January 2013

Gene Expression Signature As A Diagnostic Test For Thoracic Aortic Aneurysms: A Validation Study

Adam Sang

Gene Expression Signature as a Diagnostic Test for Thoracic Aortic Aneurysms: A Validation Study

A Thesis Submitted to the

Yale University School of Medicine

In Partial Fulfillment of the Requirements for the

Degree of Doctor of Medicine

By

Adam Xianpeng Sang

2013

GENE EXPRESSION SIGNATURE AS A DIAGNOSTIC TEST FOR THORACIC AORTIC ANEURYSMS: A VALIDATION STUDY. Adam X. Sang, Catalin C. Barbacioru, Gregory A. Kuzmik, Laura Ondere, Maryann Tranquilli, Jon K. Sherlock, John A. Elefteriades. Department of Surgery, Yale University School of Medicine, New Haven, CT.

Thoracic aortic aneurysms (TAAs) are clinically-silent diseases that predispose individuals to life-threatening aortic dissection or rupture. If detected early, TAAs can be safely treated with elective surgery. Therefore, there is a great clinical need in screening for TAAs. However, no reliable screening programs exist, and radiographic imaging is too costly and harmful for screening entire at-risk populations. We hypothesize that a novel diagnostic blood test based on the gene-expression profiles of a previously-identified panel of 41 genes (a RNA signature) is greater than 70% sensitive in detecting TAAs. Using RNA extracted from peripheral blood cells of 40 individuals (24 TAA patients and 16 spousal controls), we performed real-time PCR using customized TaqMan Array Cards to analyze the relative expression levels of this panel of genes. A 10-fold cross-validation study based on these expression levels was used to predict whether each sample belonged to a TAA patient or a spousal control. When compared with each sample's true clinical status, this RNA signature-based prediction model was 83% accurate, with a sensitivity of 88% and a specificity of 75%. Furthermore, the expression levels of the individual genes were largely consistent with their expression levels from a previous study of this RNA signature ($r^2 = 0.75$ for TAA patient samples, and $r^2 = 0.73$ for spousal control samples), supporting the reproducibility of this test. Altogether, these findings demonstrate that gene-expression profiling is an accurate, sensitive, and reliable method for detecting TAAs. If utilized as a clinical screening test, this RNA signature has the potential to detect silent TAAs, leading to earlier diagnosis and reduced mortality of this dangerous condition.

Acknowledgements

I would like to first and foremost express my gratitude towards my research mentor, Dr. John A. Elefteriades. Since our first meeting in the spring of 2009, Dr. Elefteriades has both guided my research endeavors as well as mentored me towards my career aspirations. He is also a true professional and teacher, and demonstrates for me the ideal characteristics of a surgeon.

Second, I want to thank Dr. Elefteriades' staff team for their tremendous support through the years. Maryann Tranquilli, Lorena Capone, Jodi Jacobson, Marianne McCarthy, and Carol Calini have all helped me so much since I arrived, and I share a family-like bond with every one of them.

Then, to my fellow students in the Elefteriades lab, thank you! Thank you to Adelina Hung, who started with me in lab from day one and has offered me endless support and encouragement. Thank you to Gregory Kuzmik for being my research and grant-writing buddy. Thank you to Mohammad Zafar for offering to help whenever you could!

I am indebted to Mae Geter, Donna Carranzo, and Dr. John Forrest from the Office of Student Research for their guidance and support through Summer and Short-term Research Awards.

I would also like to thank several individuals with whom this project would not have been possible: Paula Miccinesi, from Life Technologies; Karyn Ronski, from Dr. Jeff Sklar's lab; Adam Arterbery, from Dr. Clifford Bogue's lab; and Yvette Stong, from Yale Center for Clinical Investigation.

Finally, I would like to thank the two most amazing people who have helped me every step along the way, my mom and dad. This is another milestone, and it is all because of you!

Adam Sang

February 1st, 2013

Table of Contents

Introduction

Section 1: Thoracic aortic aneurysm is an asymptomatic disease

Thoracic aortic aneurysm (TAA) is a chronic disease that affects the main artery of the body, the aorta. The aorta is a large elastic artery that originates from the heart, ascends and then descends in the chest cavity (thoracic aorta), traverses the diaphragm, and continues its downward course as the abdominal aorta. Important arteries that supply the head, arms, spine, gastrointestinal tract, and kidneys all branch from the aorta. The average size of a "normal" human ascending aorta is approximately 3.0 cm for men and 2.7 cm for women, based on older studies (1, 2).

An aneurysm is an enlargement, or dilatation, of a blood vessel—the extent of dilatation for an aneurysm is usually defined as greater than 50% of the "normal" diameter of the blood vessel (3). For the ascending aorta, physicians have also adopted 4.0 cm as a hard cutoff for the upper limit of a normal (4). This thesis work focuses on ascending TAAs and arch TAAs, which make up 60% of all TAAs (5).

A great physician in the late nineteenth century once remarked that "there is no condition more conducive to clinical humility than aneurysm of the aorta" (6). This physician was Sir William Osler, one of the fathers of modern medicine, the first Professor of Medicine at Johns Hopkins Hospital, and founder of our current medical residency structure. His observation highlights one of the challenges of the management of aortic aneurysms that still persists today, namely, the initial diagnosis and detection of a disease that is a clinically-silent killer.

TAAs are asymptomatic in over 90% of cases (4, 6). They rarely produce pain. In those patients who do complain of pain from stretching of the aortic tissue or discomfort from impingement, the pattern and characteristic of the symptoms are non-specific and varied. We have published case reports in which non-specific pain portended an impending acute disaster for patients with TAAs, discovered only with surgical exploration (7-9).

Additional symptoms can arise when the aneurysm progresses to very large dimensions. If the aneurysm involves the aortic root, the aortic valves can become incompetent in preventing backflow of blood into the heart. This aortic regurgitation can produce a diastolic heart murmur on auscultation. With severe aortic regurgitation, the patient can become short of breath and develop heart failure. Occasionally, the enlarged aorta can exert a mass effect and compress on the trachea or the esophagus.

Section 2: Thoracic aortic aneurysms are likely more prevalent than we recognize

How common are thoracic aortic aneurysms?

Given their asymptomatic nature, it is difficult to truly estimate the prevalence of this disease in the general population, as many individuals can harbor TAAs without ever knowing about it. Recent studies that examined the prevalence of asymptomatic TAAs using population-wide computed tomography (CT) screening report a prevalence range of 0.16% to 0.34% (10, 11). However, these studies used a fixed cutoff diameter of 5 cm to define an aneurysm, thereby omitting potential aneurysms in the 4 to 5 cm range.

It is also interesting to consider the rate at which new cases of TAAs are being discovered and diagnosed. The annual incidence of TAAs is approximately 6 to 10 per 100,000 patient-years (12, 13). Men and women have similar incidences of TAA, although women are considerably older at the time of detection (13). This is in contrast to abdominal aortic aneurysms, which predominately affect men.

A Swedish national healthcare registry study reported that from 1987 to 2002, the incidence of thoracic aortic diseases (includes both TAAs and thoracic aortic dissections, which are described below) rose by 52% to 16.3 per 100,000 patient-years in men, and by 28% to 9.1 per 100,000 patient-years in women (14). While the increasing incidence of TAAs, which continues to modern times, is undoubtedly influenced by the aging population and increasingly frequent use of radiographic imaging, there is some evidence suggesting a bona fide increase in the true incidence of this disease (15, 16).

Altogether, aortic aneurysms of both the thorax and abdomen combine to form the 19th leading cause of death in the United States today. In addition, aortic aneurysms are the 15th leading cause of death in individuals over the age of 65 (17).

Section 3: The underlying etiology of thoracic aortic aneurysms is diverse.

TAAs can either be sporadic or genetically inherited. The majority of TAAs, to our current knowledge, are sporadic—these aneurysms are thought to be the result of a chronic degeneration of the aortic wall. Hypertension, advanced age, and smoking can accelerate this process: high blood pressure increases the wall tension of the aorta, while advanced age and

smoking cause global tissue weakening (4). The common *histological* finding associated with the degenerative changes of the aortic wall is termed cystic medial necrosis. This process involves a gradual loss of both the cellular (smooth muscle cells) and acellular (elastic fibers) elements of the middle layer of the aortic wall, the tunica media (5). This loss is in part caused by increased amounts of proteolytic enzymes, such as matrix metalloproteinases (18, 19). In addition, there are increased numbers of inflammatory cells, specifically T lymphocytes and macrophages, in the tunica media of aneurysmal aortas (20).

Other TAAs have a genetically-inherited component. The most dramatic manifestation of such TAAs can be found in connective tissue disorders. Connective tissue disorders are syndromes that affect multiple tissue and organ types, and virtually all patients with these syndromes develop dilatation of the aorta. The most common connective tissue disorder is Marfan syndrome, an autosomal dominant disease in which a mutation in the fibrillin (FBN1) gene reduces its ability to sequester transforming growth factor beta (TGF-β). The excessive TGF-β results in the weakening of several components of the extracellular matrix, including elastin. Other genetic syndromes associated with TAAs include Ehlers-Danlos syndrome (a defect in the synthesis of collagen) and the recently discovered Loeys-Dietz syndrome (a mutation in TGF-β).

TAAs associated with connective tissue disorders, however, only represent the tip of the iceberg for genetically-inherited forms of TAA. An isolated TAA, passed from generation to generation, in the absence of other medical conditions or syndromes, is increasingly recognized as a clinical entity. This “familial TAA” is likely a common manifestation of an unknown number of heterogeneous genetic mutations. Approximately 21% of patients diagnosed with TAAs have at least one family member who also has a known aneurysm (21). We can surmise that many

additional TAA patients have family members harboring unknown aneurysms. Of these familial forms of TAA, the majority (77%) appears to be inherited in an autosomal dominant manner, based on pedigree studies (21, 22). The penetrance and expressivity of familial TAAs are varied. Currently, individual mutations in five different genes (TGFβR1, TFGβR2, FBN1, ACTA2, and MYH11) have been identified as contributing to aneurysm formation (3, 23). Searching for additional mutations is an active area of research.

Several other lines of evidence support the concept that TAA is a genetic disease. First, in patients with known TAAs, there is an increased prevalence of other types of aneurysms, such as abdominal aortic aneurysms (24, 25) and intracranial aneurysms (26, 27), beyond that of the general population. This phenomenon suggests that rather than multiple, independent aneurysms forming sporadically, there is a common underlying molecular pathway, perhaps a single gene defect, that is responsible for the pathogenesis of different aneurysm types. Second, patients with bicuspid aortic valves (BAV), a congenital heart defect in which the aortic valve has two leaflets instead of the usual three, are abnormally susceptible to aortic dilatation (28). BAV is a genetic condition that appears to be inherited in an autosomal dominant manner. TAA patients with BAVs also have faster rates of aortic dilatation than TAA patients with normal tricuspid aortic valves (29). Perhaps BAV and TAA are variable manifestations of the same heritable disease (23).

Section 4: All forms of thoracic aortic aneurysms can progress to lethal outcomes

Despite their diverse etiologies, all TAAs are prone to two lethal acute events: aortic dissection and aortic rupture. Whereas TAA is a clinically-silent disease, aortic dissection and aortic rupture are dramatic and life-threatening events, and must be prevented.

Aortic dissection is a catastrophic event in which the layers of the aortic wall split apart. A tear in the tunica intima (the innermost layer of the aorta) allows blood to enter and shear apart the tunica media. Blood can continue to track along this false lumen (the space in between the layers), propagating the dissection. Eventually, important branches of the aorta, or the aorta itself, can be occluded if the dissection flap covers the true lumen of the aorta.

Aortic dissection is classically accompanied by a tearing or stabbing pain, so sudden and excruciating that most patients remember the exact moment it occurred (30). Additional symptoms are dictated by the anatomical site of dissection. Type A dissections are more surgically urgent because they can occlude the coronary arteries, the first branches of the ascending aorta, which supply blood to the heart itself, or tamponade the heart sac. These dissections can present with angina, or sudden death, depending on the degree of occlusion. Dissections in the arch of the aorta can produce the classic “pulse deficit,” in which the blood pressure of the left arm is markedly different from that of the right arm. Type B dissections and dissections of the abdominal aorta can occlude branches of the spinal arteries, splanchnic arteries, or renal arteries, producing paraplegia/paresthesia, abdominal pain, and renal failure, respectively. In reality, however, aortic dissection has such a diversity of clinical manifestations that it is commonly misdiagnosed as myocardial infarction, pericarditis, heart failure, pulmonary

embolism, or pneumothorax (31). Misdiagnoses or delayed diagnosis further exacerbates the prognosis of the disease.

An aortic rupture is a related catastrophe in which there is a complete tear or transection through all three layers of the aorta, resulting in massive internal hemorrhage. Symptoms include hypovolemic shock, multi-organ failure, and more frequently, sudden death.

Aortic dissection and aortic rupture occur with approximately the same incidence of 3.5 to 5 per 100,000 patient-years (32, 33). However, because many cases of sudden death are attributed almost reflexively to myocardial infarction, the true incidence of dissection or rupture may be higher (31). Patients with Marfan syndrome have an even higher risk of dissection, at 40% per individual (28).

The exact moment when an aorta dissects or ruptures is a function of several variables. A very important predictor is the size, or diameter, or the aorta. An aneurysmal ascending aorta grows at approximately 0.10 cm per year, whereas descending TAAs grow by 0.29 cm per year (34, 35). The annual risk of an acute event (dissection or rupture) is approximately 2% for aortas measuring 4.0 to 4.9 cm, and 3% for aortas measuring 5.0 to 5.9 cm (36). However, for ascending aortas larger than 6.0 cm, the risk of acute events increases sharply: the annual rupture or dissection rate is 6.9%, and the annual mortality is 11.8% from all causes (35, 36).

