 70 µl of 100 mM Tris-HCl buffer (pH 9.5, 0.1 mM DTPA), followed by the addition of 50 µl of 10 mM MBB. After 30 minutes, the reaction was stopped by adding 50 µl of 200 mM 5-sulfosalicylic acid. Then the sample was centrifuged and the supernatant was analyzed using a Shimadzu Prominence HPLC with fluorescence detection (λ_{ex}: 390 nm and λ_{em}: 475 nm) and an Eclipse XDB-C18 column. A separate aliquot of plasma was placed in nitric oxide preservation solution (800 mM potassium ferricyanide, 17.6 mM N-ethylmaleimide, and 6% nonidet P40) for tri-iodide NO chemiluminescence analysis. All plasma levels of H₂S or NO were quantified, respectively, with sulfide dibimane and sodium nitrite standard curves. Finally, a third plasma aliquot was used for enzyme-linked immunosorbent assay (ELISA) measurement of TSP-1, IL-6, and sICAM-1 levels using commercially available ELISA assays (R&D Systems, Cayman Chemicals and Thermo scientific respectively) per the manufacturer's instructions.

Example 6. Statistical analysis

[0033] Data were analyzed with Statistical Package for Social Sciences version 16.0 (SPSS for Windows 17 Inc, Chicago, IL, USA). A p value of <0.05 was required for statistical significance. Linear regression analysis, independent samples t-test, Chi-square test, Spearman and Pearson's correlation analysis were performed, based on the normality of the data and type of variable.

RESULTS**Example 7. Demographics and baseline characteristics**

[0034] Clinical characteristics of the enrolled patients were analyzed based on the presence or absence of vascular disease, i.e. patients with either one or both of PAD and CAD, versus those without vascular disease. The data report phenotypic characteristics and associated percentages of the cohorts for characteristics including race, gender, and traditional cardiovascular risk factors (dyslipidemia, diabetes mellitus, hypertension, obesity, smoking). Category percentages per cohort are listed in **Table 1**. **Table 2** lists the frequency with which various pharmaceutical agents (aspirin, Clopidogrel, other antiplatelet, beta blocker, ACE/ARB, statin, Cilostazol) were taken by each patient cohort.

[0035] Table 1. Patient demographic characteristics

		Diagnosis			
		No vascular disease (N=53)	Any vascular disease (N=140)	CAD alone (N=66)	PAD alone (N=13)
		Mean $\pm$ sd	Mean $\pm$ sd	Mean $\pm$ sd	Mean $\pm$ sd
Age		53 $\pm$ 8.6	57 $\pm$ 8.5	55 $\pm$ 8.7	58 $\pm$ 8.0
		Count (N%)	Count (N%)	Count (N%)	Count (N%)
Gender	Male	15 (28.3%)	85 (60.7%)	39 (59.1%)	9 (69.2%)
	Female	38 (71.7%)	55 (39.3%)	27 (40.9%)	4 (30.8%)
Race	Non-black	14 (26.4%)	66 (47.1%)	38 (57.6%)	5 (38.5%)
	Black	39 (73.6%)	74 (52.9%)	28 (42.4%)	8 (61.5%)
Dyslipidemia	No	32 (60.4%)	38 (27.1%)	19 (28.8%)	4 (30.8%)
	Yes	21 (39.6%)	102 (72.9%)	47 (71.2%)	9 (69.2%)
Diabetes mellitus	No	36 (67.9%)	85 (60.7%)	38 (57.6%)	9 (69.2%)
	Yes	17 (32.1%)	55 (39.3%)	28 (42.4%)	4 (30.8%)
Hypertension	No	10 (18.9%)	10 (7.1%)	3 (4.5%)	2 (15.4%)
	Yes	43 (81.1%)	130 (92.9%)	63 (95.5%)	11 (84.6%)
Obesity	Non-obese	17 (32.1%)	68 (48.6%)	28 (42.4%)	8 (61.5%)
	Obese	36 (67.9%)	72 (51.4%)	38 (57.6%)	5 (38.5%)
Smoking	Non- smoker	37 (69.8%)	70 (50%)	35 (53.0%)	4 (30.8%)
	Smoker	16 (30.2%)	70 (50%)	31 (47.0%)	9 (69.2%)

