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Correlations Between Intracranial Aneurysms And Thoracic Aortic Aneurysms

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Abstract

This project investigates the clinical occurrence of concurrent thoracic aortic aneurysms (TAA) and intracranial aneurysms (ICA). We hypothesized that patients with a TAA have an increased risk of harboring a concurrent ICA, and likewise that patients with an ICA have an increased risk of harboring a concurrent TAA relative to the general population. In a separate arm of this project, we hypothesized that a pre-defined gene expression profile, based on the expression levels of 41 specific genes measured in peripheral blood cells, will be exhibit a characteristic expression pattern in ICA patients and thereby have utility in detecting the presence of ICA**.**

To accomplish the first objective of this project, we reviewed the charts of patients with TAA who also had recent intracranial imaging to document the prevalence of concurrent ICA and compared this rate to the ICA prevalence in the general population. Likewise, we reviewed the charts of patients with ICA who also had recent thoracic imaging to document the prevalence of concurrent TAA**.** To investigate the gene expression profile for detecting ICA, we collected peripheral blood samples from ICA patients and non-aneurysmal controls and measured the expression levels of 39 pre-defined genes in a signature aneurysm profile using real-time PCR. The observed pattern of expression of these genes was compared to a pre-defined signature aneurysm pattern to predict the aneurysm status of each sample.

We found that 9.0% of 212 TAA patients we studied harbor a concurrent ICA. Patients with descending TAA and hypertension had significantly higher rates of concurrent ICA. We also found that 4.5% of 359 ICA patients we studied harbor a concurrent TAA. ICA patients over 70 years of age had an increased rate of concurrent TAA. We also analyzed gene expression in the blood samples of 17 ICA patients and 15 controls. By comparing the observed pattern of gene expression to a predefined signature aneurysm pattern, we were able to detect ICA from a peripheral blood test with an 88% sensitivity and overall accuracy of 63%.

In conclusion, this project finds that patients with TAA are at an increased risk relative to the general population of harboring a concurrent ICA. Likewise, patients with ICA are at an increased risk relative to the general population of harboring a concurrent TAA. Our early results show that a peripheral blood test based on the gene expression pattern of 39 genes holds promise as a sensitive screening test for ICA.

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Introduction

The Clinical and Molecular Relationships Between Intracranial Aneurysms and Thoracic Aortic Aneurysms

A marked genetic component has been noted in the development of both intracranial aneurysms (ICA) and thoracic aortic aneurysms (TAA). For example, up to 20% of patients with an ICA have a first-degree relative with this same condition.[1-3] There have been recent strides in elucidating the genetic markers associated with ICA formation. A recent multinational genome-wide association study comprising nearly 6,000 cases and over 14,000 controls identified five loci associated with ICA development.[4] Earlier work has also identified numerous additional loci that may play a role in conferring susceptibility to ICA formation.[5] The development of TAA has also been shown to be strongly influenced by genetic factors.[3,6-8] It has previously been shown that over 21% of TAA patients without a known vascular connective tissue disorder have at least one first-degree relative with TAA.[8]

ICA and TAA are known to occur together in a number of recognized inheritable disorders. Patients with Ehlers-Danlos syndrome type IV, which is caused by mutations in the gene for the collagen type III pro α-1 chain (*COL3A1*), are prone to developing both TAA and ICA.[9] Likewise, the recently described aneurysm osteoarthritis syndrome predisposes to both TAA and ICA.[10] This syndrome is caused by mutations

in *SMAD3*, a gene encoding a downstream signaling mediator of transforming growth factor-β (TGF-β) via the TGFBR1 and TGFBR2 receptors.[11] Defects in the TGF-β pathway appear to be particularly important in the development of aneurysms in multiple vascular beds, including the aorta and the cerebrovasculature.[12-15] For instance, there has been recent evidence that Loeys-Dietz syndrome, a Marfan-spectrum syndrome caused by mutations in the genes encoding the TGFBR1 and TGFBR2 receptors (*TGFBR1* and *TGFBR2* respectively), also predisposes to the development of ICA.[16]

However, even in the absence of these recognized connective tissue disorders, there is evidence for a common genetic foundation underlying the development of TAA and ICA. Common chromosomal loci important in the pathogenesis of both ICA and TAA have been identified.[17] Ruigrok *et al.* conducted a review of the literature on whole-genome linkage studies investigating genetic susceptibility loci for TAA, ICA, and abdominal aortic aneurysms (AAA) and identified three loci that may play a role in conferring increased risk for developing both TAA and ICA.[17] One of these loci contained the *TGFBR2* gene, further implicating the TGF-β pathway in ICA and TAA development.

Milewicz and colleagues recently reported on 514 families with familial thoracic aortic aneurysm/dissection syndrome (TAAD) and found that, in 15 families, 17 individuals genetically at risk for inheriting TAAD had saccular ICA.[18] This phenotype was inherited as a single gene disorder in an autosomal dominant fashion with incomplete penetrance and variable expressivity. In four families in which primarily fusiform ICA occurred, mutations in genes known to cause familial TAAD (*TGFBR1*, *TGFBR2*, and

ACTA2) were identified. In addition, a separate report on a single family with an inherited pattern of TAA, ICA, and AAA revealed a frameshift mutation in *SMAD3*.[19] However, mutations in these known TAAD genes were not observed in the majority of families studied, suggesting that mutations in an unidentified gene or additional environmental factors may be responsible for the concurrent TAA-ICA phenotype.

At the molecular level, both TAA and ICA share common pathogenic mediators, which is consistent with their shared genetic underpinnings. One such mediator that has been demonstrated to play a role in aneurysm formation is matrix metalloproteinase-9 (MMP-9; elastolytic gelatinase), a protease that targets numerous substrates in the extracellular matrix of arterial walls, including elastin, fibrillin, and collagen, and is known to mediate destructive changes in arterial wall architecture.[20] Animal models have demonstrated that these destructive tissue alterations precede aneurysm formation.[21] MMP-9 has been found to be locally upregulated in the vascular walls of both TAA and ICA.[20,22]

Likewise, in both TAA and ICA, the ratio of matrix metalloproteinases to tissue inhibitors of metalloproteinases has been found to be elevated.[20,23] Koullias *et al.* compared the levels of MMP-9 expression to that of tissue inhibitor of metalloproteinase 1 (TIMP-1) in the arterial walls of TAA specimens and found that the MMP-9:TIMP-1 ratio was elevated relative to controls, suggesting an overall proteolytic microenvironment.[20] These molecular findings promoting degradation of the extracellular matrix are consistent with the similar histopathologic findings observed in both TAA and ICA. TAA are microscopically distinguished by cystic medial

degeneration, in which the elastic fibers in the medial layer of the arterial wall are lost.[24] In ICA, the media is similarly destroyed with fragmentation of the internal elastic lamina.[1]

Clinically, TAA and ICA have been shown to occur together at high rates within certain families.[15,18,25,26] One study showed that among patients with diagnosed saccular cerebral aneurysms, 10.5% had a family history of aortic aneurysms.[25] Moreover, clustering of aortic and cerebral aneurysm disease was noted only in families with certain ethnic characteristics, further supporting a primarily genetic cause. A common mechanism in the formation of both ICA and TAA suggests that both types of aneurysm could occur in an individual with a single genetic defect.

However, reports in the literature of TAA and ICA occurring concurrently in individual patients are rare. In their study of familial TAAD, Milewicz and colleagues found 15 subjects from 12 unrelated families that had both TAA and either an ICA or a history of intracranial hemorrhage.[18] A separate case report describes a patient with concurrent TAA and ICA.[27] One aim of this project is to further investigate patients with concurrent ICA and TAA, specifically the rates at which these two types of aneurysms occur concurrently.

The Prevalence of Intracranial Aneurysms and Thoracic Aortic Aneurysms

The rates at which TAA and ICA occur concurrently in patients without known connective tissue disorders had not been previously reported prior to the data described in this project. However, in order to evaluate the elevated risk that having TAA confers for harboring a concurrent ICA or vice versa in the absence of a known connective tissue syndrome, it is important to define the background prevalence of ICA and TAA in the general population. The prevalence of ICA in the general population of the United States has been well defined and is approximately 1%. This number is based on robust data from large angiography and autopsy studies.[1,28,29]

The true prevalence of TAA in the general population is currently poorly understood, and reports in the literature on this topic are limited. There are a number of reasons for this gap in our understanding of this disease, as detailed by Elefteriades and Rizzo.[30] Primarily, identification of TAA is hindered by the fact that such aneurysms are asymptomatic in over 95% of affected patients. For this reason, TAA is often called a "silent killer" because it most commonly presents with catastrophic rupture or dissection.[31,32] Thus, most TAA remain undetected unless they are incidentally discovered by imaging studies done for other purposes or result in a symptomatic complication. Study of TAA is also complicated by referral center bias. Tertiary care centers that specialize in treating aortic disease preferentially receive referrals for patients with TAA. The number of TAA patients presenting to these specialized centers is therefore not representative of the TAA prevalence in the general population.