Activities that acutely increase systolic blood pressure are also risk factors for precipitating aortic dissection or rupture. Weight lifting and other physical exertions have been repeatedly linked to dissections and ruptures of pre-existing aneurysms (37, 38). Even emotional stress can precipitate aortic dissections, accounting for up to 40% of acute dissections in one series (30). External trauma or medical instrumentation to the aorta can also trigger the dissection event.

Regardless of cause, once a TAA dissects or ruptures, the patient's prognosis worsens rapidly. For patients with acute dissections who make it to the hospital, the overall in-hospital mortality is 27.4% (39). The in-hospital mortality for Type A dissection is 35%, and 12% for Type B dissection (39, 40).

A ruptured TAA is a more lethal event. Based on an older (1995) retrospective study from Sweden, only 41% of patients with TAA rupture reach the hospital alive, but the overall mortality from rupture is 97%, making the disease almost uniformly fatal (32). The 30-day mortality for all TAA ruptures is 71%. Even amongst those who undergo surgical treatment for rupture, the 30-day mortality is high, at 35% (14). Our ability to rescue patients with ruptured TAAs has improved over time—however, aortic rupture is still the leading cause of aneurysm-related death in TAA patients, accounting for 60% of the mortality (33). Interestingly, 79% of TAA ruptures occur in women (13).

Section 5: Diagnosis and screening for thoracic aortic aneurysms remain challenging

Given that untreated TAAs can progress to acute lethal events such as aortic dissection or aortic rupture, screening for asymptomatic TAAs before these events occur is of great clinical importance.

However, there are no standard screening programs for TAAs. Consequently, TAAs are commonly detected incidentally. In many cases, a patient might have his or her TAA detected while undergoing an echocardiogram (ECHO, or cardiac ultrasound) study or a CT scan for a completely unrelated reason. In other cases, a cluster of non-specific complaints, such as

fatigue, exercise intolerance, shortness of breath, or chest pain, might prompt the initiation of a long work-up which ultimately reveals an aortic, not cardiac, cause of the complaints. It is possible that the challenges of diagnosing TAAs, and the adverse outcomes of a missed diagnosis, are what Sir William Osler alluded to in his remarks about clinical humility.

Our advanced imaging technologies have allowed us great insight into the anatomy of the disease and provide a robust method for visualizing the aorta. However, these techniques have shortcomings as screening methods, including high cost, inconvenience, invasiveness, or harmfulness.

The issue of which imaging modality should be used to initially screen for aortic aneurysms is an ongoing area of debate. Chest X-rays are commonly used to screen for cardiac or pulmonary diseases due to their cost-effectiveness and low radiation exposure. The widening of the mediastinum, the central compartment of the thorax that contains the heart and aorta, is a classic radiographic sign of a TAA, but chest X-rays have an overall sensitivity of less than 70% for TAAs (41). Echocardiography (ECHO) is a convenient and safe method for examining aneurysms. However, ECHOs can only detect aneurysms of the aortic root or the first part of the ascending aorta due to its limited penetrance. In addition, ECHO is very operator dependent, and the accuracy of the measurements is affected by artifacts/interference, body habitus, and the presence of lung disease.

More sensitive imaging modalities have their drawbacks as well. Ultrasound applied from within the esophagus (trans-esophageal echocardiography, TEE) offers better resolution because of the proximity of the esophagus to the aorta, but it is an invasive method that requires sedation. CT scanning is widely available, easy and quick to operate, and provides excellent resolution. It is generally preferred over echocardiography for comprehensively

visualizing thoracic aortic anatomy and disease (3). As a routine screening method, however, CTs are impractical because of high cost, high radiation exposure, and toxicity from the contrast dye needed to visualize vascular structures. Magnetic resonance imaging (MRI), on the other hand, does not require exposure to radiation or harmful contrast agents, and provides excellent imaging of cardiovascular tissue. Drawbacks for MRIs include longer imaging times, incompatibility with metallic implants (e.g. pacemakers), need for sedation, very high operating costs, and lower spatial resolution than CTs.

Finally, because ascending TAAs are at much higher risk of acute events once the diameter approaches 6.0 cm, and because TAAs grow at a slow rate of 0.1 cm per year on average, obtaining precise measurements is paramount for prognosis and clinical decision-making (34, 36). And yet, there are many sources of error that can contribute to an imprecise measurement. In addition to the innate spatial resolution of the imaging modality, the measurement of the diameter is affected by whether the aorta is in systole or diastole, whether the aortic wall is included in the measurement, and whether a diameter measurement that is perfectly perpendicular to the direction of blood flow can be obtained from orthogonal views (3).

Section 6: Once diagnosed, thoracic aortic aneurysms can be safely treated with surgery

There is great clinical utility in screening for asymptomatic TAAs because there are effective treatments available. It is critically important to detect the aneurysm before it reaches dangerous diameters, as surgical treatment is safest and most effective on an elective basis, that is, before acute aortic rupture or dissection occurs.

Based on clinical studies which demonstrated that the risk of aortic rupture or dissection sharply increases as the ascending aorta approaches 6.0 cm, or as the descending aorta approaches 7.0 cm, the current guidelines recommend replacing the ascending aorta before it reaches 5.5 cm, and the descending aorta before it reaches 6.5 cm (3, 35, 42). The exact timing of surgical intervention, however, is a complex, multifactorial clinical decision that must take into account other factors, such as patient and family readiness (which includes their possible preference of living with a 2% dissection or rupture rate annually, versus facing a one-time risk of surgery), and the presence of other comorbidities. In addition, aneurysms that are symptomatic, or demonstrate a rapid rate of enlargement over recent years (> 0.5 cm/year), warrant repairs at smaller diameters (3, 9). TAAs associated with BAV or Marfan syndrome also warrant earlier repair, possibly as early as the 4.0 to 5.0 cm range (3, 15).

There are two main treatment options for TAAs on an elective basis. The first option is open surgery, in which the dilated, aneurysmal portion of the native aorta is excised, and a synthetic Dacron graft, measured for appropriate width and length, is sutured in its place. Ascending TAAs and TAAs of the aortic arch are approached through a mid-sternotomy (a midline incision through the sternum), whereas descending TAAs are approached through a lateral thoracotomy (an incision through the left rib cage). Both approaches require the use of a cardiopulmonary bypass machine.

The second, newer, option is an endovascular approach, in which a metallic stent with an intimal fabric graft is inserted through a small incision in the femoral artery at the groin, and deployed under X-ray guidance into the aneurysmal portion of the aorta. The stent is deployed against the inner surface of the aorta. Currently, endovascular approaches alone cannot address

ascending and arch disease without associated open surgical procedures; descending aneurysms and abdominal aneurysms benefit the most from endovascular approaches.

The immediate and long-term outcomes of both procedures are favorable when performed in elective settings. For surgical treatment, the 5-year survival after elective treatment is between 73 and 85%, and approaches the survival curve of a healthy, age-matched population (36, 43). By contrast, the 5-year survival after emergency surgical intervention is less favorable, at 37% (36). At experienced institutions, the perioperative mortality and complication rates, including bleeding, stroke, and paraplegia, are around 3% (43). Several trials have been initiated to examine whether endovascular approaches are as durable as open graft repairs (4). Early reports show favorable outcomes of endovascular repair of TAAs and dissections, with 30-day mortalities of 0% in an elective setting and 2.9% in an emergent setting (44). However, long-term outcomes are uncertain. Currently, ascending TAAs are still treated surgically.

Section 7: There is potential in using biomarkers to screen for aneurysm disease

Although many cases of aortic aneurysms are detected by CT or MRI, these modalities are not safe or cost-effective as screening methods for the general population. Relying on radiographic images alone for the diagnosis and management of TAAs also has two other shortcomings. First, a patient who has an aortic diameter that is smaller than the threshold for an aneurysm at the time of scanning might be declared to be aneurysm-free, but may develop an aneurysm later in life. The radiographic image is just one snapshot in time. Second, although aortic size can predict when aortic dissection or rupture may occur, as described above, the classification is not perfect, as some individuals dissect well below 5.5 cm (45).

Therefore, there is a need for better screening methods for TAA. Laboratory blood tests, thus far, have played a very limited role for aneurysm diseases. However, there are several potential benefits to using a blood test to screen for aneurysms. A blood test is cheap, safe, non-invasive, and widely available. They can be performed multiple times to monitor an aneurysm over time, without accumulating radiation or contrast toxicity. In addition, they may reveal pathologic details about the aneurysm that an image alone cannot.

Blood tests are designed to detect the biomarkers of a disease. A biomarker, in the general sense, is a molecular indicator of a biological process or state. In clinical application, biomarkers are reflective of the pathogenic activity of that disease. A biomarker must be able to be measured objectively and quantitatively. The quantity of the biomarker can correlate with the type, risk, or severity of disease. Therefore, the appropriate biomarkers may tell us not only whether an aneurysm exists, but how dangerous it is or how close it is to dissection or rupture. Biomarkers may even be able to predict aneurysm formation well before dilatation can be detected on CT.

The use of blood-based biomarkers for aneurysm disease is still in its infancy. The panel of available or investigational biomarkers is reviewed in Trimarchi et al. (46). These potential biomarkers include D-dimers, the product of cross-linked fibrin which appear to be elevated after aortic dissections; matrix metalloproteinases, which not only participate in the pathogenesis of aneurysms, but appears to be further elevated during aortic rupture and dissections (18); smooth muscle myosin, which is sensitive and specific for aortic dissections (47); and C-reactive protein, whose level actually correlates with the size of the abdominal aortic aneurysm (48).

While many of these biomarkers of aortic aneurysms have shown promise, they have not led to any clinically utilized testing programs. In many cases, the biomarker has only been shown to be of value *after* the acute aortic event. In addition, several biomarkers were tested on small patient cohorts, not tested within a clinical setting at all, or examined exclusively for *abdominal* aortic aneurysms. Finally, many of these biomarkers, including D-dimer and CRP, are very non-specific for aneurysm disease.

In 2007, Wang et al. published a study in which a comprehensive gene expression analysis of peripheral blood cells (PBCs) was conducted on blood samples from TAA patients, with spouses as controls. Using the Applied Biosystems Human Genome Survey Microarray, which represents 29,098 individual human genes, a panel of 41 classifier genes that were the most significantly over- or under-expressed in TAA samples versus control samples was identified (49). Genes in this classifier set included those integral to interleukin signaling, T-cell activation, and apoptosis. These biological pathways have all been shown to be associated with aneurysm formation (50-52).

When tested on a mixed cohort of TAA patients and spousal controls, the RNA signature succeeded in distinguishing the two groups with an overall accuracy of 78% (sensitivity = 72%, specificity = 90%). The validity of this 41-gene RNA signature was subsequently reassessed through real-time polymerase chain reaction (qPCR) using the TaqMan Gene Expression assay, which provides a more precise quantification of expression levels than microarray. This initial validation tested a small cohort of 30 samples (mixed pool of patients and controls) on a 384-well format, using the 7900HT Fast Real-Time PCR System (Applied Biosystems). This real-time PCR study demonstrated that the RNA signature was 80% accurate (sensitivity = 71%, specificity = 100%).

These results show promise for a gene-expression-based biomarker that can be used to screen for TAAs. Such a biomarker can be easily instituted as a blood test, and has proven to be both sensitive and specific for TAAs. While none of the 41 classifier genes alone are specific for aneurysm disease, the panel simultaneously examines the relative expression levels of all 41 genes, and predicts a diagnosis based on a multiple regression model. Investigation and revalidation of this new entry biomarker for TAA disease is the objective of this current study.

Statement of Purpose

There is an important role for screening tests for thoracic aortic aneurysms. TAAs are asymptomatic, lift-threatening, but treatable once diagnosed. Many patients can benefit from early detection. However, there are no standard screening tests for TAAs at present. Radiographic imaging, such as CT scans and angiograms, are the gold standards for visualizing and confirming TAAs. However, these modalities are not ideal as screening tests because of high costs, low accessibility, and harmfulness.

The purpose of this current study is to reevaluate the sensitivity and specificity of a novel screening test for TAAs. This screening test is based on relative expression levels of a panel of genes (a "RNA signature"). A previously published study demonstrated that this 41-gene RNA signature was 72% sensitive and 90% specific in distinguishing TAA patients from spousal controls (49). Further testing of this signature using TaqMan real-time PCR showed that the assay was 71% sensitive and 100% specific.

These preliminary results suggest a promising role for this RNA signature to serve as a screening test for TAA for perhaps the general population. In its maturity, this screening test would be a simple blood test that would determine a single probability of the individual having a TAA. A high probability would then warrant subsequent confirmatory studies, such as CT scan.

Specific Hypothesis

For screening tests, a high level of sensitivity is important. Based on these previous findings, we hypothesize that a revalidation study of these 41 classifier genes, using TaqMan

real-time PCR, will demonstrate a sensitivity greater than 70%, and a specificity greater than 60%, in distinguishing TAA patients from healthy, spousal controls. We will be comparing the predicted diagnosis of the blood test ("TAA" or "control") against the gold standard (the radiographic documentation of TAA).

Specific Aims

The specific aims of this study are:

1) To reproduce the findings from Wang et al., 2007, by revalidating the sensitivity and specificity of this RNA signature test in detecting TAAs on a new, independent cohort of patients;
2) To demonstrate the efficacy of a new TaqMan Array Cards (TAC) format for real-time PCR analysis of these classifier genes. The previous study by Wang et al. utilized a 384-well format.

This study will confirm whether the RNA signature is able to distinguish TAA patients from spousal controls within a mixed pool. Because this mixed pool comprises of TAA patients from Dr. John A. Elefteriades' clinical practice at Yale-New Haven Hospital, and their consenting spouses, the prevalence of TAA within this cohort is drastically higher than that of the general population. Therefore, this study will not assess the positive and negative predictive values of the RNA signature test, which are based on the true prevalence of a disease in the population.