[0036] Table 2. Patient medications taken

Home Medications		Diagnosis							
		No vascular disease		Any vascular disease		CAD alone		PAD alone	
		Count	N %	Count	N %	Count	N %	Count	N %
Aspirin	No	24	45.3%	42	30.0%	18	27.3%	3	23.1%
	Yes	29	54.7%	98	70.0%	48	72.7%	10	76.9%
Clopidogrel	No	46	86.8%	92	65.7%	46	69.7%	12	92.3%
	Yes	7	13.2%	48	34.3%	20	30.3%	1	7.7%
Other antiplatelet	No	53	100.0%	138	98.6%	65	98.5%	12	92.3%
	Yes	0	0.0%	2	1.4%	1	1.5%	1	7.7%
Beta blocker	No	21	39.6%	43	30.7%	18	27.3%	10	76.9%
	Yes	32	60.4%	97	69.3%	48	72.7%	3	23.1%
ACE/ARB	No	28	52.8%	38	27.1%	20	30.3%	5	38.5%
	Yes	25	47.2%	102	72.9%	46	69.7%	8	61.5%
Statin	No	28	52.8%	40	28.6%	18	27.3%	5	38.5%
	Yes	25	47.2%	100	71.4%	48	72.7%	8	61.5%
Cilostazol	No	53	100%	137	97.9%	66	100%	12	92.3%
	Yes	0	0.0%	3	2.1%	0	0.0%	1	7.7%

Example 8. Plasma Free H₂S and vascular disease

[0037] Linear regression analysis and independent samples t-test were employed to examine the relationship between H₂S bioavailability and specific cardiovascular disease status. Plasma free H₂S levels in patients with either vascular disease (mean = 441.0 nM) were significantly elevated compared to patients without vascular disease (mean = 368.5 nM, $p = 0.01$). Importantly, the elevated H₂S levels that were seen in patients with either vascular disease, as compared to those without vascular disease, were independent of traditional risk factors including age, race, gender, and history of diabetes mellitus, hypertension, hyperlipidemia, smoking, or obesity. Likewise, plasma free H₂S levels in patients with PAD alone (mean = 514.4 nM, $p = 0.007$) or CAD alone (mean = 443.8 nM, $p = 0.02$) were significantly greater than patients without either vascular disease (mean = 368.5 nM). However, differences in plasma free H₂S levels between patients with CAD alone versus PAD alone were not statistically significant ($p = 0.18$). Thus, plasma free H₂S alone was not able to discriminate between the two forms of vascular disease at a statistically significant level. Importantly, total NO levels were significantly reduced in patients with PAD alone as compared to patients without vascular disease. **Table 3** shows these results.

[0038] Table 3. Plasma Free H₂S and Total NO levels in vascular disease.

	No vascular disease n=53	Any vascular disease n=140	CAD alone n=66	PAD alone n=13
Plasma free H₂S (nM)	368.53 ± 20.87	441.04 ± 15.40 ($p=0.010$)	443.89 ± 21.67 ($p=0.020$)	514.48 ± 62.05 ($p=0.007$)
Total NO levels (nM)	64.71 ± 1.12	64.71 ± 1.07 ($p=0.997$)	61.56 ± 1.09 ($p=0.743$)	38.86 ± 1.20 ($p=0.034$)

All data are reported as mean ± standard error of the mean. P values are compared to the no vascular disease group.

Example 9. Free H₂S and smoking status

[0039] An independent samples t-test showed that patients who were current smokers had significantly elevated plasma free H₂S levels (mean = 452.28 nM, $p = 0.03$) as compared to those who were not current smokers (mean = 396.10 nM). However, linear regression analysis revealed that when controlled for the presence of vascular disease the difference between smokers and non-smokers was not statistically significant ($p = 0.073$). A one-way analysis of covariance (ANCOVA) between subjects was performed to examine the effect of smoking on the relationship between H₂S and vascular disease. ANCOVA revealed that patients with vascular disease had significantly higher adjusted H₂S levels (mean $\pm$ se = 438.54 $\pm$ 14.73) as compared to patients without vascular disease (mean $\pm$ se = 375.15 $\pm$ 24.11, $F(1,190) = 4.97$, $p = 0.027$).

Example 10. Inflammatory indicators and H₂S levels

[0040] Several inflammatory markers associated with cardiovascular disease were examined, including sICAM-1, IL-6, and TSP-1. There was no significant difference noted among the various inflammatory markers between the patient cohorts, although an increasing trend for IL-6 was noted in patients with PAD alone. (See **Table 4.**)

[0041] In contrast to the previous study by Smadja et al., we did not observe an increase in plasma TSP-1 levels. This is likely because our study included Caucasian and African-American males and females, whereas, the Smadja et al. study enrolled only “white men younger than 70 years of age.” We found that African-American men had significantly lower plasma free H₂S than Caucasian men.