Furthermore, traditional administrative databases such as insurance or hospital databases are typically not sufficiently detailed to distinguish various aortic pathologies (aneurysm rupture vs. dissection, for example), confounding conclusions based on these sources. Finally, many cases of fatal TAA rupture or dissection are likely misdiagnosed as myocardial infarction, thereby underestimating the true prevalence of TAA.[33]

It is currently estimated that the incidence of TAA is approximately six to ten cases per 100,000 patient-years.[34,35] However, these studies were conducted in the racially homogeneous population of Olmsted County, Minnesota, and all patients identified with TAA were Caucasian. The findings are therefore likely not generalizable to the general population. A more recent study from Itani *et al.*, reporting directly on the prevalence of TAA, examined 6,971 patients who underwent non-contrast computed tomography (CT) scans of the chest and found that 0.16% of patients had a TAA (either ascending, descending, or thoracoabdominal).[36] However, aortic aneurysms in this study were defined by an arbitrary size cut-off of aortic diameters > 5 cm, thereby overlooking potentially clinically significant aneurysms between 4 and 5 cm. The more rigorous definition of an aneurysm (a focal dilation of at least 50% greater than the normal aortic diameter) endorsed by numerous professional organizations accounts for differences in baseline aortic diameter, which is known to increase with increasing body surface area.[37-39] Kalsch *et al.* studied 4,129 patients with non-contrast CT scans in a population-based study and found that 0.34% of patients had asymptomatic TAA.[40] But TAA in this study was similarly defined by an arbitrary size cut-off. Interestingly, the incidence of TAA appears to be increasing.[35] Additional factors aside from more

frequent use of thoracic imaging may be at play.[41] Nevertheless, the true prevalence of TAA in the general population of non-syndromic individuals remains unknown.

Improved Screening is Needed for Intracranial Aneurysms

Developing a convenient and reliable screening test for ICA would be a significant clinical innovation. ICA is a predominantly asymptomatic disease and most commonly presents with rupture resulting in subarachnoid hemorrhage (SAH).[42] The incidence of SAH is approximately 1 in 10,000 per year, accounting for 27,000 ruptures annually in the United States.[42] In addition, aneurysmal SAH accounts for up to 9% of all strokes.[43] If ICA remain undetected and untreated, up to 50% will rupture during a patient's lifetime with devastating consequences.[42] SAH secondary to ICA rupture is a catastrophic event associated with high rates of morbidity and mortality: 10% of patients die before reaching a hospital, 40% of hospitalized patients die within one month, and more than 30% of survivors have persistent neurological deficits.[1,44]

Consequently, early detection of ICA and prophylactic treatment prior to rupture can be life saving. Certain patients, depending on the size and location of their ICA, may benefit from early prophylactic surgical or endovascular treatment of their aneurysm.[45] Minimally invasive endovascular coiling of ICA carries a complication risk as low as approximately 5%.[42] In other patients where immediate intervention is not warranted, early identification of ICA allows for conservative management strategies to reduce the

risk of rupture. Such strategies include strict blood pressure control, smoking cessation, avoiding heavy alcohol consumption, prohibiting stimulant medication use, and avoiding excessive straining or Valsalva maneuvers.[46]

Routine screening of certain populations for ICA is currently recommended. The American Stroke Association guidelines recommend that individuals with two or more first-degree family members with diagnosed ICA be screened on a regular basis due to their increased risk for ICA.[47] Other populations where routine screening is considered include those with prior history of SAH and patients with heritable disorders associated with ICA, such as autosomal dominant polycystic kidney disease.

Screening for ICA is currently conducted via computed tomography angiography (CTA) or magnetic resonance angiography (MRA). However these methods have a number of drawbacks. These techniques are expensive, time-consuming, often rely on contrast dyes that can cause severe kidney damage, and – in the case of CTA – expose the patient to harmful x-rays. Furthermore, even in high-risk populations, the cost-effectiveness of these methods has not been measured, and the high cost barrier of these studies may preclude broad accessibility to the general population. At this time, there is no alternative method for detecting asymptomatic ICA. An ideal modality would address all of the shortcomings of our current diagnostic tools and feature 1) low cost, 2) minimal invasiveness, 3) minimal health risks to the patient, and 4) an ability to predict aneurysm stability or impending rupture at the time of screening. The development of such a screening modality would be a significant clinical innovation, and patients with relatives

who have known aneurysms, patients with relatives who died from intracranial hemorrhage, and patients with other types of aneurysms may benefit from screening.

Using Peripheral Blood Biomarkers to Screen for Aneurysm Disease

For both aortic and intracranial aneurysms, detection of early, treatable, asymptomatic disease is difficult due to the innate characteristics of these conditions. Screening peripheral blood cells (PBCs) for markers of disease, including gene expression profiles, has an alluring role in diagnostics given the ease of testing and the diversity of molecular targets available. Such PBC-based biomarkers have been applied to other vascular diseases such as coronary artery disease, arterial hypertension, and atherosclerosis.[48-50] PBCs represent an ideal source of biomarkers for vascular disease because circulating blood cells are in constant contact with the entire vasculature.

Most aneurysm biomarkers identified up to now pertain to the diagnosis of AAA. D-dimers, for example, are breakdown products of fibrin clots, and have been found to be elevated in patients following thoracic and abdominal aortic dissection.[51] Circulating MMP-9 levels have been found to be elevated in patients with AAA versus non-aneurysmal controls.[52] Likewise, acute-phase reactants such as C-reactive peptide have also been found to be elevated in patients with AAA. Other previously-identified biomarkers include coagulation factors, tissue-specific components of smooth muscle, and immune mediators, such as IL-1, IL-6, TNF-α, and IF-γ.[53-55] However, the

exploration of genetic-based markers, such as PBC mRNA expression, for aneurysm disease may be more specific than serum markers and may have prognostic value as mRNA expression presents a "biological snapshot" of the currently active processes within the body.

The investigation of biomarkers specifically for unruptured ICA has, to date, been limited. The recent advances in elucidating genetic markers associated with ICA formation through a genome sequencing approach, while important, do not fulfill the role of a diagnostic screening test reflecting the current disease state within an individual. A number of studies have demonstrated altered gene expression profiles in ICA vessel walls.[56-58] However sampling the vessel wall itself for diagnostic purposes is unreasonable, and these profiles are unlikely to translate into easily measurable peripheral markers of disease. There have been some preliminary reports of serum markers of ICA, including elastase and lipoprotein-a, however none have been validated.[59,60] Measuring mRNA expression in PBCs as a marker of ICA has not previously been described. This approach holds promise because development of aneurysms is likely to involve an inflammatory process employing multiple components of the immune system.[55,61,62] Therefore, profiling peripheral white blood cells is an inherently rational approach for analysis of aneurysm disease.

Our group recently conducted a study evaluating an mRNA expression profile to detect TAA.[63] This initial study established the basic methodology that was used in one arm of this project. In the recently published report, a comprehensive gene expression analysis

of PBCs was conducted on blood samples from 58 TAA patients, with 36 spouses as controls. Using the Applied Biosystems Human Genome Survey Microarray, which represents 29,098 individual human genes, this study analyzed the relative expression levels of mRNA transcripts extracted from the peripheral blood sample of these patients. Genes that were most up-regulated or down-regulated in expression relative to control genes were identified. This study looked at the 41 genes that were most significantly differentially expressed between the TAA group and the control group, and found that these 41 genes were expressed in a consistent pattern in the TAA group.

This pattern was used in a classifier set of subjects to define a molecular aneurysm mRNA "signature" that was characteristic of aneurysm patients. This signature aneurysm pattern was then tested on an independent cohort of patients (22 TAA patients and 11 controls). If the observed pattern of expression of these 41 genes in a testing sample closely matched the expression pattern of the defined signature aneurysm profile, then a prediction was made that the testing sample came from an aneurysm patient. If the observed expression pattern was not a close match to the signature profile, then a prediction was made that the tested sample was a control.

This method of detecting aneurysms had a sensitivity of 72%, a specificity of 90%, and an overall accuracy of 78%. These results, based on gene expression levels measured via microarray, were reassessed through real-time PCR using the TaqMan system, which provides more precise quantification of gene expression levels than microarray. The PCR

assay data was 80% accurate (sensitivity 71%, specificity 100%) and had 89% concordance with the microarray data.

Given the documented link between TAA and ICA and the shared pathogenic mediators between the two diseases, we hypothesized that the signature RNA aneurysm profile that has been shown to be sensitive and specific in detecting TAA will have similar utility in detecting ICA.