The long-term goal of this and subsequent studies is to develop a clinical blood test for TAAs. With this goal in mind, consideration was given to the logistics of engineering and marketing a laboratory diagnostic test. One such consideration is to streamline the workflow,

from the time of phlebotomy to the acquisition of test results. Whereas the previous study of Wang et al. utilized a labor- and time- intensive 384-well format for real-time PCR that required highly skilled technicians, we are conducting this revalidation study using proprietary TaqMan Array Cards, which are customized to include primers for our specific panel of classifier genes. These TACs require minimum technical expertise, ensure a consistent quantity of primers and reagents in each well, and produce consistent PCR results using very little quantities of starting cDNA material. We believe these features will enhance the viability of the RNA signature as a future diagnostic test, and will determine in this study whether the results and outcomes from using this proprietary technology are consistent with those in Wang et al.

Methods

Selection and Enrollment of Patients and Controls

This study was approved by the Yale University Human Investigation Committee (Protocol # 0109012617).

All patients who present to the Aortic Institute at Yale-New Haven Hospital (Director, Dr. John A. Elefteriades) for consultation and who have a documented *ascending* TAA, based on definitive radiographic evidence and a physician's evaluation, are eligible for this study. Acceptable radiographic modalities include ECHO, CT, MRI, and angiogram (aortogram).

No specific selection criteria are made based on gender, age, or ethnic background. Patients who have had their TAAs surgically resected are also included, as long as there is at least one year's time between the time of surgery and time of blood specimen collection.

Patients with TAAs as part of a connective tissue disorder, such as Marfan syndrome, were excluded. Patients with active leukemia or other blood dyscrasias that may distort the constituents of the peripheral blood sample collected, or patients treated with chemotherapy within the past year that may likewise distort the blood sample, were excluded as well.

Spouses were enrolled as controls because they generally share similar age, diet, geography, and ethnicity as the enrolled patients. All patients and controls have given advanced written consent for inclusion in this study. A questionnaire was administered to participants to collect data on age, height, weight, medications, medical history, and family history.

Selection of Target Classifier Genes

The 41 classifier genes that comprise the RNA signature panel were identified through microarray analysis, as described in Wang et al. (49). Briefly, peripheral blood was collected from 58 TAA patients and 36 spousal controls using the PAXgene Blood RNA Tubes (Qiagen, Valencia, CA). Total RNA was extracted from each sample (PAXgene Blood RNA Kit, PreAnalytiX, Qiagen), and used as template for the synthesis of digoxigenin-UTP labeled cRNA using the Chemiluminescent RV-IVT Labeling Kit v 1.0 (Applied Biosystems, Foster City, CA). The hybridization was performed using the Human Genome Survey Microarray v 2.0 (Applied Biosystems), which contains probes for 29,098 individual human genes. Array hybridization was performed for 16 hours at 55^{o}C. Chemiluminescence detection, image acquisition, and analysis were performed using the Chemiluminescence Detection Kit and 1700 Chemiluminescent Microarray Analyzer (Applied Biosystems). The significance analysis of microarrays (SAM) software (Stanford, Palo Alto, CA) was used to identify potential signature genes.

The microarray analysis to identify potential signature genes, as described above, was not performed or repeated in this current study.

RNA Collection and Extraction (PAXgene)

2.5 mL of whole blood was collected in PAXgene Blood RNA Tubes (Qiagen) from enrolled patients and spouses using standard phlebotomy techniques (using the BD Vacutainer Safety-Lok Blood Collection Set (Becton, Dickinson and Company, Franklin Lakes NJ)). PAXgene Blood RNA Tubes contain a proprietary reagent that protects RNA from degradation by RNases, and minimizes in-vitro (post-phlebotomy) alterations in gene expression. Blood samples were allowed to incubate at room temperate (25^{o}C) for 2 to 3 hours for lysis of blood cells, and then stored at 4^{o}C overnight for 24 hours. Blood tubes were then stored at – 80^{o}C for long-term

storage. Prior to RNA extraction, the blood specimen was thawed at 4^{o}C for 24 hours, and then equilibrated to room temperature for 2 to 3 hours (per kit protocol).

RNA extraction was performed using the PAXgene Blood RNA Kit (PreAnalytiX, Qiagen). PAXgene Blood RNA Tubes containing patient samples were centrifuged at 3000 g for 10 minutes. The supernatant was decanted. 4 mL of RNase-free water was added to the pellet, and vortexed until the pellet dissolved. The tube was centrifuged again at 3000 g for 10 minutes. The supernatant was decanted. 350 µl of Buffer BR1 (provided with kit) was added to pellet, and vortexed. The entire sample was then transferred by pipet to a 1.5 mL microcentrifuge tube. 300 µl of Buffer BR2 and 40 µl of proteinase K were added to the sample. The sample was then incubated for 10 minutes at 55^{o}C using a shaker-incubator at 400 rpm.

The lysed sample was subsequently transferred to a PAXgene Shredder spin column placed in a 2 mL processing tube, and centrifuged for 3 minutes at 13400 g. The flow-through containing the sample was transferred to a new 1.5 mL microcentrifuge tube. 350 µl of 100% ethanol was added to the flow-through, and vortexed. The sample was then transferred into the PAXgene RNA spin column placed in a 2 mL processing tube, and centrifuged for 1 minute at 13400 g (samples were centrifuged in the PAXgene RNA spin column in two allotments, and the flow-through was discarded for each). 350 µl of Buffer BR3 was added to the PAXgene RNA spin column, and centrifuged for 1 minute at 13400 g. The flow-through was decanted. A mixture of 10 µl of reconstituted DNase I and 70 µl of Buffer RDD was added to the spin column, and the sample was incubated at room temperature for 15 minutes.

The PAXgene RNA spin column was then washed with 350 µl of Buffer BR3 (centrifuged for 1 minute at 13400 g, and the flow-through was decanted). Afterwards, the spin column was washed twice with 500 µl of Buffer BR4 (centrifuged for 1 minute and 3 minutes, at 13400 g).

After the last flow-through was decanted, the spin column was centrifuged again for 1 minute for 13400 g to spin out residual buffer. The spin column containing the extracted RNA was then transferred to a new 1.5 mL microcentrifuge tube. 40 µl of Buffer BR5 was added to the column, and allowed to sit for 1 minute at room temperate. The spin column was then centrifuged for 1 minute at 13400 g to elute the RNA. The spin column subsequently was treated again with 40 µl of Buffer BR5, and eluted again. The final eluate (80 uL) was incubated for 5 minutes at 65^{o}C in a shaker-incubator without shaking. The eluate containing total RNA sample was immediately placed on ice prior to subsequent processing.

RNA Collection and Extraction (Tempus)

3.0 mL of whole blood was collected in Tempus Blood RNA Tube (Applied Biosystems) from enrolled patients and spouses using standard phlebotomy techniques. The Tempus Blood RNA Tubes contains an additive that stabilizes the RNA and in-situ transcription profile. After blood collection, the tube was shaken vigorously for 10 seconds to disperse the stabilizing reagent within the sample, per protocol recommendations. Blood tubes were then allowed to incubate at room temperate (25^{o}C) for 2 to 3 hours for lysis of blood cells, and then stored at 4^{o}C overnight for 24 hours. Blood tubes were then stored at – 80^{o}C for long-term storage. Prior to RNA extraction, the blood specimen was thawed at 4^{o}C for 24 hours, and then equilibrated to room temperature for 2 to 3 hours.

RNA extraction was performed using the Tempus Spin RNA Isolation Kit (Applied Biosystems). The blood sample in the Tempus Blood RNA Tube was poured into a clean 50 mL conical tube (Ambion, Life Technologies, Carlsbad, CA). Approximately 3 mL of 1X Phosphate-buffered saline (PBS) was added to the solution to bring the total volume to 12 mL. The conical tube was then vortexed at maximum speed for 30 seconds, and centrifuged for 30 minutes at

4°C and 3000 g. The supernatant was decanted, and the conical tube (with the pellet) was left inverted for 1 minute. Afterwards, 400 µl of RNA Purification Resuspension Solution was added to the pellet, and vortexed.

A purification filter was inserted into a collection tube. 100 µl of RNA Purification Wash Solution 1 was pipetted onto the membrane of the purification filter for pre-wetting the membrane. The resuspended RNA was then transferred onto the purification filter, and centrifuged for 30 seconds at 13400 g (flow-through was discarded). 500 µl of RNA Purification Wash Solution 1 was added to the purification filter, and centrifuged for 30 seconds at 13400 g (flow-through was discarded). 500 µl of RNA Purification Was Solution 2 was added to the purification filter, and centrifuged for 1 minute at 13400 g (flow-through was discarded). A mixture of 10 µl of reconstituted DNase I and 70 µl of Buffer RDD was added to the purification filter, and the sample was incubated at room temperature for 15 minutes.

After DNase I treatment, 500 µl of RNA Purification Wash Solution 2 was added to the purification filter, incubated at room temperature for 5 minutes, and then centrifuged for 30 seconds at 13400 g (flow-through was discarded). Another 500 µl of RNA Purification Wash Solution 2 was added to the purification filter for a second wash (centrifuged for 30 seconds at 13400 g; flow-through was discarded). The purification filter was then centrifuged by itself for 30 seconds at 13400 g to dry the filtration membrane. The purification filter was subsequently transferred to a new collection tube. 100 µl of Nucleic Acid Purification Elution Solution was added to the purification filter, and incubated for 2 minutes at 70°C. The purification filter was then centrifuged for 30 seconds at 13400 g. The eluate containing the RNA was pipetted back into the same purification filter, and centrifuged for 2 minutes at 13400 g. The eluate containing

total RNA sample was then transferred to a clean 1.5 mL microcentrifuge tube, and placed on ice prior to subsequent processing.

RNA Quantification/Quality Assessment

5 µl of total RNA for each sample was sent to the Yale Center for Genome Analysis (formerly Keck Microarray Core Facility) for RNA quantification and quality assessment (300 Heffernan Drive, B36 West Haven, CT 06516). RNA quantification was performed using the NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA integrity was determined by electrophoresis using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

RNA quantification and quality assessment were performed at a Core Facility, and not by the authors of this current study.

cDNA Synthesis

Total RNA was reverse-transcribed into cDNA for real-time PCR analysis using the QuantiTect Reverse Transcription Kit (Qiagen). 2 µl of 7X gDNA Wipeout Buffer was added to 200 ng of each RNA sample, and the total volume was brought to 14 µl with RNase free water. The mixture was incubated for 2 minutes at 42^{o}C, and immediately placed on ice.

After incubation, the following was added to each sample: 1 µl Quantiscript Reverse Transcriptase, 4 µl Quantiscript RT Buffer (5X), and 1 µl RT Primer Mix, bringing the total reaction volume to 20 uL. The reaction mixture was incubated for 30 minutes at 42^{o}C. The reaction mixture containing cDNA was then deactivated at 95^{o}C for 2 minutes, and stored on ice.

Preparing the TaqMan Array Cards

The TaqMan Array Cards (Production Number: 1198801 and 1198802; Sales Order: 186436998 and 186437213) were generously provided by Applied Biosystems. Each TaqMan Array Card (TAC) is an 8-lane system; each lane (fill reservoir) branches off to 48 different wells. Each well contains the TaqMan Gene Expression Assay, a pre-loaded and desiccated mixture of sequence-specific primers (TaqMan MGB Probes) customized for our panel of classifier genes (**Fig 1**). The exact sequences of the primers are proprietary and not currently available.

20 µl of template cDNA was mixed with 80 µl of RNase/DNase-free water and 100 µl of TaqMan Gene Expression Master Mix (2X) (Applied Biosystems), producing a total volume of 200 µl per sample. The TaqMan Gene Expression Master Mix contains AmpliTaq Gold DNA Polymerase, Uracil-DNA Glycosylase, deoxyribonucleotide triphosphates, ROX Passive Reference, and buffer solution. The sample was gently vortexed.

The TACs, stored at 4^{o}C, were allowed to reach room temperate. 100 µl of each sample, containing cDNA synthesized from approximately 100 ng of total RNA (for each sample), was loaded into each lane on the TAC. Two replicates (two lanes) were run per sample, for a total of four samples per TAC. Proper attention was given to loading each lane port without air bubbles, and without damaging the foil at the base of the port.

Once all eight lanes were loaded with cDNA/Master Mix (100 µl per lane), the TAC was loaded onto array holders and Sorvall Custom Buckets for centrifugation using the Sorvall Legend XT (Thermo Scientific). Generous permission was received from the laboratory of Dr. Jeffrey L. Sklar, Department of Pathology, Yale University School of Medicine, for the use of the centrifuge. The centrifugation process allows the samples to be distributed from the fill reservoir to each of the 48 reaction wells branching off of each lane. The TACs were centrifuged

on a custom setting (#15679) for two consecutive 1 minute segments at 331 g. After centrifugation, the fill reservoir of TAC was examined manually to ensure uniform and consistent distribution of sample.

The TACs were then individually sealed using a sliding carriage contraption to seal the sample fluidics within each well. Proper care was taken for correct orientation, placement, and removal of the TAC prior to and after sealing. The fill reservoirs were then trimmed off the TACs using generic scissors.

Real-Time Polymerase Chain Reaction

Real-time PCR was performed using the 7900HT Fast Real-Time PCR System thermocycler (Applied Biosystems). Generous permission was received from the laboratory of Dr. Clifford W. Bogue, Department of Pediatrics, Yale University School of Medicine, for the use of the thermocycler. Prior to use, a custom block and precision plate holder was installed on the 7900HT thermocycler to ensure proper physical fit, cycling conditions and fluorescence detection of the TACs.