[0042] Finally, Pearson correlation analysis did not reveal any significant association between plasma free H₂S and levels of IL-6, sICAM-1, or TSP-1.

[0043] Table 4. Plasma sICAM-1, IL-6, and TSP-1 levels in vascular disease.

	No vascular disease n=53	Any vascular disease n=140	CAD alone n=66	PAD alone n=13
sICAM-1 ng/mg	365.04 ± 1.05	389.16 ± 1.03 (p=0.324)	375.40 ± 1.04 (p=0.704)	378.80 ± 1.16 (p=0.766)
IL-6 ng/mg	12.83 ± 1.20	17.53 ± 1.12 (p=0.156)	13.24 ± 1.17 (p=0.900)	25.89 ± 1.37 (p=0.093)
TSP-1 ng/mg	40.95 ± 2.25	37.09 ± 1.37 (p=0.143)	37.33 ± 1.95 (p=0.230)	32.43 ± 4.05 (p=0.093)

All data are reported as mean ± standard error of the mean. P values are compared to no vascular disease group.

Example 11. NO and vascular disease

[0044] The Kleinbongard et al study reported lower plasma nitrite levels in the presence of a higher number of vascular disease risk factors. Interestingly, we found that patients with no vascular disease had considerably lower plasma total NO levels (mean = 64.7 nM) as compared to previously reported 'normal' NO levels of healthy adults (~150-300 nM). However, the patients without clinically-diagnosed vascular disease still had numerous risk factors (**Table 1**) that are known to contribute to endothelial cell dysfunction and reduced NO levels; thus these results are not inconsistent. Linear regression analysis revealed that plasma total NO levels were only significantly lower only in patients with PAD alone (mean=38.86 nM, p=0.034) as compared to subjects without vascular disease. Together, these data highlight that plasma NO bioavailability was considerably diminished in our study subjects as compared to normal healthy subjects, and that patients with PAD alone had a significant deficit in total NO bioavailability.

Example 12. Ratio of H₂S to NO and vascular disease

[0045] We examined the relationship between plasma free H₂S and NO. We analyzed the data to discriminate between different forms of vascular disease, but

Spearman correlation analysis between plasma NO and free H₂S in all patients with vascular disease did not reveal a significant correlation. We next calculated plasma H₂S/NO concentration ratios for each cohort and analyzed the ratio using linear regression analysis. Importantly, the plasma H₂S/NO ratio was significantly elevated in the PAD-only group ($p = 0.031$), both as compared to patients with CAD alone, and as compared to those without vascular disease.

[0046] **Figure 2** depicts the molar ratio of plasma free H₂S to total NO, for each of the vascular disease cohorts. The free H₂S/NO ratio was significantly elevated in patients with PAD alone over all other comparison cohorts, $*p < 0.05$. No vascular disease, $n = 53$; CAD alone, $n = 66$; PAD alone, $n = 13$; and any vascular disease, $n = 140$. Together, these data revealed the novel finding that patients with PAD alone have significantly greater amounts of plasma free H₂S as compared to NO. The data are shown in **Table 5**. An [H₂S] : [NO] molar ratio above about 6.5 is suggestive of PAD. The higher the ratio (greater than 8, greater than 10), the more likely the patient has PAD alone.

[0047] **Table 5. Ratio of plasma H₂S to plasma NO in patient groups.**

Group	No.	Plasma [H ₂ S] : [NO] molar ratio	P value (compared to control)
Control (no CAD or PAD)	53	5.26 ± 1.14	
All PAD	74	5.87 ± 1.13	0.513
PAD alone	13	12.06 ± 1.23	0.004**
PAD or CAD or both	140	6.23 ± 1.08	0.260
PAD and CAD	61	5.05 ± 1.14	0.830
CAD alone	66	6.62 ± 1.10	0.174

**PAD alone compared to CAD alone: P-value = 0.031.

**PAD alone compared to PAD and CAD: P-value = 0.002

[0048] Possible therapies that could be employed following a diagnosis of decreased plasma H₂S levels include treatment with one or more of the following: statin(s), cilostazol, a suitable NO-based drug or NO prodrug, cardiac catheterization, vascular stent placement, vascular angioplasty, vascular bypass surgery, supervised exercise therapy, or other treatments known in the art for PAD or CAD.