Specific Aims

This project investigated the link between TAA and ICA via three approaches. The first arm aims to investigate the occurrence of concurrent ICA in patients with TAA. We hypothesize that patients with TAA will harbor concurrent ICA at a rate higher than the general population. Our specific aim for this approach is the following:

- To document the rate of concurrent ICA within a TAA patient population

The second arm aims to investigate this relationship of concurrent aneurysms in the opposite direction. We hypothesized that patients with ICA will harbor concurrent TAA at rates higher than that found in the general population. Our specific aim for this approach is the following:

- To document the rate of concurrent TAA within an ICA patient population

The third arm aims to evaluate whether the signature aneurysm RNA expression profile, defined in a previous study from our research group, that has been shown to be highly accurate, sensitive, and specific in detecting TAA is also able to detect ICA from a peripheral blood sample.[63] We hypothesize that this signature peripheral RNA expression profile, based on the expression levels of 41 specific genes, will detect the presence of ICA with a high degree of accuracy, sensitivity, and specificity. Our specific aims for this approach are the following:

- To analyze the RNA expression levels of 41 predefined genes using custom-made real-time PCR arrays in peripheral blood samples of ICA patients and controls
- To classify each sample as either an ICA case or a non-ICA control using previously defined formulas based on the pattern of gene expression of the 41 genes in our signature aneurysm profile
- To evaluate the accuracy, sensitivity, and specificity of ICA detection using the signature aneurysm profile by comparing the expression profile-predicted ICA classification to the known clinical ICA status of the subjects

Methods

Arm 1: Concurrent ICA in a TAA Patient Population

We retrospectively reviewed patient records from 1997 to 2009 in the thoracic aortic aneurysm database maintained by Dr. John Elefteriades to identify patients with TAA who also had available preoperative high-quality intracranial images by either CTA or MRA. Patients with a diagnosis of a connective tissue disorder, such as Ehlers-Danlos syndrome or Loeys-Dietz syndrome, were excluded. We identified 212 patients the met inclusion criteria out of approximately 1560 patients who underwent TAA repair during that time period.

The cerebral imaging scans in these 212 patients were obtained for one of two purposes. Imaging studies in 160 (75%) patients were obtained by our thoracic aortic team specifically for the purpose of ICA screening prior to surgical TAA repair. These patients were termed the "prospective group." The remaining 52 (25%) patients were termed the "non-prospective group" and underwent intracranial imaging for non-specific neurologic symptoms (e.g. headache, neck pain) or oncologic reasons. Forty-five patients in the non-prospective group (87%; 21% of the entire cohort) had the brain scans in the past for largely non-specific neurologic symptoms unrelated to the aneurysm (dizziness, weakness, headache in 37 patients) or for oncologic screening (4 patients). In addition, 1 patient in the non-prospective group had a history of stroke, 1 patient had a history of

transient ischemic attack, and 1 patient had a history of mental status changes. Four non-prospective patients also had a known ICA at the time of TAA repair. All brain images were reviewed by a staff neuroradiologist at Yale-New Haven Hospital. The 1,348 patients who did not receive any preoperative cerebral imaging (and thus excluded from this study) were not screened for a variety of reasons including emergent surgical circumstances, lack of compliance, inability to travel, and cost or lack of insurance coverage.

Data was collected from each patient record including age (at time of surgical TAA repair), gender, ethnicity, blood pressure status, smoking status, and the characteristics of aneurysms present (diameter, anatomic location, and rupture status). Patients were divided into the ascending or descending TAA group based on the most clinically significant portion of aneurysmal aorta. Arch aneurysms were classified with the portion of the aorta to which they were most closely related anatomically. The prevalence of ICA in the general population was identified through literature review, and was based on thousands of autopsies and angiographies which served as our comparison population.[1,28,29] Statistical comparison of ICA-positive and ICA-negative patients groups in various categories was done with Fisher's exact test using SPSS software (version 19; IBM Corporation). All statistical tests were two-tailed and significance was defined at the 0.05 level. This study protocol was approved by the Yale University Human Investigation Committee (#0509000633).

Arm 2: Concurrent TAA in an ICA Patient Population

We retrospectively reviewed the 1,224 medical records of all patients presenting to the neurosurgery service at Yale-New Haven Hospital over 6 years (from July 2005 to July 2011) for evaluation or treatment of ruptured or unruptured ICA. The presence of an ICA was confirmed by a staff neuroradiologist at Yale-New Haven Hospital. We evaluated the radiographic records of these patients to identify those with high-quality thoracic imaging including trans-thoracic echocardiography (TTE), trans-esophageal echocardiography (TEE), CT of the chest, or MRA of the chest that allowed for assessment of aortic diameter. These imaging modalities were considered reliable assessments of only specific portions of the thoracic aorta: TTE for the ascending aorta, TEE for the descending aorta, and both CT and MRA for the entire thoracic aorta. A total of 359 patients presenting with ICA were found to have high-quality thoracic imaging and were included in this study. Patients with connective tissue disorders known to predispose to both TAA and ICA, such as Loeys-Dietz syndrome or Ehlers-Danlos syndrome, were excluded from this study, as were those with mycotic intracranial aneurysms. Thoracic imaging studies in these patients were performed as part of a pre-operative work-up for ICA treatment (either microsurgical clipping or endovascular coiling) in 64% of patients, or for unrelated reasons such as trauma or cancer screening in the remaining 36%.

Data from eligible patient records were collected including age (at time of presentation with ICA), gender, ethnicity, blood pressure status, smoking status, and the characteristics of aneurysms present (diameter, anatomic location, and rupture status).

We recorded the number of these patients with concurrent TAA. TAA was defined by official radiology reports documenting a focal aortic dilation relative to the adjacent vessel diameter rather than by arbitrary size cut-offs.

Bivariate statistical comparison of various characteristics between the TAA-positive and TAA-negative patient groups was done using Fisher's exact tests for categorical variables and Wilcoxon rank-sum test for continuous variables. Using a p-value of 0.1 in bivariate logistic regression as cut-off for inclusion in a multivariate model, age in years and ICA size were selected to be included in the multivariate logistic regression model. These analyses were conducted using SAS (version 9.2; SAS Institute, Inc.) by collaborators at the Yale Center for Analytical Sciences at the Yale School of Public Health (Maria M. Ciarleglio, PhD and Xiangyu Cong, PhD MPH). All statistical tests were two-tailed and significance was defined at the 0.05 level. This study protocol was approved by the Yale University Human Investigation Committee (#0509000633).

Arm 3: Detecting ICA via a Peripheral Blood mRNA Expression Profile

We prospectively enrolled patients who presented for evaluation and/or treatment of diagnosed ICA to the neurovascular clinic of the Department of Neurosurgery at Yale-New Haven Hospital. The presence of ICA in patients was confirmed using standard imaging modalities (CTA, MRA, or formal angiography). All imaging studies were read and verified by a staff neuroradiologist or neurosurgeon at Yale-New Haven Hospital.

Any patient who presented with at least one radiographically verified ICA, regardless of rupture or treatment status, was eligible to participate in this study. Exclusion criteria included a diagnosis of active leukemia or other blood dyscrasias that may distort the constituents of the peripheral blood sample collected, treatment with chemotherapy within the past year that may likewise distort the peripheral blood sample, or the known presence or history of aortic aneurysms.

Questionnaires and review of medical records were utilized to collect subjects' demographic information and aneurysm risk factors, including age, gender, sex, race, smoking status, blood pressure status, medications, and family history of aneurysms or cardiovascular disease. Aneurysms size, location, and detection modality were also recorded.

Spouses of TAA patients served as non-aneurysmal controls (these subjects were enrolled and their blood was collected and processed by my classmate Adam X. Sang). A standard questionnaire addressing symptoms and history of TAA or ICA was used to exclude the presence of these aneurysms in controls (necessary to avoid confounding the results of our analysis, given the above-described ability of the RNA signature to detect TAA). Given the low prevalence of both types of aneurysms in the general population, and the risks and costs inherent in subjecting spousal controls with CT or MRA, a non-invasive questionnaire was deemed suitable for these purposes. As in the ICA patients enrolled, questionnaires were utilized to collect the same demographic, medical history, and aneurysm risk factor information from controls.

A power calculation was performed to estimate the necessary sample size for this study. We hypothesized that the signature peripheral expression profile will have a 70% sensitivity and 95% specificity in detecting ICA based on the results of our prior study in TAA patients. Using an α of 0.05 and a β of 0.10, corresponding to a 90% power level, and conservatively assuming a 4:1 case:control ratio consistent with our prior study experience, a necessary total sample size of 24 patients was calculated using a standard formula[64]:

$$N = \frac{\{z_\alpha\sqrt{[P(1-P)(1/q_1 + 1/q_2)]} + z_\beta\sqrt{[P_1(1-P_1)(1/q_1) + P_2(1-P_2)(1/q_2)]}\}^2}{(P_1 - P_2)^2}$$

where N is the total number of subjects required in the study, z_α is 1.96 (for an α of 0.05), z_β is 1.282 (for a β of 0.10), P_1 is the expected rate of a test result indicating aneurysm in the case group (0.7 given an estimated 70% sensitivity), P_2 is the expected rate of a test result indicating aneurysm in the control group (0.05 given an estimated 95% sensitivity), q_1 is the anticipated proportion of subjects in the case group (0.75), q_2 is the anticipated proportion of subjects in the control group (0.25), and $P = q_1P_1 + q_2P_2$.