SDS v2.3 software (Applied Biosystems) was used to operate the real-time PCR cycling procedure. The real-time PCR settings were selected as follows:

- Assay Type: ΔΔCt (RQ)
- Container: 384 Wells TaqMan Low Density Array
- Template: Blank Template

An .SDS setup file was provided by Applied Biosystems accompanying our specific TAC and gene layout. This setup file was imported into the SDS software prior to the start of real-time PCR to configure the well-specific array data (including the gene name of each well

location). The sample name and ID of each loading port was entered manually. The remainder of the settings used was default.

The default thermal cycling conditions were used:

- 50.0°C for 2 minutes →
- 94.5°C for 10 minutes →
- Forty (40) cycles of (97.0°C for 30 seconds, then 59.7°C for 1 minute).

RQ Manager v1.2 software (Applied Biosystems) was used to analyze the raw data of real-time PCR. The .SDS files containing data for the real-time PCR runs for each TAC were sent electronically to molecular biostatisticians at Life Technologies.

The ΔΔCt (RQ) analysis for all samples to quantify the relative expression of each of the classifier genes was performed by biostatisticians at Life Technologies.

Classification Model and Statistical Analysis

The model for classifying each sample as "TAA Patient" or "Spousal Control" was based on previously applied methods (49). The relative expression levels of the classifier genes were used to classify each sample as either a patient or a control using the comparative ΔCt method (ΔΔCt) and a 10- fold cross-validation test.

For each gene in the panel, the output from the TaqMan real-time PCR analysis is a relative expression level of that gene versus the expression level of an endogenous control (PPIA gene), expressed as ΔCt. The ΔCt was averaged across replicates to remove sample input errors and natural biological variation.

The ΔC_T values that constitute the expression profile of each individual gene in sample X = (x1,…,x41) was inserted into the defined linear classifier regression. This classifier is based on the two discriminant scores:

$$\delta_k(x^*) = \sum_{i=1}^{p} \frac{(x_i^* - \overline{x_{ik}}')^2}{(s_i + s_0)^2} - 2\log(\pi_k)$$

where $\overline{x_{ik}}'$ is the average expression level of gene *i* in class k of previously used samples, s_i is the pooled within-class standard deviation for gene *i*, s_0 is the median value of the s_i over all genes, and π_k is prior probability of class k (the overall frequency of class k in the population), where $\pi_1 + \pi_2 = 1$.

The regression predicts that sample X* is a case (k = 1) if $\delta_1(x^*) < \delta_2(x^*)$ and control otherwise.

Discriminant scores were used to estimate class probabilities, by analog to Gaussian linear discriminant analysis:

$$\hat{p}_k(x^*) = \frac{e^{-1/2\delta_k(x^*)}}{e^{-1/2\delta_1(x^*)} + e^{-1/2\delta_2(x^*)}}$$

Based on these two scores and a 10-fold cross-validation study, using reiterative bins of four random samples (3 TAA samples and 1 control, 2 TAA samples and 2 controls, etc), each sample was given a probability of being a TAA (case) sample. This probability was used to classify each sample as either “case” (TAA patient) or “control” (no aneurysm); a sample was classified as a case if its probability of being a case exceeded 50% (and vice versa). The predicted “case” and “control” status of each sample was then compared to the sample’s actual status, as

defined by their clinical diagnosis, to compute an absolute number of true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN). The sensitivity (TP/(TP+FN)), specificity (TN/(FP+TN)), and accuracy ((TP+TN)/total) were then calculated.

The ΔΔCt (RQ) analysis for all samples and the multiple regression algorithms used to predict the clinical status of each sample ("aneurysm" or "no aneurysm") was performed by biostatisticians at Life Technologies.

Results

Patient Demographics

Twenty-four patients with confirmed TAAs and sixteen spouses were enrolled in this validation study. **Table 1** summarizes their clinical features. Of note, two spousal controls were lost to follow-up, before their clinical information could be obtained.

Most of the TAA patients (79%) were men, whereas only 19% of the spousal controls were men. Patients and controls share similar age (59 years) at time of blood collection, but TAA patients were slightly taller, likely secondary to the gender disparity. The patient and control groups had similar rates of hypertension (63% versus 64%), cigarette smoking (25% versus 29%), and diabetes (8.3% versus 7.1%). TAA patients had a higher prevalence of cardiac comorbidities than spousal controls (50% versus 7.1%); these comorbidities include coronary artery disease, arrhythmias, and heart failure.

All 24 TAA patients had their aneurysms in the ascending portion of the aorta. Five of these patients had aneurysms that additionally extended to the aortic arch. The average size of the TAAs was 5.18 cm, ranging from 4.5 cm to 6.9 cm. Seven of the 24 samples were obtained from TAA patients post-operatively. A high percentage (54%) of TAA patients also had bicuspid aortic valves. 58% of TAA patients had a family history positive for aortic aneurysms or presumed “cardiac” sudden death, as compared to only 7.1% of spousal controls.

Comparison of RNA Extraction Methods

The first 17 blood samples were collected using PAXgene Blood RNA Tubes (Qiagen), based on the previous study by Wang et al (49). However, for economic reasons, subsequent samples were collected using the Tempus Blood RNA Tubes (Applied Biosystems).

A comparison was made between the quantity (concentration) as well as the quality of the RNA isolated with each system (**Appendix 1**). The average RNA concentration of samples isolated with the PAXgene system was 95.7 ng/µL, versus 135.94 ng/µL for RNA samples isolated with the Tempus system ($p < 0.01$, two-tailed T-test).

The 260/280 absorption ratio, a traditional measure of the protein contamination of nucleic acid samples, was assessed between the PAXgene samples and the Tempus samples. While the 260/280 ratios for samples isolated with either method were greater than 2, signifying a "pure" sample, the PAXgene samples had an average 260/280 ratio of 2.14, compared with 2.07 for the Tempus samples ($p < 0.01$).

Additional RNA quality control was performed using the 2100 Bioanalyzer, which uses microfluidic electrophoresis to determine RNA quality (**Appendix 2**). The Bioanalyzer system generates a RIN, or "RNA Integrity Number," ranging from 0 to 10, to reflect the purity and quality of RNA samples. Samples with higher RINs are considered to be of higher quality. The PAXgene samples had an average RIN of 7.53, whereas the Tempus samples had an average RIN of 6.36 ($p < 0.05$).

TaqMan Real-Time PCR Assay Validation

Customized TaqMan Array Cards incorporating the panel of classifier genes (the RNA signature) identified in Wang et al. were generously provided by Life Technologies. An accompanying array map (**Fig 1**) displays the identity of the genes in each well. In the 48-well array used in this study, four wells are empty, one well contains the endogenous control gene (PPIA), and 43 wells contain classifier genes. After cross-matching the list of genes with those found in the original study, only 39 of the 41 genes identified in Wang et al. were found to be incorporated in this current TaqMan Array Card (see **Table 2** for a list of the 39 genes).

During real-time PCR, six of these 39 genes showed poor amplification after standard cycling conditions, across all 40 blood RNA samples (24 patients, 16 controls). For the 33 genes that amplified successfully, the average number of cycles needed to amplify the target past the preset fluorescence detection threshold (the Ct value) across samples ranged from 16 to 29 (**Fig 2A**). By contrast, for the six genes that failed to amplify, no appreciable or consistent fluorescence was detected by cycle 40 (**Fig 2B**). Therefore, the final prediction algorithm is based on the relative expression levels of 33 genes. The coefficients of the six genes that failed to amplify are 0, and therefore do not contribute to the prediction model.

After a 10-fold cross-validation test of the 24 TAA samples and 16 control samples, using the delta-delta-Ct values of each gene for each sample and the previously developed prediction algorithm (49), a cross-validation probability (0-100%) was calculated for each sample; this probability reflects the likelihood that the particular sample is from a TAA patient (**Fig 3**). Based on this cross-validation, 25 samples were classified as “TAA” and 15 samples were classified as “controls”.

When each sample's predicted diagnosis was compared with the sample's true clinical diagnosis (the actual status of the individual donating the blood sample), the RNA signature test was 83% accurate:

		True Clinical Status		
		TAA	Control	
Predicted Status (Cross-Validation)	TAA	**21**	**4**	25
	Control	**3**	**12**	15
		24	16	40

The sensitivity of the test was 88%, and the specificity of the test was 75%. **Table 3** compares the characteristics and testing parameters of the current validation study with that of the previously published study.

Comparison of Gene Expression Patterns with Previous Study

The current RNA signature assay is based on the relative expression levels of 33 different genes in the PBCs of TAA patients versus spousal controls. To test for the consistency and reproducibility of the assay, we compared the expression patterns of these 33 genes between the current and previous studies.

To assess whether the expression profiles of the TAA patient samples and spousal control samples in our current assay reflect or mirror those of the previously published assay, we first compared the average Ct values for each of the 33 classifier genes acquired between the previous test and the current test. This analysis was further broken down into two separate comparisons, one for TAA patient samples, and one for control samples (**Fig 4**).

For TAA patient samples, the previous real-time PCR study was able to amplify all classifier genes, on average, before the 35th cycle (**Fig 4A**). The average Ct values from the previous study ranged from 16 to 35 (x-axis). When plotted against the average Ct values of the same genes as determined by the current real-time PCR study, most of the points lie above the y=x, signifying that for most of the classifier genes, it took more cycles (higher Ct values) to amplify the gene (past the threshold of detection) in our current study than in the previous study. Furthermore, six genes failed to amplify, as mentioned above. However, there was good correlation between Ct values from the previous study and Ct values from the current study (r^2 = 0.75), suggesting that the individual genes behave in a somewhat consistent manner between the two studies. For example, a gene that was expressed in low amounts in TAA patient samples, and thus had a high Ct value (requiring more cycles to amplify) in the previous study, also had a high Ct value as determined by the current study.

For spousal control samples (**Fig 4B**), the same pattern was observed: the overall correlation was good (r^2 = 0.73), but most points lied above the y=x line, again signifying that these genes required more cycles to amplify in our current study than in the previous study.

Next, the relative expression levels (the fold-change) of the classifier genes between cases and controls were analyzed to determine if they were preserved between the previous study and the current study. In other words, if the previous real-time PCR study determined that gene X was expressed three times more abundantly in the PBCs of TAA patients relative to spousal controls, the fold-change for gene X would equal 3, and we wanted to assess whether the fold-change value for gene X in our current study also approximates 3.

The panel of classifier genes originally identified in Wang et al. included genes that were both over-expressed in TAA patients versus spousal controls, as well as under-expressed in TAA

patients versus spousal controls (**Fig 5A**; also see *Table 2* in Wang et al., 2007). The fold-change values (in log 2) were both positive and negative. By contrast, in the current study, the majority of the same panel of genes appears to be over-expressed in TAA patients relative to spousal controls (**Fig 5B, Fig 6**). Quantitatively, the average fold-change (log 2) in expression levels of control samples relative to TAA samples (for all genes) in the current study is -0.328, denoting a relative *under-expression* of the panel of 33 classifier genes in control patients (**Table 2**). The previous study, on the other hand, demonstrated that the average fold-change (log 2) for the same 33 genes is +0.034, signifying a very slight trend towards *over-expression* in control patients. The eight genes that either did not amplify in the current assay, or were not included in the TaqMan Array Cards, were excluded from this calculation. Altogether, the newly designed assays for the same set of genes show a bias for up regulation in TAA samples relative to controls.

Discussion

The clinical features of thoracic aortic aneurysms render the disease sorely in need of a screening test. TAAs are clinically-silent, life-threatening, and treatable with surgery. With timely diagnosis, we eliminate the risk of TAAs becoming a “silent killer”, and can offer patients options in living with or treating this condition.

There are currently no screening programs for TAA. In this era of molecular medicine, many technologies have become available to help identify biomarkers of disease and promising therapeutic targets. Molecular diagnostics have not yet been utilized clinically for TAAs, but there is mounting evidence that gene expression profiles might play a role in screening for and detecting TAAs. A previously published study from our institution utilized microarray technology to identify genes that were over-expressed or under-expressed in circulating leukocytes in a consistent, detectable manner in TAA patients versus spousal controls (49). A panel of 41 classifier genes, identified through this method, was engineered into a “RNA signature” that was 80% accurate for detecting TAA. Many of these genes are involved in the inflammatory process or pathogenesis pathways of TAAs, providing a mechanistic basis for why this signature might be able to detect TAAs.

Our current investigation is a validation study, with the purpose of demonstrating whether this RNA signature is reproducibly accurate, and whether this signature has potential to become a clinical laboratory test for TAA. We elaborate the findings of the Wang et al. study in two ways: 1) our testing samples come from a newly recruited cohort of TAA patients and

spouses and are independent from the samples used in the original microarray analysis, and 2) our blood RNA samples are tested on a commercialized TAC platform that more closely resembles the real world configuration of an actual diagnostic test, instead of a 384-well format commonly used in basic research settings.

Forty individuals were recruited for this current study—24 patients with radiographically documented TAAs, and 16 healthy, spousal controls. Analysis of the peripheral white blood cell RNA signature for these individuals, using the TaqMan real-time PCR system, generated a probability for each individual having TAA disease. When these probabilities were converted to categorical classifications of "TAA patient" or "spousal control" for each sample, the signature was 83% accurate in discriminating TAA samples from the control samples. The signature was 88% sensitive and 75% specific.

The overall accuracy of the assay is comparable to that of the previous study (83% versus 80%). The current TAC format with 33 viable classifier genes has a higher sensitivity (88% versus 71%) but lower specificity (75% versus 100%), when compared with the results of the previous study (**Table 3**).