Example 13. Plasma total sulfide and bound sulfane sulfur indicate cardiovascular disease status based on race

[0049] The relationship between plasma sulfide bioavailability and cardiovascular disease may depend on an individual's race or sex. Figures 3A – 3C illustrate measured levels of plasma total sulfide and bound sulfane sulfur from American males of either African (A) or European (C) ancestry, grouped as having coronary artery disease (CAD), peripheral artery disease (PAD), any form of vascular disease (AVD), or controls without vascular disease. CAD was diagnosed by angiography. PAD was diagnosed by ankle-brachial index measurements. Figure 3A depicts that plasma bound sulfane sulfur was significantly reduced in individuals of African ancestry with CAD alone, PAD alone, or both CAD and PAD, as compared to individuals of African ancestry without cardiovascular disease (control). By contrast, in individuals of European ancestry plasma bound sulfane sulfur levels were significantly reduced only for individuals with PAD alone. Figure 3B depicts that bound sulfane sulfur was significantly reduced in individuals of African ancestry with AVD. Figure 3C illustrates that, for individuals of African ancestry but not for individuals of European ancestry, plasma total sulfide levels were significantly reduced for individuals with AVD as compared to control. Together, these data demonstrated that decreased plasma bound sulfane sulfur and decreased total sulfide levels are significantly associated with the presence of cardiovascular disease in males of African ancestry. Comparable studies for females will be conducted in the future.

[0050] The complete disclosures of all references cited in the specification are hereby incorporated by reference in their entirety, as is the complete disclosure of priority application serial number 61/717,758. In the event of an otherwise irresolvable conflict, however, the disclosure of the present specification shall control.

CLAIMS

What is claimed:

1. A method for treating or preventing vascular disease in a vertebrate patient, said method comprising the steps of:

(a) assaying plasma free H_2S concentration in a blood sample from the patient; and identifying the patient as having vascular disease or as being at risk for vascular disease if the concentration of plasma free H_2S in the patient is substantially higher than the mean level in a control population having no vascular disease; and

(b) administering a therapeutic for or prophylaxis against vascular disease to the patient if the patient is thus identified as having vascular disease or as being at risk for vascular disease.

2. A method for treating or preventing peripheral artery disease in a vertebrate patient, said method comprising the steps of:

(a) assaying plasma free H_2S concentration and plasma NO concentration in a blood sample from the patient; and identifying the patient as having peripheral artery disease or as being at risk for peripheral artery disease if the ratio of the concentration of plasma free H_2S in the patient to the concentration of plasma NO in the patient is substantially higher than the mean ratio in a control population having no peripheral artery disease; and

(b) administering a therapeutic for or prophylaxis against peripheral artery disease to the patient if the patient is thus identified as having peripheral artery disease or as being at risk for peripheral artery disease.

3. A method for treating or preventing vascular disease in a human patient of African ancestry, said method comprising the steps of:

(a) assaying the bound sulfane sulfur concentration in a blood sample from the patient; and identifying the patient as having vascular disease or as being at risk for vascular disease if the concentration of bound sulfane sulfur in the patient is substantially lower than the mean level in a control population of African ancestry of the same sex as the patient but having no vascular disease; and

(b) administering a therapeutic for or prophylaxis against vascular disease to the patient if the patient is thus identified as having vascular disease or as being at risk for vascular disease.

4. A method for treating or preventing vascular disease in a human patient of African ancestry, said method comprising the steps of:

(a) assaying the total sulfide concentration in a blood sample from the patient; and identifying the patient as having vascular disease or as being at risk for vascular disease if the concentration of total sulfide in the patient is substantially lower than the mean level in a control population of African ancestry of the same sex as the patient but having no vascular disease; and

(b) administering a therapeutic for or prophylaxis against vascular disease to the patient if the patient is thus identified as having vascular disease or as being at risk for vascular disease.