Peripheral blood samples (3 ml) from each case and control were obtained using collection tubes specifically formulated for preserving RNA (Tempus Blood RNA tubes, Life Technologies). Blood draws for the ICA patients were carried out by staff phlebotomists at the Yale Physicians Building; blood draws for the non-aneurysmal

controls were carried out by Adam X. Sang. Following collection, blood samples were kept at room temperature for 2 hours to allow for thorough blood cell lysis. Samples were then stored at -80°C until RNA extraction.

The Tempus blood tubes were subsequently thawed on ice and the contents transferred to a centrifuge-compatible tube with 3 ml of 1x phosphate-buffered saline. This mixture was then centrifuged at 4°C at 3000 g for 30 minutes. The resulting supernatant was discarded and the pellet containing the total RNA was resuspended in 400 μL of RNA Purification Resuspension Solution (Applied Biosystems). Total RNA was extracted from the PBCs following the protocol of the Tempus Spin RNA Isolation Kit (Life Technologies).

Integrity of the extracted RNA was assessed by measuring the RNA integrity number (RIN) using an institutional 2100 Bioanalyzer (Agilent); this was carried out by the staff at the Yale West Campus core facilities and analyzed by me. A RIN of 7.0 was used as the minimum threshold for sample inclusion (on a total scale of 1 to 10, where 1 represents high RNA degradation and 10 represents fully intact RNA).[65] RNA concentration and purity was measured using a Nanodrop spectrophotometer (Thermo Scientific); this was carried out by the staff at the Yale West Campus core facilities and analyzed by me. The A260/A280 ratio was used to assess sample purity. The wavelength of maximum absorbance for RNA is 260 nm, while absorbance at 280 nm is used to assess for protein concentration. An A260/A280 ratio of 1.8 was used as the minimum threshold for sample purity in this study. Low quality samples were noted and excluded prior to the real-time PCR stage (one sample in this study was excluded for this reason).

400 nanograms of the extracted total RNA were taken to synthesize complimentary DNA (cDNA) following the protocol of the QuantiTect Reverse Transcription Kit (Qiagen). The collected cDNA was used to perform real-time quantitative PCR (qPCR) in order to measure gene expression of our genes of interest in the signature aneurysm profile. We used the TaqMan platform (Life Technologies) for all qPCR steps of this project. TaqMan probes contain both a fluorophore and a quencher molecule and are individually designed to be complementary to a region of the target cDNA flanked by the amplification primers. The fluorophore will only fluoresce once the probe is bound to its specific target and subsequently degraded by the 5' to 3' exonuclease activity of the TaqMan DNA polymerase (thereby separating the fluorophore from the quencher). The fluorescence resonance energy transfer (FRET) feature of TaqMan reporter probes thereby reduces false-positive fluorescence and provides enhanced specificity over traditional intercalating fluorophore-based qPCR.

The qPCR reactions in this study were conducted using custom premade TaqMan arrays supplied by Life Technologies. Each array has 384 wells in which an individual qPCR reaction is carried out. Each well on these custom cards has a reaction primer for one of the genes in the signature aneurysm profile or a control gene, and each array can analyze up to eight blood samples. At the time of this study, it was noted that the custom-made TaqMan array cards held primers for only 39 of the 41 genes (including control genes) in the original signature aneurysm profile. Therefore, further analysis was carried out using the expression levels of these 39 genes only (Table 1). Four additional genes, *CDH13*,

IL10, *JUN*, *MMP15*, were also included on the TaqMan array cards because they have been hypothesized to be related to TAA, but the expression levels of these genes were not analyzed as part of this study.

The qPCR reactions were carried out using a 7900HT TaqMan thermal cycler/fluorescence detector (Life Technologies). SDS v2.3 software (Applied Biosystems) was used with the manufacturer-recommended default settings to operate the thermocycler. The cDNA synthesized from 200 ng of total RNA was added to each lane of the TaqMan array cards per the manufacturer recommendations. Each sample was assayed in duplicate to minimize technical variation, thereby consuming of total of 400 ng of total RNA per sample. 50 μL of the TaqMan real-time PCR gene expression master mix (Life Technologies) containing the TaqMan DNA polymerase, nucleotides, and reference dye for the qPCR system was also added to each lane of the TaqMan cards. A variable volume of sterile water was also added to each lane of the TaqMan cards to bring the up volume in each lane to an even 100 μL per the manufacturer recommendations. The TaqMan array cards were then centrifuged for two 1-minute intervals at 331 g to distribute the loaded cDNA sample and mastermix into each well of the card. The expression levels of the endogenous control genes *ACTB*, *B2M*, *GAPDH*, and *PPIA* (cyclophilin A) were measured for each sample, as in our prior study, to normalize the measured expression levels across all samples.[63] This gene were chosen because they have relatively constant expression levels in all blood cells.

The expression pattern of the 39 genes was then used to classify each sample as either an ICA case or non-ICA control using the methods previously validated in our group's prior study.[63] Relative gene expression and statistical analysis using the $\Delta\Delta C_T$ method was conducted by a collaborating statistician at Life Technologies (Catalin Barbacioru).[66] In this process, the raw qPCR output for each of the 41 genes in our signature profile is a relative expression level of that gene versus the expression level of an endogenous control gene, expressed as ΔC_T. The ΔC_T is thus a normalized expression level that is averaged across replicates to remove sample input errors and natural biological variation. The ΔC_T value of each gene for each sample is then compared to the average ΔC_T value of that same gene in the signature profile. The resulting $\Delta\Delta C_T$ of a gene will be the relative quantity (relative-fold expression level) of that gene compared to the "expected" expression level of that gene. The $\Delta\Delta C_T$ values for each gene in each sample are then entered into a multiple regression model (the classifier formula) that predicts whether the sample is an ICA case or a non-ICA control based on how closely the observed pattern of expression matches the pre-defined signature aneurysm pattern of expression.

We then compared the algorithm-predicted "case" and "control" classification to the actual clinical aneurysm status of each subject 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) of this method of ICA detection were calculated to determine overall efficacy of the profile. This study protocol was approved by the Yale University Human Investigation Committee (#0109012617).

Results

Arm 1: Concurrent ICA in a TAA Patient Population

In our cohort of 212 patients with intracranial imaging, there were 141 males (67%) and 71 females (33%). Ages ranged from 18 to 92 years old (mean 62 years). There were 197 patients with ascending TAA (93%) and 15 patients with descending TAA (7%). Nineteen patients within the cohort (9.0%) harbored a concurrent ICA (Table 2). The ICA ranged from 1.0 to 11.0 mm in size (mean 3.7 mm, standard deviation 2.6 mm). Among only the prospective patient group, 10 patients (6.3%) had a concurrent ICA. Of the nine patients with concurrent ICA from the non-prospective group, five patients had cerebral imaging due to non-specific symptoms of headache or neck pain and the ICA was an incidental finding. Among the other four patients, two had undergone ICA clipping in the past, one had a prior ICA rupture, and one was screened by clinicians outside our study due to a family history of ICA.

Patients with descending TAA had a higher prevalence of ICA (5/15, 33%) than patients with ascending TAA (18/197, 7.1%) ($p = 0.006$) (Figure 1A). Considering only the prospective patient group, there was a strong trend in the same direction, but statistical significance was not achieved in this smaller sample ($p = 0.08$). Patients with hypertension also had a higher prevalence of ICA (18/153, 11.8%), compared to patients

without hypertension (1/59, 1.7%) ($p = 0.03$). Considering just the prospective group, there was a trend in the same direction, but statistical significance was not achieved.

Thirteen of 105 (12.4%) patients with a current or past history of smoking harbored an ICA, while 6/107 (5.6%) non-smokers had an ICA ($p = 0.096$) (Figure 1B). Among just the prospective group, 8/76 (10.5%) of TAA patients with a current or past history of smoking had a concurrent ICA, while 2/84 (2.4%) of patients without a smoking history had an ICA ($p = 0.05$). Patients were also divided into two groups based on age: younger and older than 60 years of age. There were 4/88 (4.5%) patients younger than 60 with an ICA, while 15/124 (12.1%) patients older than 60 had an ICA ($p = 0.09$). Among the prospective group, there was no significant difference in age distribution between ICA-positive and ICA-negative individuals.