Several additional comparisons can be made between the current results and the results of the previous study. First, as shown in **Figure 4**, for most genes that were successfully amplified, the current TAC platform required a higher number of cycles of amplification before the gene product can be detected (as reflected by the higher Ct values), compared with the 384-well platform. This systematic shift in the Ct values is likely a function of the smaller amount of starting cDNA used in the TAC-based real-time PCR amplification. Because the baseline amount of cDNA is lower, more cycle numbers are required to amplify the gene targets past the set threshold of detection. The fact that a smaller amount of starting cDNA template (and therefore,

starting RNA quantity) can produce the same high level of diagnostic accuracy is an advantage of the TAC platform, and is an inherent feature of the microfluidics design of the system. The microfluidics allow for very small amounts of sample to uniformly populate dozens of wells and produce consistent amplification of the targets. No pre-amplification step is needed. In a clinical laboratory setting, this test can theoretically be accurate even with a small quantity of blood.

Second, several gene targets did not amplify during real-time PCR in our current study, whereas they did amplify in the previous study. One possibility is that there is a manufacturing error with the TACs, such that some wells did not contain proper amounts of reagents. Another possibility, based on the findings in **Figure 4A**, is that the genes that failed to amplify in the current study were those that had high Ct values in the previous study, and therefore were genes that were expressed in low levels. In our current assay, because we used smaller amounts of starting material, the quantity of these targets could have been too low for detection even after 40 cycles.

In addition, two of the original 41 classifier genes did not even appear in our custom-made commercial TaqMan Array Cards. Again, this can reflect a manufacturing error or an error in the ordering process for these Array Cards.

Regardless, the consequence is that our current assay relies on the coefficients and contributions of only 33 genes, instead of the full panel of 41 classifier genes as identified in the previous study. However, the overall accuracy of the assay was not negatively affected by having eight fewer genes. Interestingly, the sensitivity actually improved, whereas the specificity worsened. As a screening test, a high sensitivity is a critical parameter, even at the cost of high specificity. The finding that removing eight genes actually improves the sensitivity of the assay suggests the need for future studies to examine the receiving operating characteristic (ROC)

curve as a function of number of genes (and which genes) are included in the assay. Such ROC curves can help determine the optimal number of genes that should appear in the final version of the RNA signature assay.

Third, as shown in **Figure 5** and **Figure 6**, most of the genes in our current assay appear to be *over-expressed* in the PBCs of TAA patients compared to controls. By contrast, approximately half of the genes in the previous assay were under-expressed in TAA patients, while the other half of the genes were over-expressed. It is unclear what the underlying cause of this discrepancy is. One possibility is that several genes in our RNA signature assay are not, as we have hypothesized, consistently over- or under-expressed in the peripheral blood cells of TAA patients versus controls. Instead, they can be confounder genes that only appear to be expressed in consistent manners in the original cohort of samples, but actually are not in fact a "signature" of TAA disease. Nonetheless, our current assay can still distinguish TAA patients from controls with a high level of accuracy.

Altogether, we have demonstrated that a modified 33-gene RNA signature assay accurately detects TAA from healthy, spousal controls, using a sample of blood alone. This assay has strong potential to be the basis of a clinical laboratory test for TAAs. This RNA signature assay for TAA is safe, non-invasive, and cost-effective. Individuals who are tested "positive" by this assay will then be subsequently referred for more extensive work-up, including radiographic imaging. Individuals at higher risk for harboring or developing a later aneurysm, including older individuals and those with a family history of TAAs, can be repeatedly monitored with this assay over time, without the risk of accumulating radiation or contrast toxicity.

The customized, prefabricated TaqMan Array Cards used in this assay allow for considerable streamlining of work, eliminating the need to manually preload dozens of

individual wells with standardized amounts of gene-specific primers. The single-loading-port system also distributes samples evenly to all wells. In addition, the TaqMan system is a highly *accurate* method for quantification of expression levels of genes, because the fluorescent reporter is covalently attached to each sequence-specific primer. The report will only fluoresce once the nearby quencher molecule, also attached to the primer, is digested away during the polymerization step. This reduces the incidence of background, noise, and false-positive fluorescence. Lastly, the TaqMan Array Cards can be easily modified in the future to add or remove genes, and can be easily mass produced for clinical use across the country. In summary, the TaqMan platform allows for high-throughput surveillance of the expression levels of multiple genes in a convenient enough manner to allow utilization in most regular diagnostic laboratories, instead of requiring a highly sophisticated research laboratory and staff. This is ideal for a clinical blood test.

There were several limitations to this validation study. While using spouses as controls is ideal for matching age, ethnicity, diet, and geography, it does not control for gender. Since there are possible gender-based differences in the expression levels of one or more of our genes of interest, the ideal setup would have similar numbers of male and female patients (and controls). However, we have limited our study sample to patients with *ascending* TAAs, which may be more prevalent in men than in women (4).

Secondly, we made the assumption that all spousal controls are free from TAA disease, based on 1) the relatively low population prevalence of the disease, and 2) the self-report of all spouses. However, none of the enrolled spouses had radiographic evidence that definitively rules out thoracic aneurysms. We felt that imposing such an inclusion criterion for controls would result in either low enrollment of spouses, or enrollment of spouses with other

comorbidities, since healthy individuals generally do not have ECHOs, CT scans, or MRI scans. In addition, requesting that spouses obtain imaging just for the purposes of this study would be prohibitively expensive, and medically contraindicated.

We also enrolled a few TAA patients who have already had their aneurysms replaced. All such patients had their surgeries more than one year prior to the time of blood collection, and we expect that any blood products they received during their hospitalization had washed out. We presume that an underlying "aneurysm" molecular biology persists systemically even after aneurysm resection. However, it is possible that either the stress and inflammatory response associated with surgery, or the presence of a foreign body, permanently altered the expression profiles of these patients' circulating leukocytes. Since we are developing a screening test, our ideal test population would all be pre-operative patients.

Lastly, we used two different blood collection and RNA stabilization systems, the PAXgene and the Tempus Blood RNA tubes. We had initially believed that because we are measuring the *relative,* and not absolute, expression levels of a panel of genes, the quality and quantity of RNA extracted from blood would not affect the outcome. However, published studies have demonstrated that samples isolated with PAXgene and Tempus systems amplify differential subsets of genes, including some interleukins (which are found in our current assay of genes) (53). Fortunately, the overall accuracy of our assay in detecting TAA did not appear to be negatively affected. Future studies must utilize a single and uniform RNA isolation system.

Future studies will focus on generalizing these current findings to a cohort that more closely resembles the population-to-be-screened. A true prospective validation study in which consecutive individuals with unknown TAA statuses are tested with this RNA signature assay, and then compared to a gold standard, will be the ultimate proof of the clinical utility of this

assay. However, this setup will require a prohibitively high number of participants before enough de novo TAAs can be detected with this assay. Thus, such an experiment would be unfeasible for TAAs. A more feasible prospective study would involve a small subset of the general population in which the prevalence of TAA is already high, such as the family members of TAA patients.

Subgroup analysis is a natural extension of this current study. For example, we can interrogate the assay with just male patients against male controls, or examine whether the assay performs well for TAA patients with bicuspid valves. An evaluation of whether this assay is sensitive for TAAs in patients with connective tissue disorder, whom we have intentionally excluded from this current study, is already underway by our group.

In order to demonstrate the specificity of this assay for TAAs, as opposed to being able to detect any cardiovascular, inflammatory, or aneurysmal disease, future studies can evaluate whether the assay can distinguish TAA patients from patients with coronary artery disease, intracranial aneurysms, and abdominal aortic aneurysms, or, possibly, whether the signature can detect these other aneurysm types as well. In our current cohort, significantly more TAA patients have other cardiac conditions than spousal controls (**Table 1**), again raising the question of whether our RNA signature is specific for TAAs. We must rule out the possibility that the RNA signature detects cardiac disease in general.

Finally, the thermocycler used for real-time PCR in this study, the 7900HT Fast Real-Time PCR System thermocycler (Applied Biosystems), is being phased out of use in research and diagnostic laboratories, and will be replaced with the ViiA7 Real-Time PCR System (Applied Biosystems), and possibly even newer models, by the time this assay is ready for clinical use.

Such is the rapid pace of molecular genetics in the current era. Therefore, a validation study should be repeated using the latest line of real-time thermocyclers.

We envision that the widespread availability of such thermocyclers, even in a few laboratories per state, and the widespread distribution of the TACs customized with our RNA signature assay, can make this screening test for TAA available nationwide. We hope that our promising findings regarding the accuracy of this RNA signature in detecting TAA, and future commercialization of the assay, will produce a powerful tool in preventative cardiology. Such a tool will lead to earlier diagnosis of silent thoracic aneurysms and earlier access to treatment, thereby reducing the mortality of this disease.

Figures

	Case	Control
Sample Size	24	16
Percent Male	79%	19%
Mean Age (Range)	59 (26-81)	59 (46-80)
Mean Height in Inches (Range)	69 (58-75)	66 (62-71)
Mean Weight in Pounds (Range)	182 (90-247)	185 (120-295)
Aneurysm Type: Ascending Alone	19/24	-
Aneurysm Type: Ascending and Arch	5/24	-
Average Size in cm (Range)	5.18 (4.5 - 6.9)	-
Post-operative Sample	7/24 (29%)	-
Bicuspid Aortic Valve	13/24 (54%)	-
Family History of Aortic Aneurysms	14/24 (58%)	1/14 (7.1%)
Hypertension	15/24 (63%)	9/14 (64%)
Smoker	6/24 (25%)	4/14 (29%)
Hyperlipidemia	15/24 (63%)	6/14 (43%)
Diabetes	2/24 (8.3%)	1/14 (7.1%)
Non-Valvular Heart Disease	12/24 (50%)	1/14 (7.1%)

Table 1. Summary of clinical characteristics of enrolled patients (case) and spouses (control).

GeneSymbol	AssayID	Median Ct (TAA)	Median Ct (Control)	FC(Control/TAA)	FC(Control/TAA) from Wang et al.
AKR1B1	Hs01091556_g1	20.342	20.872	-0.530	0.527
APOA1BP	Hs00603256_m1	20.653	20.910	-0.257	0.000
ATAD3A	Hs01587333_m1	23.593	24.553	-0.960	0.077
ATP5G1	Hs00829069_s1	19.754	19.742	0.012	0.707
C10orf99	Hs01379644_m1	35.000	35.000	0	-1.292
C14orf138	Hs00757319_s1	22.495	22.527	-0.031	-0.216
C15orf63	Hs00742666_s1	24.186	25.038	-0.853	-0.283
CDK4	Hs00364847_m1	20.865	20.760	0.105	0.043
CUTA	Hs00360405_g1	18.856	18.807	0.049	0.200
EDF1	Hs00610154_g1	18.144	17.957	0.187	0.355
ENPP4	Hs00208830_m1	24.787	25.645	-0.858	-0.190
HMOX2	Hs01558389_m1	20.831	21.440	-0.609	0.389
IL18R1	Hs00977687_m1	25.215	26.575	-1.360	-0.625
IMPDH2	Hs01021357_g1	23.726	23.814	-0.088	0.369
MED6	Hs00902624_m1	21.840	21.841	-0.001	-0.199
MIF	Hs00236988_g1	17.166	16.873	0.293	0.200
hCG19900	Hs00202945_m1	28.582	27.598	0.984	-0.071
hCG1773879	Hs01000283_s1	25.521	26.506	-0.985	0.157
hCG2042278	Hs01039755_s1	24.561	25.488	-0.927	-0.063
hCG2036771	Hs01568938_m1	35.000	35.000	0	-0.653
NOSIP	Hs01119990_g1	18.815	18.917	-0.102	0.694
NR1I2	Hs01114267_m1	35.000	35.000	0	0.155
NUDC	Hs00702452_s1	19.810	19.714	0.096	0.548
NUDT5	Hs00274029_m1	21.413	21.662	-0.248	-0.011
PCDHA12	Hs00259025_s1	35.000	35.000	0	-0.718
PHB	Hs00855044_g1	22.289	22.471	-0.182	-0.100
PPAN	Hs00220301_m1	22.139	22.177	-0.038	-0.236
RGS3	Hs00367777_m1	20.497	21.219	-0.722	-0.153
RUVBL1	Hs01031910_m1	23.359	23.913	-0.553	0.466
SBSN	Hs00415561_m1	35.000	35.000	0	-0.280
SERF2	Hs00428481_m1	18.246	17.991	0.255	-0.927
SLC25A24	Hs00534808_s1	23.216	23.137	0.079	-0.772
SNRPC	Hs00853882_g1	19.132	18.719	0.413	0.914
SOS2	Hs01127279_m1	21.544	21.637	-0.094	-0.915
SSU72	Hs00204394_m1	18.506	18.514	-0.008	-0.090
SYNGAP1	Hs01006404_m1	26.886	29.710	-2.824	0.455
THOC6	Hs00738018_g1	35.000	35.000	0	0.571
VKORC1	Hs00829655_s1	21.999	22.520	-0.520	-0.163
VPS72	Hs00195618_m1	23.033	23.571	-0.538	0.049

Table 2. Fold-change in expression level of control samples over TAA samples for each gene. Genes that had fold-changes equal to 0 correspond to those that did not amplify (no fluorescence detected by the 35^{th} cycle).