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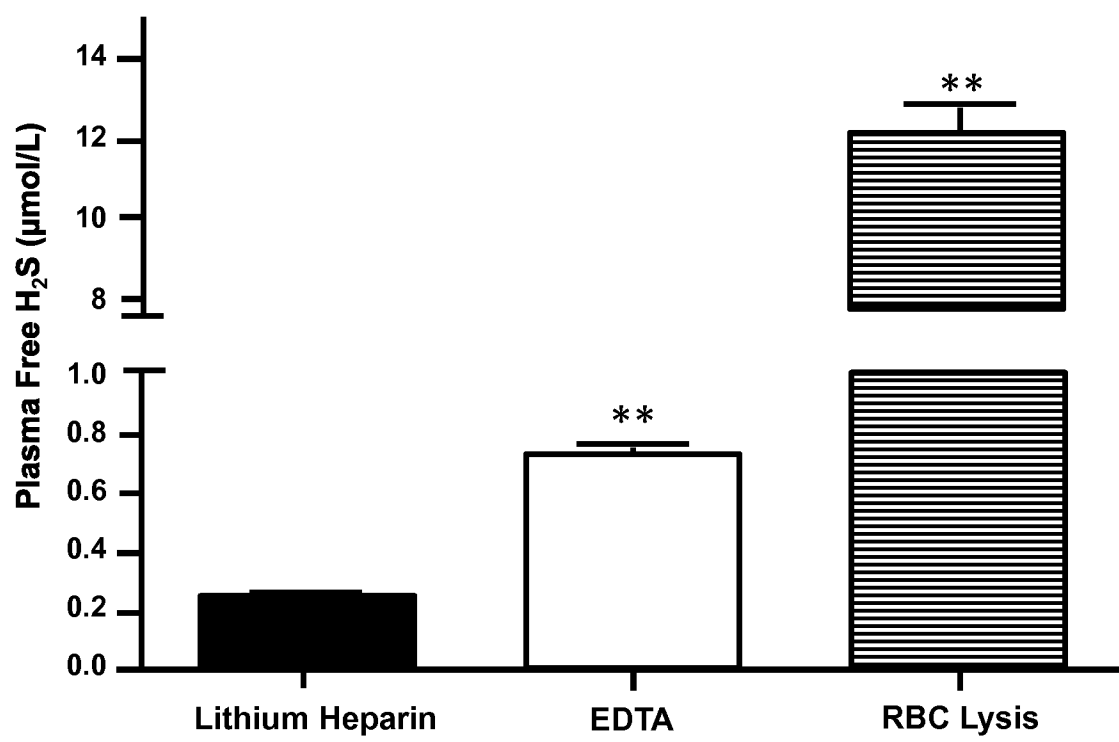