Eight of 71 (11.3%) females had an ICA, while 11/141 (7.8%) males had an ICA ($p = >0.2$). Among the prospective group, there was also no significant difference in gender distribution between ICA-positive and ICA-negative individuals. Sixteen of 185 (8.6%) of Caucasians had an ICA, while 3/16 (18.7%) of African Americans had an ICA ($p = 0.18$). Eleven individuals of other races were included in this study, none of which had an ICA. Among the prospective group, there was also no significant difference in race distribution between ICA-positive and ICA-negative individuals.

The data in Arm 1 of this project has been previously published.[67]

Arm 2: Concurrent TAA in an ICA Patient Population

In our cohort of 359 patients with ICA and recent thoracic imaging, thoracic imaging modality was TTE in 205 patients (57%), TEE in 7 (2%), CT in 146 (41%), and MRA in one patient. Additional patient characteristics are listed in Table 3. Of the 359 patients, a total of 16 (4.5%) were found to have concurrent TAA. Thoracic imaging modality was CT in nine of these patients revealing six ascending TAA, two aortic arch TAA, and three descending TAA (two patients had multiple TAA). The remaining seven of these patients had TTE and were found to have ascending TAA. Characteristics of patients with concurrent TAA are also given in Table 3.

Of the 18 total TAA identified, 13 (81%) TAA were located in the ascending aorta (mean diameter 4.4 cm, range 3.7 – 6.3 cm), two (13%) in the aortic arch (mean diameter 5.4 cm, range 4.8 – 6.0 cm), and three (19%) in the descending aorta (mean diameter 3.7 cm, range 3.2 – 4.3 cm). The prevalence of ascending TAA among patients with thoracic imaging modalities suited to detect lesions is this location (TTE, CT, MRA) was therefore 3.7% (13/352). Among patients with imaging suitable to select descending TAA (TEE, CT, MRA), the rate of concurrent descending TAA was 1.9% (3/154). Similarly, the prevalence of aortic arch TAA among patients with adequate arch imaging (CTA, MRA) was 1.4% (2/147).

One patient with an ascending TAA also had a known abdominal aortic aneurysm, and a second patient with an ascending TAA was found to have a bicuspid aortic valve. One patient with an aortic arch TAA was also diagnosed with a diverticulum of Kommerell at the origin of the left subclavian artery. One patient with a descending TAA also had a history of descending aortic dissection and a left main coronary artery aneurysm. In all other patients with concurrent TAA and ICA, there were no known additional aneurysms or vascular abnormalities. None of the patients carried a diagnosis of a connective tissue disease.

Patients were categorized according to their age (> 70 years old or ≤ 70 years old), and patients over 70 years of age had a significantly higher rate of concurrent TAA (9.5%) compared to patients ≤ 70 years old (3.2%, $p = 0.03$) based on univariate analysis (Figure 2A). Older age analyzed as a continuous variable was likewise significantly correlated with increased rate of concurrent TAA ($p = 0.016$). Patients were further categorized according to the size of their ICA (> 4.0 mm and ≤ 4.0 mm), and patients with ICA > 4.0 mm had a higher prevalence of concurrent TAA (6%) compared to those with ICA ≤ 4.0 mm (1.7%) (Figure 2A). This difference represented a trend towards higher prevalence of concurrent TAA with large ICA diameter, but statistical significance was not reached ($p = 0.10$). Gender ($p > 0.2$), ethnicity, ($p > 0.2$), hypertension ($p = 0.20$), smoking status ($p > 0.2$), and the presence of multiple ICA ($p > 0.2$) did not significantly affect the risk of concurrent TAA (Figure 2B). The proportion of patients presenting with ruptured ICA was not significantly different between the TAA (56.3%) and non-TAA (64.1%) groups

($p > 0.2$). Likewise, ICA location within the cerebrovasculature was not significantly associated with the prevalence of concurrent TAA (Table 3).

Univariate logistic regression identified age (in years), age (dichotomized as ≤ 70 and >70 years), and ICA size (dichotomized as ≤ 4.0 mm and >4.0 mm) as significantly associated with the odds of TAA at the 0.10 level. Including age (as a continuous variable) and ICA size together in a multivariate model (Table 4), the effect of ICA size is not significant at the 0.05 level after controlling for age in the model ($p = 0.13$), although age remains statistically significant ($p = 0.03$) with an odds ratio of 1.05.

The data in Arm 2 of this project as been previously presented at a national meeting.[68]

Arm 3: Detecting ICA via a Peripheral Blood mRNA Expression Profile

To date, 17 blood samples from ICA patients and 15 samples from non-aneurysmal controls have been analyzed. Mean age of ICA patients was 60 years old (range 37-83 years), and 15 patients (89%) were female (consistent with the known female predominance among ICA patients). Mean age of the control group was 59 years, and 80% were female. The ICA group had 20 ICA among the 17 patients. Aneurysm locations are tabulated in Table 5. Two patients had bilateral internal carotid artery aneurysms, and one patient had ICA of both the middle cerebral artery and the posterior communicating artery. Mean aneurysm size was 5.8 mm (range 1 to 20 mm). Four

patients (24%) in the cohort had previously treated ICA, three with endovascular coiling and one with endovascular flow-diverting stent placement. Two patients (12%) had previously ruptured ICA, both of which had their aneurysms treated via endovascular coiling. Nine patients (53%) had a history of hypertension, six patients (35%) had a history of smoking, and six patients (35%) had a positive family history of ICA. No patients had a known connective tissue disorder. Eight patients (47%) were being treated with an anti-inflammatory medication at the time of blood sample collection. These medications included aspirin, prednisone, hydroxychloroquine, and statin medications.

Total RNA was extracted and analyzed from 18 ICA patient samples. All included samples had a RIN above 7.0 (minimum 7.7) and an A260/280 ratio above 1.8 (minimum 1.99). Mean RNA concentration was 137.8 ng/ul (range 74.8 to 395.2 ng/ul), mean RIN was 8.7 (range 7.7 to 9.7), and mean A260/A280 ratio was 2.07 (range 1.99 to 2.11). These values are individually tabulated for each sample in Table 6. One sample was excluded from these calculations and prior to cDNA synthesis for insufficient RNA concentration (a total nucleic acid concentration of only 5.5 ng/µl as measured by spectrophotometry; a RIN was not calculated and the A260/280 ratio was disregarded).

Once gene expression levels for each sample were collected and analyzed by our collaborating statisticians, the classifier algorithm successfully predicted that 15 of the 17 ICA samples were aneurysm patients (88% sensitivity). The classifier algorithm predicted that 10 of the 15 control patients were also aneurysm patients (67% false positive rate) (Figure 3). This resulted in a specificity of 33% and an overall accuracy of

63%. Of the four patients with previously treated ICA, three were predicted to be in the ICA group. The one treated ICA patient that was predicted by the classifier algorithm to be a control had a previously ruptured ICA that was treated by endovascular coiling. The other false negative patient had a small 3mm ICA of the posterior inferior cerebellar artery.

Discussion

Concurrent Intracranial and Thoracic Aortic Aneurysms

Arm 1 of this study illustrates that patients with TAA have a higher prevalence of ICA than the general population. In our overall cohort, 9.0% of TAA patients had concurrent ICA. Considering only the prospective group, the prevalence of ICA was 6.3%. This rate was compared to the background prevalence of ICA in the general population, drawn from >10,000 autopsies and angiograms reported in multiple studies.[1,28,29] These studies found the prevalence of ICA to be approximately 1% in the general population. The prevalence of ICA in our cohort of patients with TAA thus far exceeds the expected ICA prevalence in the general population.

Patients in the prospective group, who underwent cerebral imaging solely for the purpose of this study, were analyzed separately to reduce possible selection bias in our results.

Nonetheless, to completely exclude those patients in the non-prospective group would inevitably undercount the true prevalence of ICA in our TAA patient population. We believe the introduction of bias from including the non-prospective patients is minimal, as most symptoms prompting the prior scans were non-specific and not likely directly attributable to an ICA. Nonetheless, we performed analysis excluding the patients from the non-prospective group to obviate this concern. Even the most conservative estimate (from analysis of the prospective group only) finds that 6.3% of TAA patients harbor a concurrent ICA– indicating that the risk of TAA patients for developing ICA is manyfold higher than that of the general population.