	Wang et al.	Wang et al.	Current Study
Method	Microarray	qPCR (384-well plate)	qPCR (TACs)
No. of Genes	41	41	33
TAA Samples (N)	22	21	24
Control Samples (N)	11	9	16
Accuracy (%)	78	80	83
Sensitivity (%)	72	71	88
Specificity (%)	90	100	75

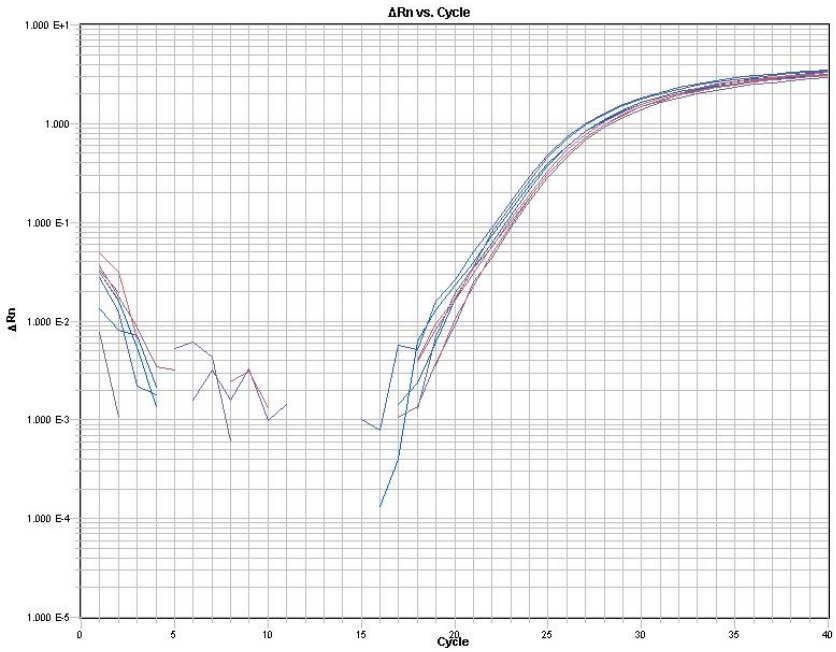
Table 3. Comparison of the testing characteristics of the three validation studies.

(TAC: TaqMan Array Card)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	PHB	N/A	SERF2	VKORC1	SNRPC	C10orf99	SBSN	C14orf138	N/A	PPIA	GAPDH	B2M	ACTB	RGS3	PCDHA12	ATAD3A	N/A	NUDT5	MED6	APOA1BP	ATP5G1	CDK4	SLC25A24	MIF
2	N/A	PPAN;PPAN-P2RY11	NUDC	NR1I2	AKR1B1	EDF1	HMOX2	IL18R1	IMPDH2	NOSIP	NOSIP	RUVBL1	SOS2	VPS72	C15orf63	ENPP4	SSU72	CUTA	SYNGAP1	THOC6	CDH13	JUN	IL10	MMP15
3	PHB	N/A	SERF2	VKORC1	SNRPC	C10orf99	SBSN	C14orf138	N/A	PPIA	GAPDH	B2M	ACTB	RGS3	PCDHA12	ATAD3A	N/A	NUDT5	MED6	APOA1BP	ATP5G1	CDK4	SLC25A24	MIF
4	N/A	PPAN;PPAN-P2RY11	NUDC	NR1I2	AKR1B1	EDF1	HMOX2	IL18R1	IMPDH2	NOSIP	NOSIP	RUVBL1	SOS2	VPS72	C15orf63	ENPP4	SSU72	CUTA	SYNGAP1	THOC6	CDH13	JUN	IL10	MMP15
5	PHB	N/A	SERF2	VKORC1	SNRPC	C10orf99	SBSN	C14orf138	N/A	PPIA	GAPDH	B2M	ACTB	RGS3	PCDHA12	ATAD3A	N/A	NUDT5	MED6	APOA1BP	ATP5G1	CDK4	SLC25A24	MIF
6	N/A	PPAN;PPAN-P2RY11	NUDC	NR1I2	AKR1B1	EDF1	HMOX2	IL18R1	IMPDH2	NOSIP	NOSIP	RUVBL1	SOS2	VPS72	C15orf63	ENPP4	SSU72	CUTA	SYNGAP1	THOC6	CDH13	JUN	IL10	MMP15
7	PHB	N/A	SERF2	VKORC1	SNRPC	C10orf99	SBSN	C14orf138	N/A	PPIA	GAPDH	B2M	ACTB	RGS3	PCDHA12	ATAD3A	N/A	NUDT5	MED6	APOA1BP	ATP5G1	CDK4	SLC25A24	MIF
8	N/A	PPAN;PPAN-P2RY11	NUDC	NR1I2	AKR1B1	EDF1	HMOX2	IL18R1	IMPDH2	NOSIP	NOSIP	RUVBL1	SOS2	VPS72	C15orf63	ENPP4	SSU72	CUTA	SYNGAP1	THOC6	CDH13	JUN	IL10	MMP15
9	PHB	N/A	SERF2	VKORC1	SNRPC	C10orf99	SBSN	C14orf138	N/A	PPIA	GAPDH	B2M	ACTB	RGS3	PCDHA12	ATAD3A	N/A	NUDT5	MED6	APOA1BP	ATP5G1	CDK4	SLC25A24	MIF
10	N/A	PPAN;PPAN-P2RY11	NUDC	NR1I2	AKR1B1	EDF1	HMOX2	IL18R1	IMPDH2	NOSIP	NOSIP	RUVBL1	SOS2	VPS72	C15orf63	ENPP4	SSU72	CUTA	SYNGAP1	THOC6	CDH13	JUN	IL10	MMP15
11	PHB	N/A	SERF2	VKORC1	SNRPC	C10orf99	SBSN	C14orf138	N/A	PPIA	GAPDH	B2M	ACTB	RGS3	PCDHA12	ATAD3A	N/A	NUDT5	MED6	APOA1BP	ATP5G1	CDK4	SLC25A24	MIF
12	N/A	PPAN;PPAN-P2RY11	NUDC	NR1I2	AKR1B1	EDF1	HMOX2	IL18R1	IMPDH2	NOSIP	NOSIP	RUVBL1	SOS2	VPS72	C15orf63	ENPP4	SSU72	CUTA	SYNGAP1	THOC6	CDH13	JUN	IL10	MMP15
13	PHB	N/A	SERF2	VKORC1	SNRPC	C10orf99	SBSN	C14orf138	N/A	PPIA	GAPDH	B2M	ACTB	RGS3	PCDHA12	ATAD3A	N/A	NUDT5	MED6	APOA1BP	ATP5G1	CDK4	SLC25A24	MIF
14	N/A	PPAN;PPAN-P2RY11	NUDC	NR1I2	AKR1B1	EDF1	HMOX2	IL18R1	IMPDH2	NOSIP	NOSIP	RUVBL1	SOS2	VPS72	C15orf63	ENPP4	SSU72	CUTA	SYNGAP1	THOC6	CDH13	JUN	IL10	MMP15
15	PHB	N/A	SERF2	VKORC1	SNRPC	C10orf99	SBSN	C14orf138	N/A	PPIA	GAPDH	B2M	ACTB	RGS3	PCDHA12	ATAD3A	N/A	NUDT5	MED6	APOA1BP	ATP5G1	CDK4	SLC25A24	MIF
16	N/A	PPAN;PPAN-P2RY11	NUDC	NR1I2	AKR1B1	EDF1	HMOX2	IL18R1	IMPDH2	NOSIP	NOSIP	RUVBL1	SOS2	VPS72	C15orf63	ENPP4	SSU72	CUTA	SYNGAP1	THOC6	CDH13	JUN	IL10	MMP15

Figure 1. TaqMan Array map of the RNA signature. In this example of a 48-well TaqMan Array Card format, each card has loading lanes for eight (8) samples. Each lane will distribute the sample to two adjacent rows, each containing 24 wells. Each colored box represents a different gene in this customized TaqMan Array Card. “N/A” represents empty wells. PPIA was used as the endogenous control. Real-time PCR using the 7900HT Fast Real-Time PCR System utilizes such a map file to assign a gene identity to the fluorescence amplification curves emerging from each well.

A.



B.

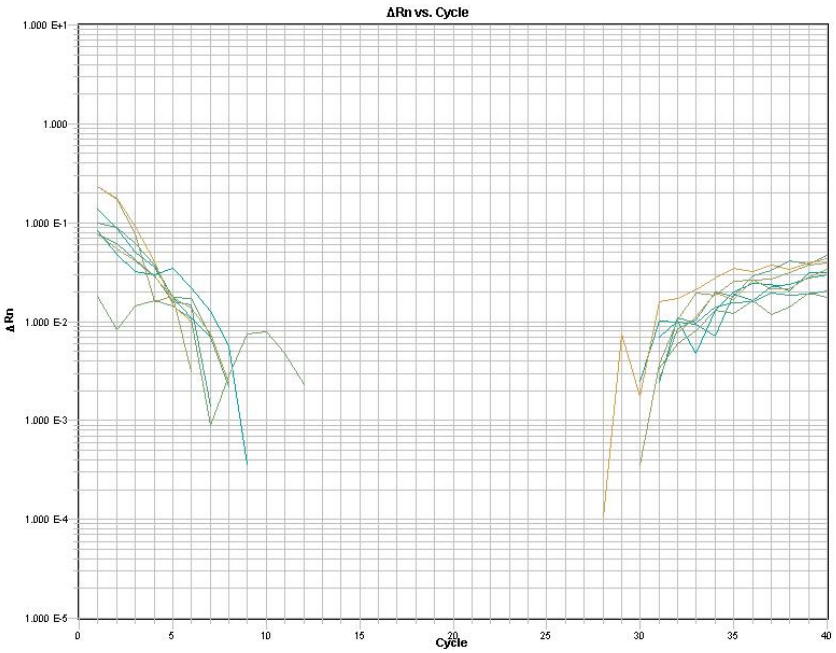


Figure 2. Real-time PCR amplification curves. (A). Amplification curve of a single gene across eight samples. Each sample is represented by a different colored curve. The fluorescence of each sample (y-axis) is shown as a function of cycle number (x-axis). Note the efficient logarithmic phase of the amplification starting at cycle 18, followed by a plateau phase. **(B)**. Six individual genes, however, failed to amplify by the end of the 40-cycle run. This amplification curve shows the failed amplification of one of these genes. Note the lack of a logarithmic phase for all eight samples in this representative assay.

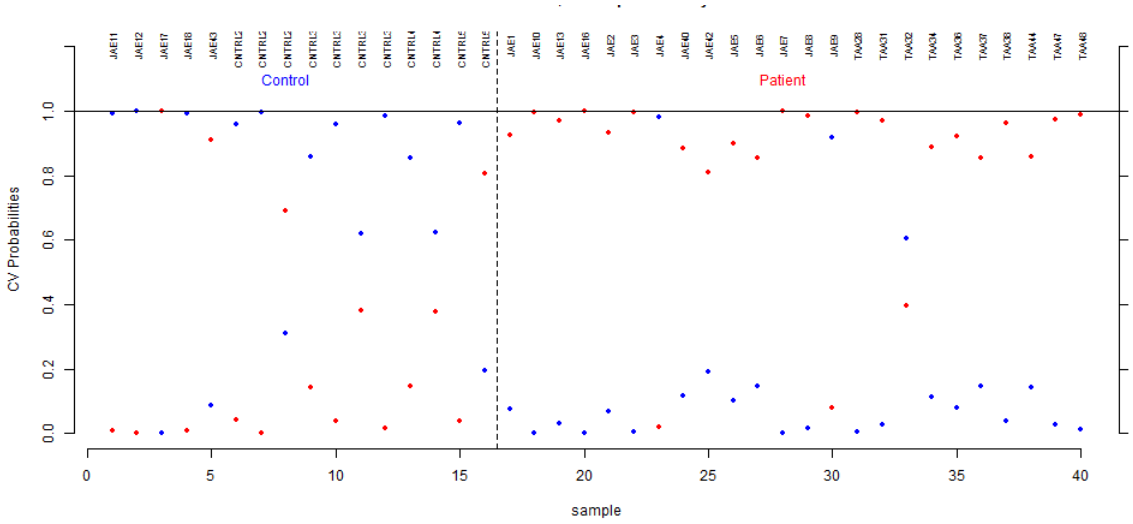


Figure 3. 10-fold cross-validation of the 33-gene RNA signature in predicting aneurysm status. After a 10-fold cross-validation study, the probability of each of the 40 samples (vertical column) originating from a "TAA patient" was calculated (red dots). This probability ranged from 0 to 100%, depending on the prediction from the cross-validation. The samples are grouped according to their true clinical status (16 Controls, and 24 Patients). The RNA signature is successful in predicting the correct clinical status for most samples.