Fig. 1A

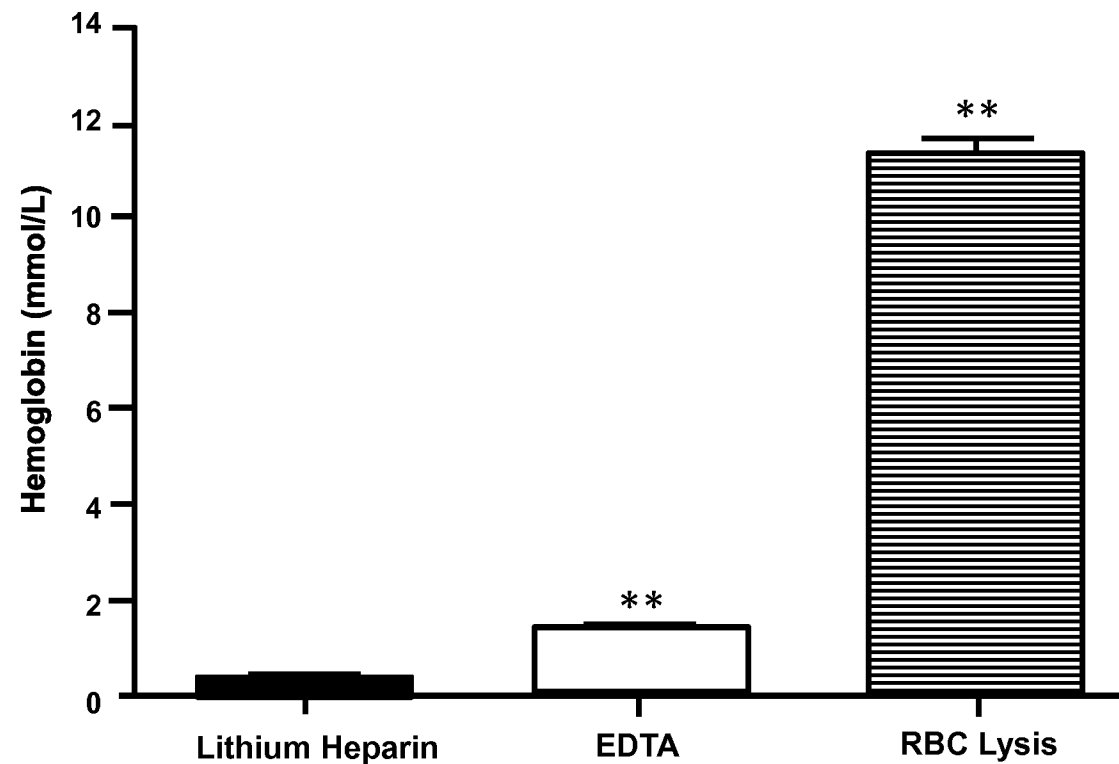
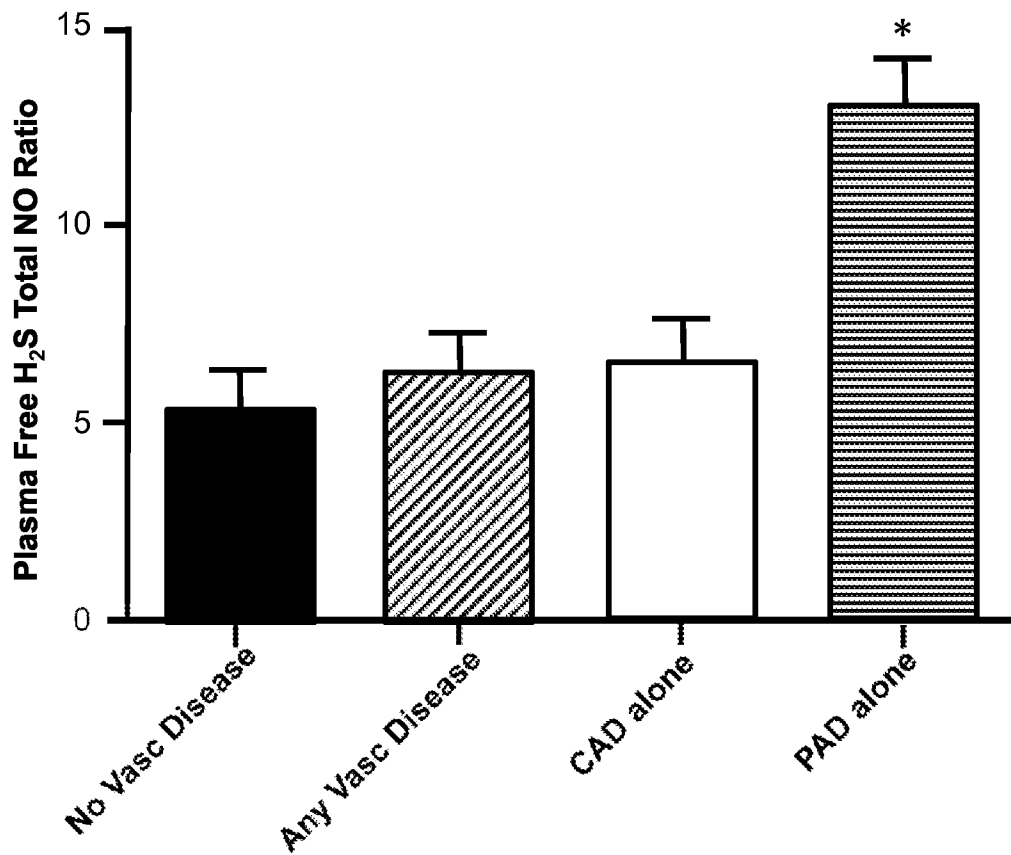