This association between ICA and TAA is consistent with the common proteolytic mechanisms that underlie these two conditions.[20,22] MMPs have been implicated in the proteolytic activity that precedes aneurysm formation and rupture in multiple vascular trees.[21,69] MMP-9, in particular, exhibits elevated local expression in both ICA and TAA.[20,22] Our results are also consistent with observations by Milewicz and colleagues that there may be a common genetic basis for both ICA and TAA.[25]

In this study, we found a higher prevalence of ICA in patients with descending TAA than with ascending TAA. Such differences are consistent with our group's prior genetic observations that ascending and descending aneurysms are different diseases.[8] It is thought that the embryology, pathophysiology, and clinical manifestations of aortic aneurysms divide TAA into 2 diseases based on location in the aorta: proximal to the ligamentum arteriosum (ascending) and distal to the ligamentum (descending and

thoracoabdominal).[70] One salient clinical difference, supporting the notion the ascending TAA fundamentally differ from descending TAA, is the finding that ascending aneurysms tend to be arteriosclerosis-free, while those located in the descending, thoracoabdominal, and abdominal aorta tend to be arteriosclerotic.[71] Thus, it appears reasonable that there could be differences in the relative prevalence of ICA between ascending and descending TAA patients, as found in our study. Interestingly, a similar finding was recently reported in a study correlating aortic coarctation to ICA.[72] The authors found that patients with ascending aortopathy had lower rates of concurrent ICA than those without ascending aortic disease.

We also found that systemic hypertension significantly increased the prevalence of ICA in TAA patients. There are multiple studies that document the relation between hypertension and aneurysms.[2,73,74] Therefore it is not surprising that this risk factor contributes to ICA formation in a patient population that has already proven to be aneurysm-prone. It is important to note, however, that hypertension is an important risk factor for SAH, a condition with a mortality rate of up to 50%.[73,75,76] Hypertensive TAA patients may therefore be at particularly increased risk for the consequences of SAH.

Additionally, we found that TAA patients with a current or past history of smoking have an elevated prevalence of concurrent ICA. Among patients in the prospective group, smokers had a significantly higher prevalence of concurrent ICA. Among all patients, there was a strong trend in the same direction, although statistical significance was not

achieved. While this anomaly is likely a result of sample size limitation, the data strongly suggest that TAA smokers are at high risk for ICA. Moreover, smoking is a known risk factor for ICA and SAH.[1] Our results additionally suggest that individuals greater than 60 years of age may have an elevated prevalence of concurrent ICA, but the data do not reach statistical significance.

One limitation of our analysis of concurrent ICA in the TAA patient population is our relatively small sample size, particularly limiting the number of patients available for sub-group analysis. Further study in larger populations will be necessary to determine if the trend toward higher prevalence of concurrent ICA in the elderly and in smokers is validated. Our findings should also be interpreted with caution due to the small event size of ICA. Another apparent shortcoming of the study is our comparison to the prevalence of ICA in the general population derived from the literature. Our study population may not be identical to the population in which the baseline ICA prevalence was determined due to the age, atherosclerosis, and hypertension associated with TAA. However, the studies in the literature from which the general population ICA prevalence was determined are comprehensive and based on thousands of patients. It would not be possible to recreate such investigations into the rate of ICA in the general population as part of this project. In fact, such data from the literature is likely much more powerful than any small comparison group that might be identified in our own institution.

Another concern, discussed above, is the possibility of selection bias – that is, that among our overall group of TAA patients, those with prior neurologic symptoms were more

likely to have a brain image available, making it more likely for them to be included in this study, and thus skewing the results towards a higher prevalence of concurrent ICA. We feel that our separate analysis of the prospective patients mitigates this concern, as our finding of elevated ICA prevalence among TAA patients was upheld in this more selected group. In addition, only four patients had intracranial imaging for a known ICA, while the neurological symptoms that prompted intracranial imaging in the non-prospective group were non-specific and likely unattributable to an ICA.

Another weakness of this study was the lack of confirmation by angiography of the ICA detected by MRA or CTA. This is salient in light of the fact that these modalities may have substantial false-positive rates. In the recently published experience of Pradilla *et al*., CTA had a false-positive rate of 20.5%.[77] Likewise, the same group reported that MRA may have a false-positive rate of up to 38%.[78] Accordingly, our findings should be interpreted with caution and will require further investigation for confirmation.

In Arm 2 of this project, we document the rate of concurrent TAA in an ICA patient population, finding that 4.5% of our cohort harbored concurrent TAA. We used data drawn from population-based studies as a comparison group.[36,40] While the prevalence of TAA in the general population remains poorly defined, the available evidence suggests that it is less than 1%. Therefore, our findings suggest that ICA patients are at substantially increased risk for harboring a concurrent TAA. Similar conclusions have been recently reported. In a study of patients with aortic coarctation, Curtis *et al.* report that a group of ICA patients had a higher rate of concurrent TAA than

a non-ICA group (17% vs. 10% rate of concurrent TAA), although this difference was not statistically significant.[72]

Identification of groups at high risk for TAA is an important piece of clinical knowledge that can aid in the early detection and treatment of such aneurysms. Because an unrecognized TAA that remains untreated can result in the lethal events of aortic rupture or dissection, it is important to identify TAA early before they grow to reach a dangerous diameter. Identifying populations that are at elevated risk for developing TAA therefore has particular clinical significance and has the potential to save lives through early detection of these typically asymptomatic aneurysms.

In this study, the majority (81%) of concurrent TAA in the ICA patient population were found in the ascending aorta. This contrasts with the data from Arm 1 of this project in which concurrent ICA were more strongly linked with descending TAA. This may be a reflection of the disproportionate use of TTE imaging in 57% of our study population that is unable to adequately visualize descending TAA. When analyzing just the subgroup of patients with thoracic imaging modalities adequate to visualize the descending aorta (TEE, MRA, CT), the prevalence of concurrent TAA was 1.9% (3/154). While smaller than the TAA prevalence in our overall cohort, this number is still likely greater than that of the general population. We also found that increasing age was significantly related to increased risk of concurrent TAA. This is logical given the advanced age at which TAA typically develop compared to ICA. There was a strong trend towards ICA diameter >4.0 mm being associated with increased risk of concurrent TAA, however statistical

significance was not achieved. No other factors measured were found to be significantly associated with increased prevalence of concurrent TAA.

One weakness of this study is the lack of a selected comparison group to define the prevalence of TAA in the non-ICA patient population. However, it was not feasible to select a suitable control group of sufficient size from a single hospital radiology database to determine a background TAA prevalence. Selecting a set of thoracic imaging scans from a tertiary-care hospital database would have been subject to considerable selection bias that would likely overestimate the prevalence of TAA. Furthermore, a very large comparison group would be required given the rarity of TAA in the general population. We believe an overall 4.5% rate of concurrent TAA in the ICA patient population is clinically relevant given the overall rarity of TAA and the substantial consequences of leaving an unrecognized TAA untreated.

Detecting ICA via a Peripheral Blood mRNA Expression Profile

Taking only a peripheral blood sample from a standard blood draw, and using automated methods with standard laboratory techniques for measuring gene expression, we achieved an 88% sensitivity in detecting ICA. This approach holds promise as an initial screening test for ICA due to its high sensitivity, convenience, relatively low cost compared to CTA or MRA, lack of health risks associated with those radiographic modalities, and the automated high-throughput nature of the TaqMan array cards that will make this

approach easily translatable to a clinical setting. Nonetheless, these early results should be interpreted with caution and will require further investigation to be validated. The current primary limitation is that our sample size remains very small. Additionally, the specificity of our approach is currently unacceptably low. We anticipate that this may improve with a larger sample size given the high specificity of this approach in the initial study in which the signature aneurysm profile was defined.[63]

Another potential benefit of this approach is that an mRNA profile has the potential to shed light on specific biological processes at play in the pathogenesis of ICA. Prior studies have demonstrated the viability of this approach in elucidating novel pathogenic mechanisms of disease.[49] Other studies have used this approach to validate targeted disease therapies. One study of patients with atherosclerosis utilized gene expression analysis in PBCs to identify the genes that were differentially expressed between patients with the disease and controls.[50] The authors identified the *FOS* gene as being significantly upregulated in atherosclerosis patients. They were subsequently able to rationally select a small molecular inhibitor of the FOS protein and found that this targeted inhibitor reduced monocyte activation, thereby modulating an important pathogenic mechanism of the disease.

One unanswered question regarding the gene expression profile detected in PBCs is whether it reflects the body's detection of, response to, or primary cause of a dilated, weakened, and aneurysmal vessel wall. The identity of a number of the genes identified whose expression level was associated with the presence of an aneurysm suggests that the signature aneurysm profile may reflect the biological processes that lead to aneurysm

formation. Genes in the 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.[55,62,79,80] Accordingly, the signature aneurysm profile holds promise not just for screening for aneurysms, but for identifying a predisposition to later developing ICA. This finding highlights the utility of this methodology for elucidating candidate genes and biological pathways that may underlie the pathogenesis of aneurysm disease and may be future therapeutic targets.

One limitation of this study was that ICA patients were not radiographically screened to conclusively rule out the presence of a TAA. The presence of a TAA may have resulted in a false positive prediction by the classifier algorithm based on its demonstrated ability to detect TAA. However, given the low prevalence of TAA in the general population (as well as the ICA population) as discussed above, the impact of this potentiality would be low and was outweighed by the costs, risks, and logistical limitations of screening all ICA patients for TAA.