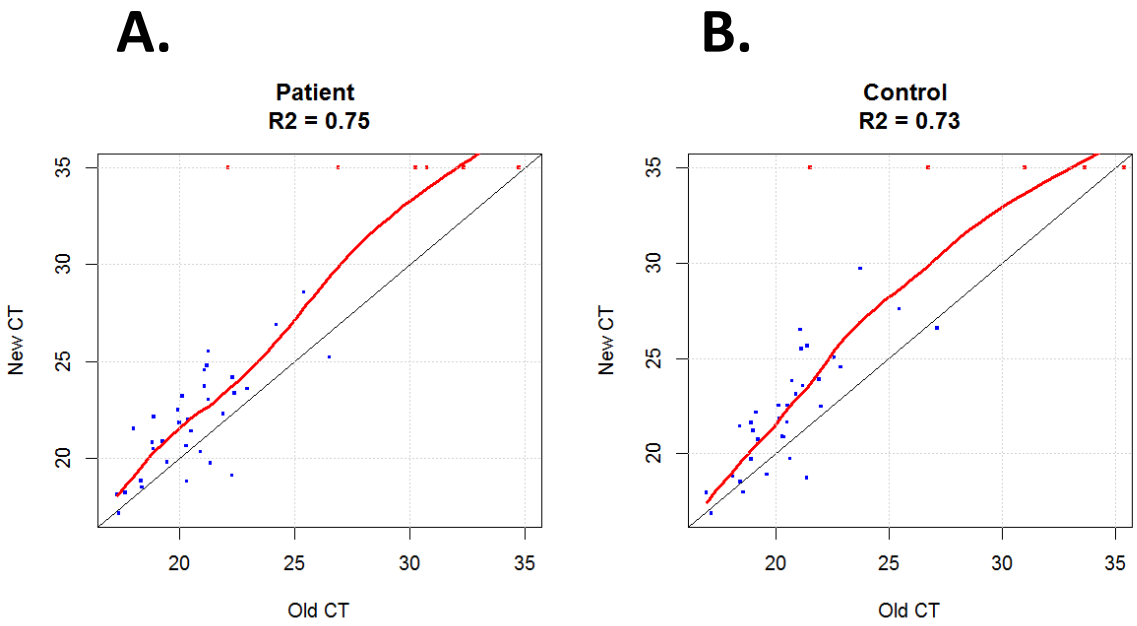


Figure 4. Comparison of Ct values between current and previous assay studies. Each gene in the assay is represented by a point in the scatterplot. Red points represent the genes that failed to amplify in the current assay. **(A).** For each gene, the Ct values are averaged across all TAA patients in the prior assay ("Old Ct") and across all TAA patients in the current assay ("New Ct"). Each gene is then plotted in the appropriate coordinate, at the intersection of the "Old Ct" value and "New Ct" value. The y=x line shows an ideal relationship in which the average Ct value for a gene is identical between the current and prior assays. The red line represents the loess approximation of the measurements. The coefficient of determination (r^2) value, listed above the scatterplot, describes the linear correlation of the Ct values between the current and prior assays. The genes that failed to amplify in the current assay (red dots) did not have fluorescence levels that exceeded the threshold even by cycle 35. **(B).** Similar scatterplot showing the correlation between "Old Ct" and "New Ct" values for each gene across all spousal controls.

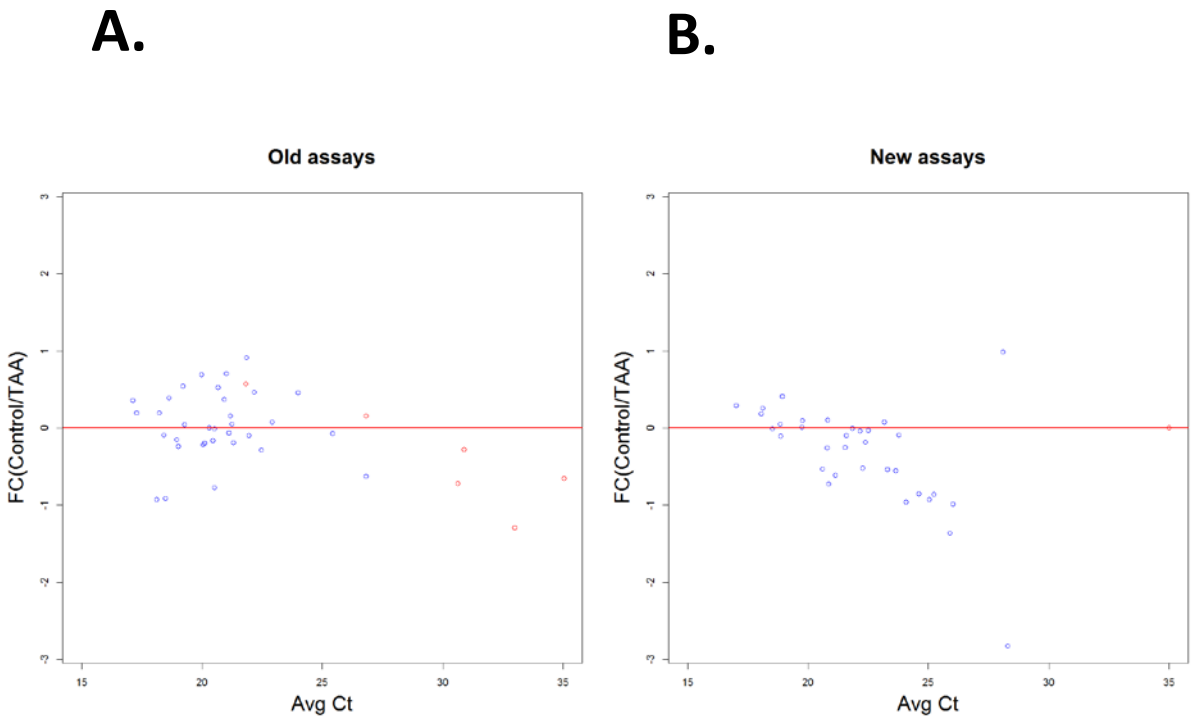


Figure 5: Analysis of the fold-change in gene expression between TAA patients and spousal controls. Each gene in the assay is represented by a point in the graph. For each gene, the relative expression level of that gene in TAA (case) samples versus spousal (control) samples is expressed as a fold-change. Genes near the red (y = 0) line are those that are not expressed in significantly different amounts between cases and controls. Genes located above the red line are those that are over-expressed in controls relative to TAA patients. Likewise, genes located below the red line are those that are under-expressed in controls relative to TAA patients. Red points represent genes that failed to amplify in the current study ("New assays"). The y-axis is in log 2. **(A).** In the prior assays ("Old assays"), there are genes that are both over-expressed and under-expressed in TAA patients. **(B).** By contrast, the current assays ("New assays") shows that most genes are over-expressed in TAA patients relative to spousal controls.

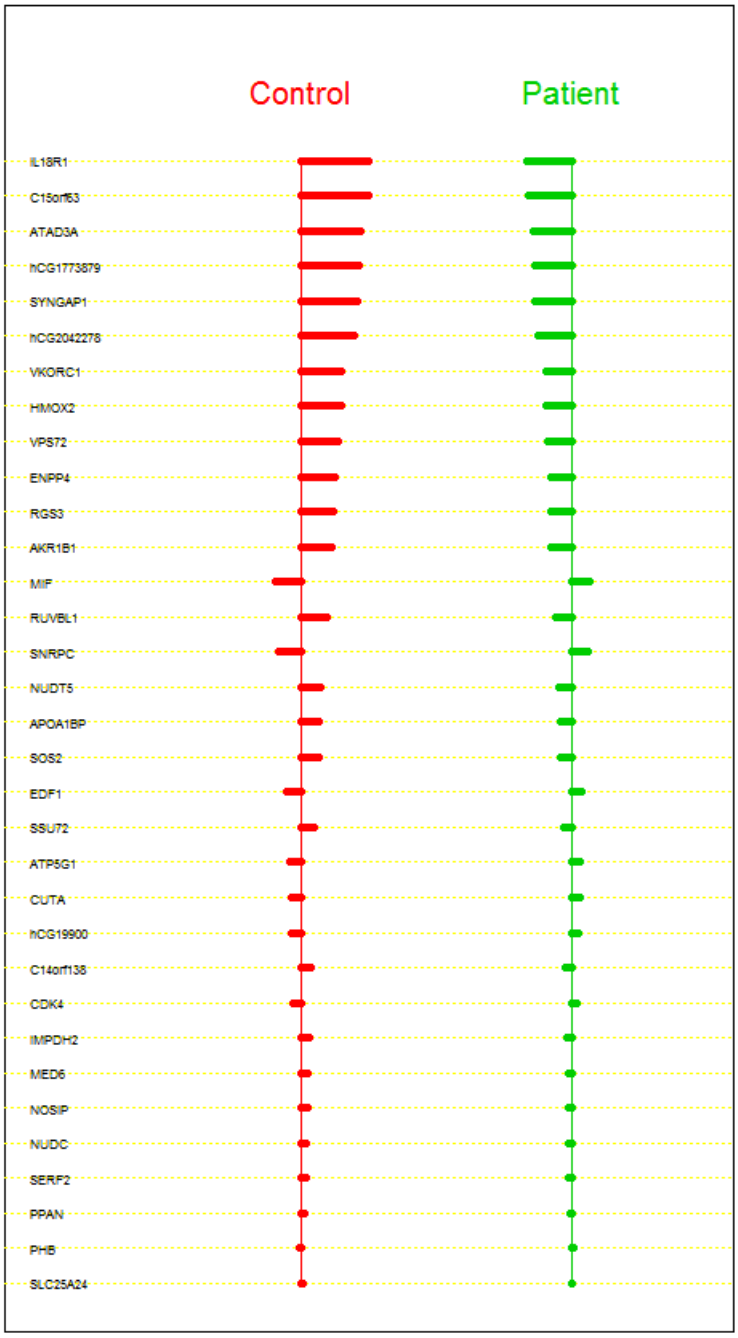
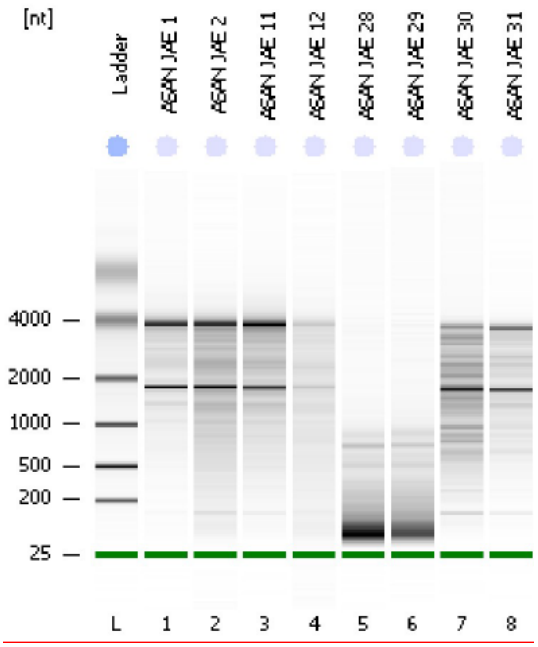


Figure 6: Nearest shrunken centroid profiles across the 33 classifier genes. The relative expression levels of each of the 33 classifier genes between TAA patient samples and control samples are shown as a centroid profile. Left-ward bars represent over-expression, and right-ward bars represent under-expression. The genes are arranged in descending order of magnitude in fold-change expression levels between TAA samples and control samples. As shown in this centroid profile, the most discriminatory classifier genes appear to be over-expressed in TAA patients relative to control samples.

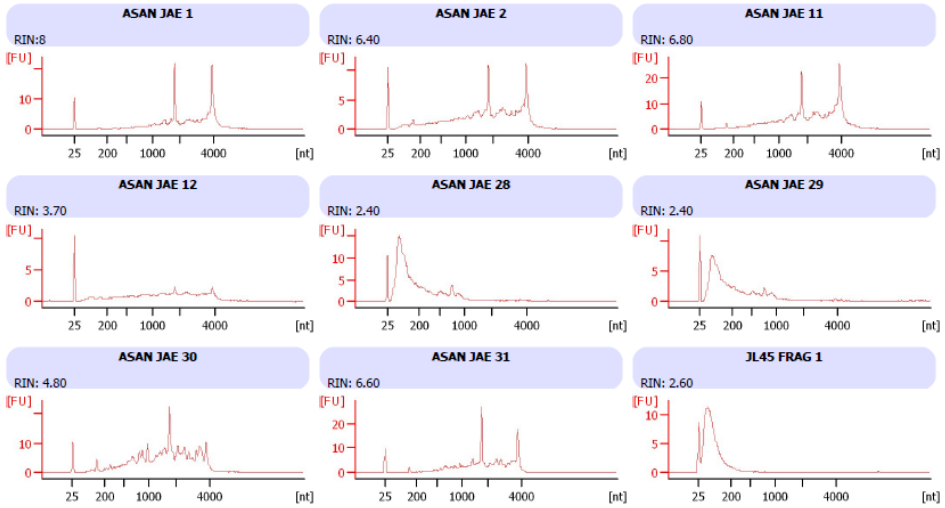
	RNA (ng/uL)			260 /280 Ratio			RIN	
	PaxGene	Tempus		PaxGene	Tempus		PaxGene	Tempus
	96.92	154.82		2.15	2.07		8	2.4
	100.64	87.45		2.07	2.09		6.4	2.4
	107.9	190.41		2.13	2.04		7.7	6.6
	62.2	115.32		2.15	2.06		8.5	6.6
	140.9	87.84		2.11	2.13		8	6.8
	76.9	142.18		2.13	2.11		6.8	7
	154.1	109.35		2.10	2.12		7.7	6.7
	49.4	166.98		2.23	2.08		8.3	7
	97.9	131.01		2.16	2.12		8.1	2.5
	159.59	164.70		2.09	2.06		6.8	3
	46.63	70.58		2.23	2.13		3.7	8.8
	123.1	132.00		2.10	2.09		7.4	8.1
	67.5	142.7		2.11	2.05		8	7.2
	113.5	118.6		2.08	2.05		8.9	8.4
	100.2	138.4		2.11	2.05		8.7	8
	48.65	84.7		2.21	2.04			5.3
	80.44	124.5		2.15	2.02			7
		164.5			2.04			8.1
		78.6			2.04			4.1
		105.5			2.04			7.8
		166.7			2.05			8.6
		313.8			2.06			7.5
Count	17	22		17	22		15	22
Mean	95.67	135.94		2.14	2.07		7.53	6.36
p-value (T-test)	0.0065505			0.0000613			0.0425528	

Appendix A. Comparison of PAXgene and Tempus RNA isolation methods. The quantitative yield, 260/280 purity ratio, and the RNA Integrity Number (RIN) are listed for each sample isolated with either method. All three measures of the performance of an RNA isolation kit are statistically significantly different between the PAXgene and Tempus systems.

A.



B.