Fig. 1B

2 / 4**Fig. 2**

3 / 4

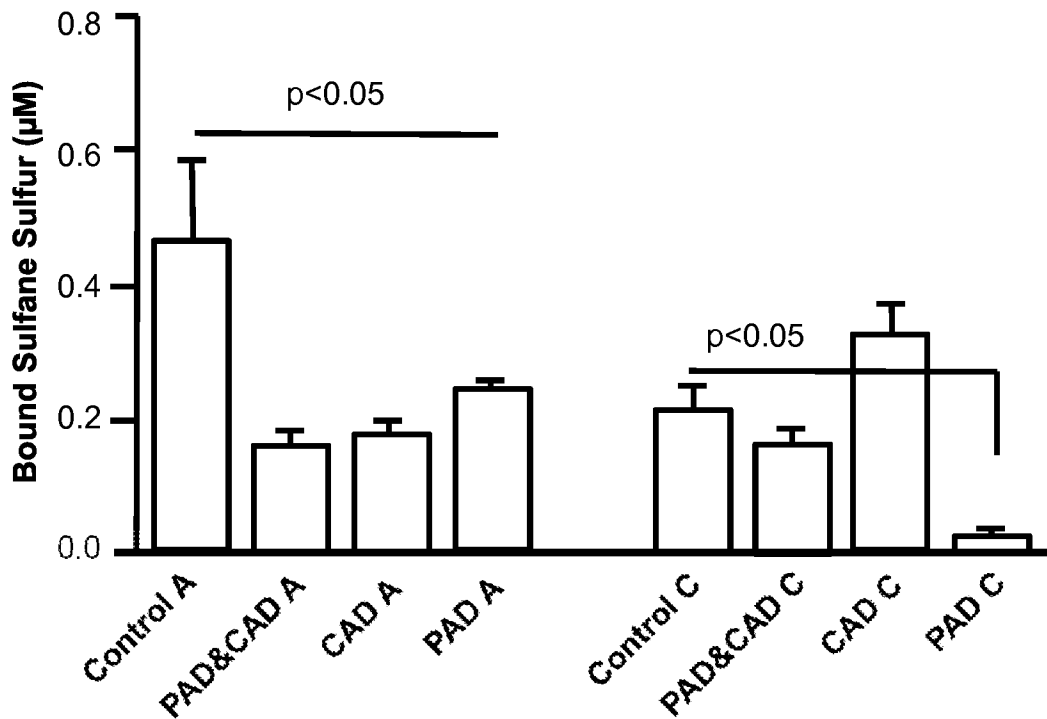


Fig. 3A

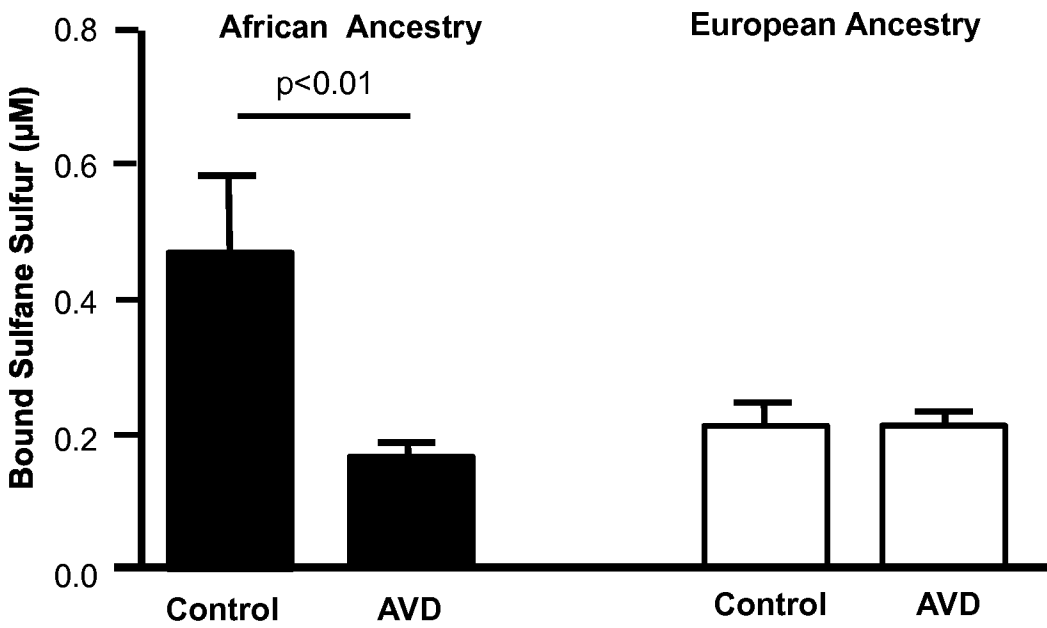


Fig. 3B

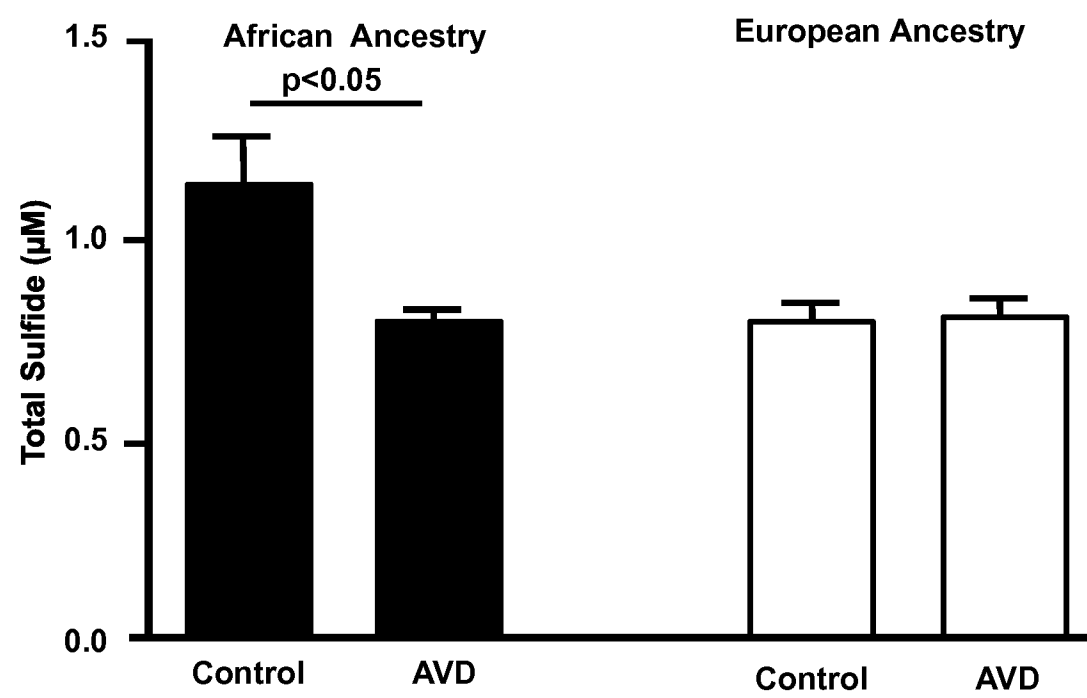


Fig. 3C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2013/066012

A. CLASSIFICATION OF SUBJECT MATTER		
G01N 33/84 (2006.01) G01N 33/50 (2006.01) G01N 33/96 (2006.01) According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
G01N 33/50, 33/48, 33/84, 33/96		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
EAPATIS, RUPAT, Patentscope, DWPI, NCBI (PubMed), SpringerLink		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2007/0072798 A1 (OY JURILAB LTD) 29.03.2007, abstract, paragraphs [0001]-[0003], [0038]-[0039], [0043]-[0044], [0050], [0166], table 2, 3, claims 1-7, 29	1, 2
Y	CALVERT J W. et al. Novel insights into Hydrogen sulfide-mediated cytoprotection, Antiox. Redox signaling, 2010, Vol.12, no.10, pp. 1203-1216, especially abstract, fig.1	1, 2
Y	US 7708989 B2 (NITROMED, INC.) 04.05.2010, abstract, claims, fig. 1, col. 10, lines 44-col. 11, lines 1-2, col. 11, lines 35-col. 12, lines 1-18	1- 4
Y	TOOHEY JI. Possible involvement of sulfane sulfur in homocysteine-induced atherosclerosis. Med Hypotheses, 2001, 56(2), pp. 259-261, (abstract), [online]. Retrieved from PubMed, PMID: 11425300	3,4
Y	DRAGAN KOVACIC et al. Total Plasma Sulfide in Congestive Heart Failure. Journal of Cardiac Failure, 2012, Vol. 18, no. 7, pp.541-548	3, 4
☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
* Special categories of cited documents:	“T”	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
“A” document defining the general state of the art which is not considered to be of particular relevance	“X”	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
“E” earlier document but published on or after the international filing date	“Y”	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
“L” document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	“&”	document member of the same patent family
“O” document referring to an oral disclosure, use, exhibition or other means		
“P” document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search	Date of mailing of the international search report	
18 December 2013 (18.12.2013)	13 February 2014 (13.02.2014)	

Name and mailing address of the ISA/ FIPS Russia, 123995, Moscow, G-59, GSP-5, Berezhkovskaya nab., 30-1 Facsimile No. +7 (499) 243-33-37	Authorized officer N. Chudina Telephone No. 8(495)531-65-15
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Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2013/066012

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

According to Rule 13 of the Regulations under the PCT the claimed inventions does not meet the requirement of unity of invention *a priori*, therefore independent claims 1-4 form several groups of inventions, which are not so linked as to form a single general inventive concept.

The special technical feature of independent claims 1 and 2 is assaying plasma free H₂S concentration in a blood sample.

The special technical feature of independent claims 3 is assaying the bound sulfur concentration in a blood sample.

The special technical feature of independent claims 4 is assaying the total sulfide concentration in a blood sample.

Hence, claims comprise 3 groups of inventions, namely:

1 invention – claims 1-2

2 invention – claim 3

3 invention – claim 4

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.