Another weakness of this study is the use of spouses of TAA patients as controls. Limitations of using this control population include possible gender- and age-based differences in the expression levels of one or more of our genes of interest and differences in comorbidities that are not gender- or age-neutral (for example, atherosclerosis). However, even though patients and controls in this study were not formally matched, both the gender ratio (89% female in the ICA group vs. 80% of controls) and mean age (60 years in the ICA group vs. 59 years among controls) were

comparable, thereby minimizing any differences based on gender and age. Control subjects enrolled in this study were likewise not formally screened for the presence of TAA and ICA aside from a medical history questionnaire. While undetected aneurysms have the potential to confound our results, the low prevalences of ICA and TAA in the general population minimize this potential. Given the risks and costs inherent in subjecting healthy spousal controls to CTA or MRA to screen for aneurysms, the non-invasive questionnaire was deemed appropriate.

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Figures

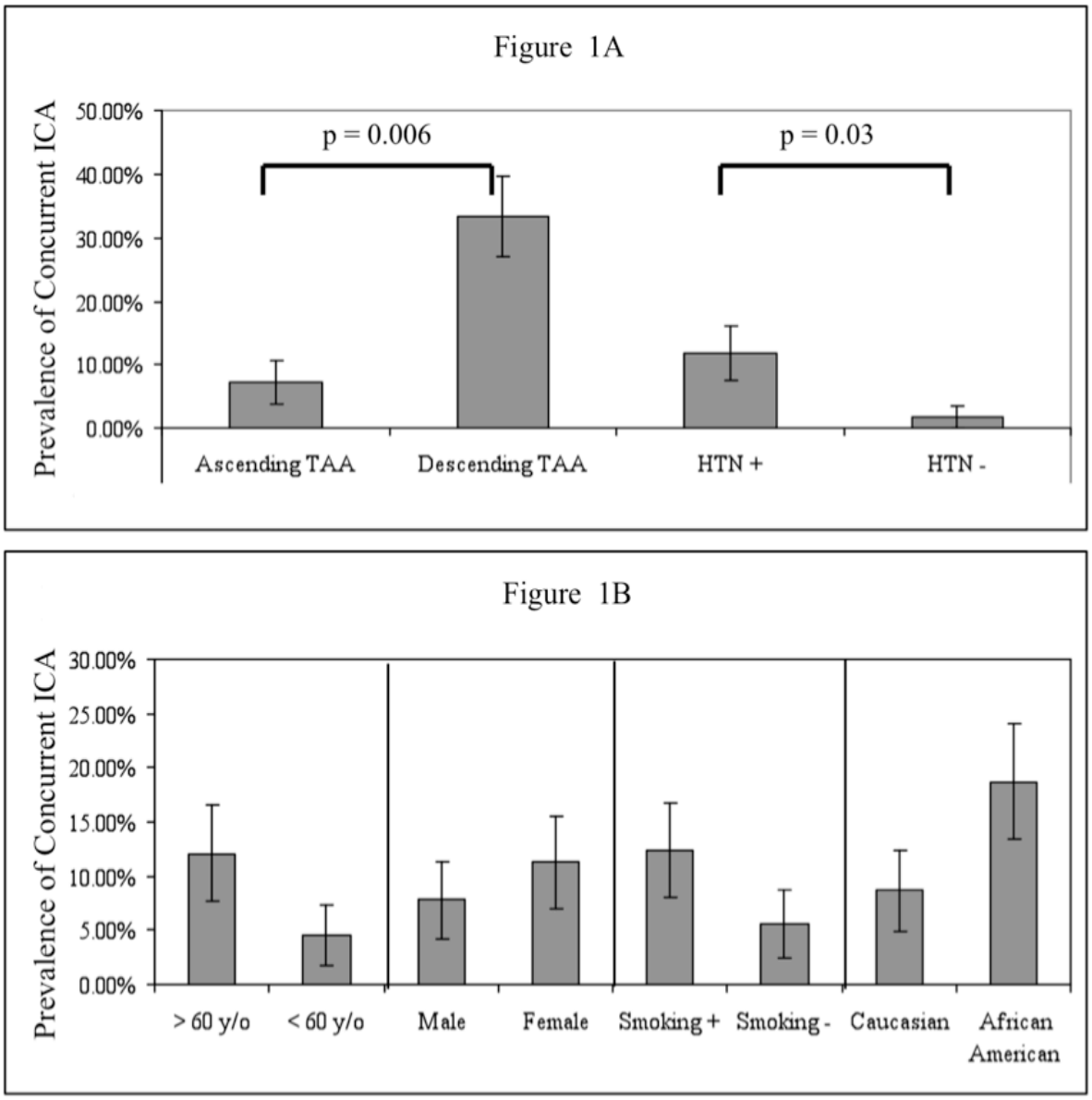

Figure 1. Rates of concurrent ICA among different TAA patient subgroups. Error bars represent 95% confidence intervals. (A) Patients with descending TAA had significantly greater incidence of ICA than patients with ascending TAA, and hypertensive patients had significantly greater incidence of ICA than normotensive patients. (B) Age, gender, smoking status, and ethnicity did not have a significant effect on rates of concurrent ICA among all patients. HTN = hypertension. Adapted with permission from reference 67.

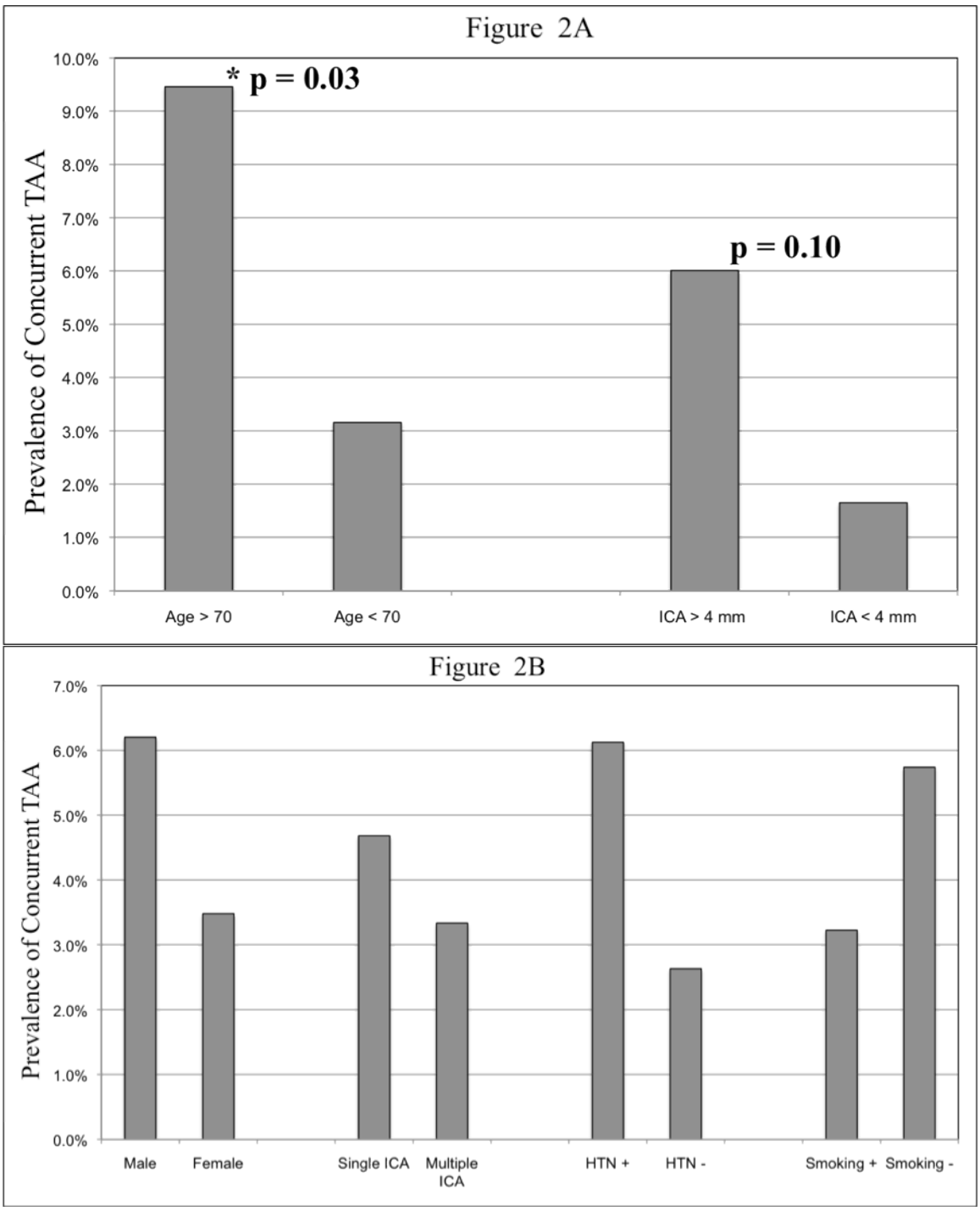

Figure 2. Rates of concurrent TAA among different ICA patient subgroups. (A) Rates of concurrent TAA based on age and size of maximal ICA diameter. Patients over 70 years old had a significantly higher rate of TAA. There was a strong trend towards a higher rate of TAA in patients with ICA larger than 4.0 mm. (B) Rates of concurrent TAA based on gender, number of ICA, blood pressure status, and smoking status. These characteristics did not significantly differ between the TAA and non-TAA groups. HTN = hypertension.