Appendix B. A representative output from the 2100 Bioanalyzer. (A). The integrity and quality of each RNA sample is evaluated through electrophoresis. Samples are run against a RNA ladder. "High quality" samples show a two characteristic bands, around 4000 and 1800 nt, with very little contamination or smear outside of these bands. In this set of samples, lanes 5 and 6 are degraded samples (no bands at 4000 and 1800 nt), lane 4 contains low amounts of RNA (low-intensity bands), and lane 7 contains contamination (extraneous bands). **(B).** The peak fluorescence as a function of molecular size, for the same eight samples. Note the characteristic twin peaks, representing the 18S and 28S subunits of RNA.

References

1. Johnston, K.W. 1991. Suggested standards for reporting on arterial aneurysms. *Journal of vascular surgery* 13:452.
2. Roman, M.J. 1989. Two-dimensional echocardiographic aortic root dimensions in normal children and adults. *The American journal of cardiology* 64:507.
3. Hiratzka, L.F., Bakris, G.L., Beckman, J.A., Bersin, R.M., Carr, V.F., Casey, D.E., Eagle, K.A., Hermann, L.K., Isselbacher, E.M., Kazerooni, E.A., et al. 2010. 2010 ACCF/AHA/AATS/ACR/ASA/SCA/SCAI/SIR/STS/SVM Guidelines for the Diagnosis and Management of Patients With Thoracic Aortic Disease: Executive Summary. *Journal of the American College of Cardiology* 55:1509-1544.
4. Elefteriades, J.A., Olin, J.W., and Halperin, J.L. 2013. Diseases of the Aorta (Chapter 106). In *Hurst's The Heart*. V. Fuster, R.A. Walsh, and R.A. Harrington, editors.
5. Isselbacher, E.M. 2005. Thoracic and abdominal aortic aneurysms. *Circulation* 111:816-828.
6. Elefteriades, J. 2007. *Acute Aortic Disease*. New York: Informa Healthcare.
7. Elefteriades, J.A., Tranquilli, M., Darr, U., Cardon, J., Zhu, B.Q., and Barrett, P. 2005. Symptoms plus family history trump size in thoracic aortic aneurysm. *Ann Thorac Surg* 80:1098-1100.
8. Kuzmik, G., Sang, A., Cai, G., Tranquilli, M., and Elefteriades, J. 2012. Respecting Symptoms in Thoracic Aortic Aneurysm Management: A Case of Symptomatic Necrotizing Granulomatous Aortitis. *International Journal of Angiology* 21:151-154.
9. Elefteriades, J.A. 2010. Indications for aortic replacement. *J Thorac Cardiovasc Surg* 140:S5-9; discussion S45-51.
10. Itani, Y., Watanabe, S., Masuda, Y., Hanamura, K., Asakura, K., Sone, S., Sunami, Y., and Miyamoto, T. 2002. Measurement of aortic diameters and detection of asymptomatic aortic aneurysms in a mass screening program using a mobile helical computed tomography unit. *Heart and Vessels* 16:42-45.
11. Kalsch, H., Lehmann, N., Mohlenkamp, S., Becker, A., Moebus, S., Schmermund, A., Stang, A., Mahabadi, A.A., Mann, K., Jockel, K.H., et al. 2011. Body-surface adjusted aortic reference diameters for improved identification of patients with thoracic aortic aneurysms: Results from the population-based Heinz Nixdorf Recall study. *Int J Cardiol*.
12. Bickerstaff, L.K., Pairolero, P.C., Hollier, L.H., Melton, L.J., Van Peenen, H.J., Cherry, K.J., Joyce, J.W., and Lie, J.T. 1982. Thoracic aortic aneurysms: a population-based study. *Surgery* 92:1103-1108.
13. Clouse, W.D., Hallett, J.W., Jr., Schaff, H.V., Gayari, M.M., Ilstrup, D.M., and Melton, L.J., 3rd. 1998. Improved prognosis of thoracic aortic aneurysms: a population-based study. *JAMA* 280:1926-1929.
14. Olsson, C., Thelin, S., Stahle, E., Ekbom, A., and Granath, F. 2006. Thoracic aortic aneurysm and dissection: increasing prevalence and improved outcomes reported in a nationwide population-based study of more than 14,000 cases from 1987 to 2002. *Circulation* 114:2611-2618.
15. Elefteriades, J.A., and Rizzo, J. 2007. Epidemiology, prevalence, incidence, trends. In *Acute Aortic Disease*. J. Elefteriades, editor. New York: Informa Healthcare. 89-98.
16. Elefteriades, J.A., and Farkas, E.A. 2010. Thoracic aortic aneurysm clinically pertinent controversies and uncertainties. *J Am Coll Cardiol* 55:841-857.

17. Control, C.f.D.C.a.P.C.a.N.C.f.I.P.a. 2010. 20 Leading Causes of Death, United States. Atlanta.
18. Barbour, J.R., Spinale, F.G., and Ikonomidis, J.S. 2007. Proteinase systems and thoracic aortic aneurysm progression. *J Surg Res* 139:292-307.
19. Koullias, G.J., Ravichandran, P., Korkolis, D.P., Rimm, D.L., and Elefteriades, J.A. 2004. Increased tissue microarray matrix metalloproteinase expression favors proteolysis in thoracic aortic aneurysms and dissections. *Ann Thorac Surg* 78:2106-2110; discussion 2110-2101.
20. He, R., Guo, D.C., Sun, W., Papke, C.L., Duraisamy, S., Estrera, A.L., Safi, H.J., Ahn, C., Buja, L.M., Arnett, F.C., et al. 2008. Characterization of the inflammatory cells in ascending thoracic aortic aneurysms in patients with Marfan syndrome, familial thoracic aortic aneurysms, and sporadic aneurysms. *J Thorac Cardiovasc Surg* 136:922-929, 929 e921.
21. Albornoz, G., Coady, M.A., Roberts, M., Davies, R.R., Tranquilli, M., Rizzo, J.A., and Elefteriades, J.A. 2006. Familial thoracic aortic aneurysms and dissections--incidence, modes of inheritance, and phenotypic patterns. *Ann Thorac Surg* 82:1400-1405.
22. Coady, M.A. 1999. Familial Patterns of Thoracic Aortic Aneurysms. *Archives of Surgery* 134:361-367.
23. Silberbach, M. 2009. Bicuspid aortic valve and thoracic aortic aneurysm: toward a unified theory. *J Am Coll Cardiol* 53:2296-2297.
24. Larsson, E., Vishnevskaya, L., Kalin, B., Granath, F., Swedenborg, J., and Hultgren, R. 2011. High frequency of thoracic aneurysms in patients with abdominal aortic aneurysms. *Ann Surg* 253:180-184.
25. Lee, J.J., Dimick, J.B., Williams, D.M., Henke, P.K., Deeb, G.M., Eagle, K.A., Stanley, J.C., and Upchurch, G.R. 2003. Existence of abdominal aortic aneurysms in patients with thoracic aortic dissections. *Journal of Vascular Surgery* 38:671-675.
26. Kim, D.H., Van Ginhoven, G., and Milewicz, D.M. 2005. Familial Aggregation of Both Aortic and Cerebral Aneurysms: Evidence for a Common Genetic Basis in a Subset of Families. *Neurosurgery* 56:655-661.
27. Kuzmik, G.A., Feldman, M., Tranquilli, M., Rizzo, J.A., Johnson, M., and Elefteriades, J.A. 2010. Concurrent intracranial and thoracic aortic aneurysms. *Am J Cardiol* 105:417-420.
28. Friedman, T., Mani, A., and Elefteriades, J.A. 2008. Bicuspid aortic valve: clinical approach and scientific review of a common clinical entity. *Expert Rev Cardiovasc Ther* 6:235-248.
29. Davies, R.R., Kaple, R.K., Mandapati, D., Gallo, A., Botta, D.M., Jr., Elefteriades, J.A., and Coady, M.A. 2007. Natural history of ascending aortic aneurysms in the setting of an unreplaced bicuspid aortic valve. *Ann Thorac Surg* 83:1338-1344.
30. Hatzaras, I.S., Bible, J.E., Koullias, G.J., Tranquilli, M., Singh, M., and Elefteriades, J.A. 2007. Role of exertion or emotion as inciting events for acute aortic dissection. *Am J Cardiol* 100:1470-1472.
31. Elefteriades, J.A., Barrett, P.W., and Kopf, G.S. 2008. Litigation in nontraumatic aortic diseases--a tempest in the malpractice maelstrom. *Cardiology* 109:263-272.
32. Johansson, G., Markström, U., and Swedenborg, J. 1995. Ruptured thoracic aortic aneurysms: A study of incidence and mortality rates. *Journal of Vascular Surgery* 21:985-988.
33. Clouse, W.D., Hallett, J.W., Schaff, H.V., Spittell, P.C., Rowland, C.M., Ilstrup, D.M., and Melton, L.J. 2004. Acute Aortic Dissection: Population-Based Incidence Compared With Degenerative Aortic Aneurysm Rupture. *Mayo Clinic Proceedings* 79:176-180.

34. Rizzo, J.A., Coady, M.A., and Elefteriades, J.A. 1998. Procedures for Estimating Growth Rates in Thoracic Aortic Aneurysms. *Journal of Clinical Epidemiology* 51:747-754.
35. Coady, M.A., Rizzo, J.A., Hammond, G.L., Mandapati, D., Darr, U., Kopf, G.S., and Elefteriades, J.A. 1997. What is the appropriate size criterion for resection of thoracic aortic aneurysms? *The Journal of Thoracic and Cardiovascular Surgery* 113:476-491.
36. Davies, R.R., Goldstein, L.J., Coady, M.A., Tittle, S.L., Rizzo, J.A., Kopf, G.S., and Elefteriades, J.A. 2002. Yearly rupture or dissection rates for thoracic aortic aneurysms: simple prediction based on size. *The Annals of Thoracic Surgery* 73:17-28.
37. Elefteriades, J.A., Hatzaras, I., Tranquilli, M.A., Elefteriades, A.J., Stout, R., Shaw, R.K., Silverman, D., and Barash, P. 2003. Weight lifting and rupture of silent aortic aneurysms. *JAMA* 290:2803.
38. Hatzaras, I., Tranquilli, M., Coady, M., Barrett, P.M., Bible, J., and Elefteriades, J.A. 2007. Weight lifting and aortic dissection: more evidence for a connection. *Cardiology* 107:103-106.
39. Hagan, P.G. 2000. The International Registry of Acute Aortic Dissection (IRAD): New Insights Into an Old Disease. *JAMA: The Journal of the American Medical Association* 283:897-903.
40. Tsai, T.T., Fattori, R., Trimarchi, S., Isselbacher, E., Myrmel, T., Evangelista, A., Hutchison, S., Sechtem, U., Cooper, J.V., Smith, D.E., et al. 2006. Long-term survival in patients presenting with type B acute aortic dissection: insights from the International Registry of Acute Aortic Dissection. *Circulation* 114:2226-2231.
41. Kodolitsch, Y.v., Nienaber, C.A., Dieckmann, C., Schwartz, A.G., Hofmann, T., Brekenfeld, C., Nicolas, V., Berger, J., and Meinertz, T. 2004. Chest radiography for the diagnosis of acute aortic syndrome. *The American Journal of Medicine* 116:73-77.
42. Coady, M.A., Rizzo, J.A., and Elefteriades, J.A. 1999. Developing Surgical Intervention Criteria for Thoracic Aortic Aneurysms. *Cardiology Clinics* 17:827-839.
43. Achneck, H.E., Rizzo, J.A., Tranquilli, M., and Elefteriades, J.A. 2007. Safety of thoracic aortic surgery in the present era. *Ann Thorac Surg* 84:1180-1185; discussion 1185.
44. Iyer, V.S. 2006. Early outcomes after elective and emergent endovascular repair of the thoracic aorta. *Journal of vascular surgery* 43:677.
45. Pape, L.A. 2007. Aortic Diameter $\geqslant$ 5.5 cm Is Not a Good Predictor of Type A Aortic Dissection Observations From the International Registry of Acute Aortic Dissection (IRAD). *Circulation* 116:1120.
46. Trimarchi, S., Sangiorgi, G., Sang, X., Rampoldi, V., Suzuki, T., Eagle, K.A., and Elefteriades, J.A. 2010. In search of blood tests for thoracic aortic diseases. *Ann Thorac Surg* 90:1735-1742.
47. Suzuki, T. 2000. Diagnostic implications of elevated levels of smooth-muscle myosin heavy-chain protein in acute aortic dissection. The smooth muscle myosin heavy chain study. *Annals of Internal Medicine* 133:537.
48. Vainas, T. 2003. Serum C-reactive protein level is associated with abdominal aortic aneurysm size and may be produced by aneurysmal tissue. *Circulation* 107:1103.
49. Wang, Y., Barbacioru, C.C., Shiffman, D., Balasubramanian, S., Iakoubova, O., Tranquilli, M., Albornoz, G., Blake, J., Mehmet, N.N., Ngadimo, D., et al. 2007. Gene expression signature in peripheral blood detects thoracic aortic aneurysm. *PLoS One* 2:e1050.
50. Tang, P.C., Yakimov, A.O., Teesdale, M.A., Coady, M.A., Dardik, A., Elefteriades, J.A., and Tellides, G. 2005. Transmural inflammation by interferon-gamma-producing T cells correlates with outward vascular remodeling and intimal expansion of ascending thoracic aortic aneurysms. *FASEB J* 19:1528-1530.

51. Shimizu, K. 2006. Inflammation and cellular immune responses in abdominal aortic aneurysms. *Arteriosclerosis, thrombosis, and vascular biology* 26:987.
52. Dawson, J. 2007. Aortic aneurysms secrete interleukin-6 into the circulation. *Journal of vascular surgery* 45:350.
53. Asare, A.L., Kolchinsky, S.A., Gao, Z., Wang, R., Raddassi, K., Bourcier, K., and Seyfert-Margolis, V. 2008. Differential gene expression profiles are dependent upon method of peripheral blood collection and RNA isolation. *BMC Genomics* 9:474.