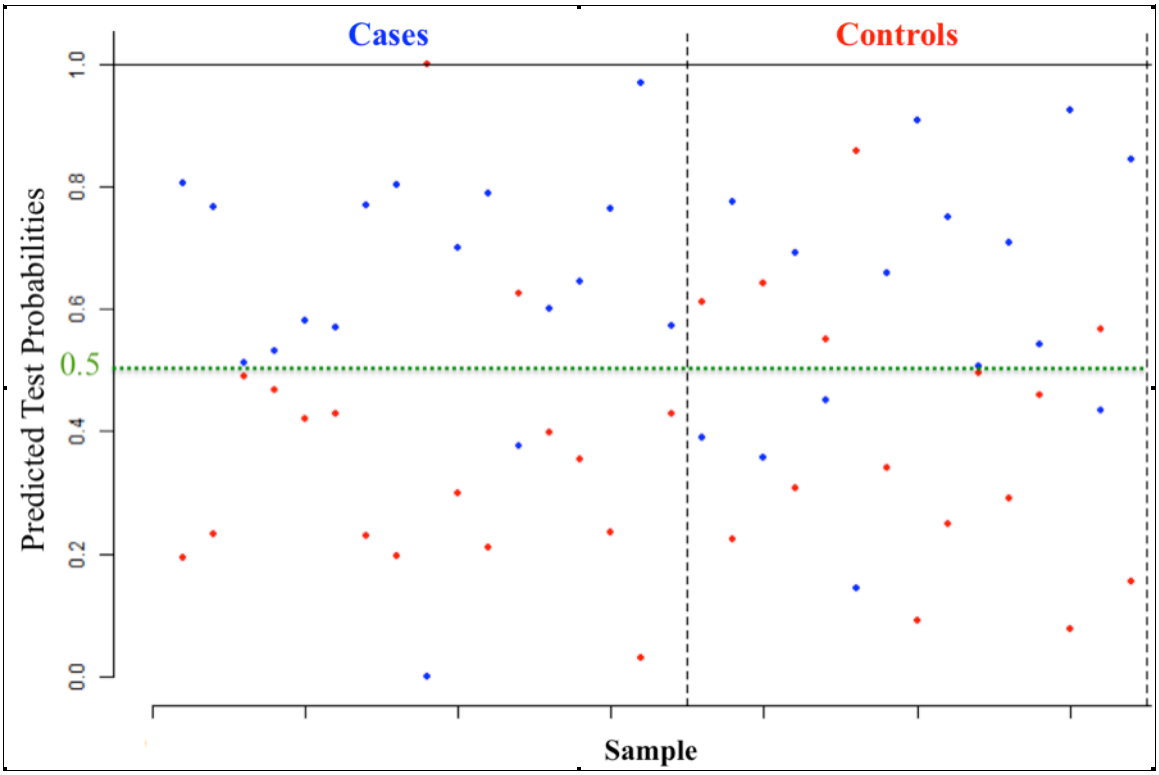

Figure 3. Output of the classifier algorithm for each ICA case (left) and non-ICA control (right). Each blue data point represents the probability that a given sample has an ICA (i.e. is a close match to the signature aneurysm profile; a value of 1 on the y-axis represents a perfect match to the signature aneurysm profile). The red data points represent 1 minus the probability that a given sample has an ICA. Any sample with a predicted ICA probability (blue data point) greater than the 0.5 threshold line is predicted by the algorithm to be an ICA case, and is classified as such. Samples with blue data points below the 0.5 threshold are predicted to be non-ICA controls.

Tables

Table 1. Gene symbols of the 39 genes included in the signature aneurysm profile.

ACTB	NR1I2
AKR1B1	NUDC
APOA1BP	NUDT5
ATAD3A	PCDHA12
ATP5G1	PHB
B2M	PPAN
C10orf99	**PPIA**
C14orf138	RGS3
C15orf63	RUVBL1
CDK4	SBSN
CUTA	SERF2
EDF1	SLC25A24
ENPP4	SNRPC
GAPDH	SOS2
HMOX2	SSU72
IL18R1	SYNGAP1
IMPDH2	THOC6
MED6	VKORC1
MIF	VPS72
NOSIP	

Genes used as controls are indicated in bold.

Table 2. Characteristics of TAA patients with concurrent ICA

Age (years)	Gender	TAA Location	ICA Location	Intracranial Imaging Modality	HTN	Smoker	Ethnicity
36	M	Des	ACA	MRA	+	0	AA
56	F	Asc	Left IC	CTA	+	+ [quit]	C
57	F	Asc	Left MCA	CTA	+	+	AA
59	M	Des	MCA	CTA	+	0	C
61	M	Asc	Basilar	CTA	+	+	C
64	M	Asc	Left vertebral	CTA	0	+	C
64	F	Asc	Left IC	CTA	+	+	C
66	F	Des	Left IC	MRA	+	+	AA
67	M	Asc	Left IC	CTA	+	+	C
67	M	Asc	Left IC	CTA	+	+	C
68	F	Asc	Right IC	CTA	+	+	C
71	M	Asc	Right ACA	MRA	+	0	C
75	M	Asc	Right IC	CTA	+	0	C
77	F	Asc	Left IC	CTA	+	+	C
77	F	Asc	Left IC	CTA	+	0	C
79	M	Des	Right IC	MRA	+	+	C
81	M	Des	Basilar	MRA	+	+	C
82	M	Asc	ACA	CTA	+	0	C
86	F	Asc	Right MCA	CTA	+	+	C

AA = African American; ACA = anterior cerebral artery; Asc = ascending aorta, C = Caucasian; Des = descending aorta; HTN = hypertension; IC = internal carotid artery; MCA = middle cerebral artery. A "+" indicates the patient had a history of hypertension or smoking. A "0" indicates the patient did not have a history of hypertension or smoking. Adapted with permission from reference 67.

Table 3. Characteristics of ICA patients with concurrent TAA

	Non-TAA patients (n = 343)		**TAA patients (n = 16)**		**p value**
Method of Thoracic Imaging	CT: 137 (40%) TTE/TEE: 205 (60%) MRA: 1 (< 1%)		CT: 9 (56%) TEE: 7 (44%)		>0.2
Mean Age	58.0 years		66.4 years		0.014
Gender	65% Female		50% Female		>0.2
Ethnicity	69% Caucasian 31% Non-Caucasian		63% Caucasian 37% Non-Caucasian		>0.2
Blood Pressure	54% Hypertensive		75% Hypertensive		0.20
Smoking	57% Smokers		42% Smokers		>0.2
ICA Presentation	64% Ruptured		56% Ruptured		>0.2
Mean ICA Size	6.53 mm		7.62 mm		>0.2
Multiple ICA	74 (21.6%)		2 (12.5%)		>0.2
ICA Location (75 patients with multiple ICA; 472 Total ICA)	33 ACA (7%) 88 Acom (20%) 30 Basilar (6%) 105 IC (22%) 106 MCA (23%) 3 PCA (<1%)	64 Pcom (14%) 4 PICA (1%) 8 SCA (2%) 13 Vert (3%) 2 Other (1%)	1 ACA (6%) 6 Acom (38%) 1 IC (6%) 4 MCA (25%)	2 Pcom (13%) 2 Vert (13%) 2 Other (13%) (anterior spinal & ophthalmic arteries)	>0.2

ACA = anterior cerebral artery; Acom = anterior communicating artery; IC = internal carotid artery; MCA = middle cerebral artery; PCA = posterior cerebral artery; Pcom = posterior communicating artery; PICA = posterior inferior cerebellar artery; SCA = superior cerebellar artery; Vert = vertebral artery

Table 4. Multiple logistic regression including age (as a continuous variable) and ICA size (4.0 mm as cut-off) measuring the risk of concurrent TAA

Characteristics	**Odds Ratio of Concurrent TAA (95% CI)**	**p value**
Age (in years)	1.05 (1.01 – 1.09)	0.025
ICA size (in mm)		0.127
$\leq$ 4.0mm	0.31 (0.07 – 1.40)	
> 4.0mm	Ref.	

Table 5. Characteristics of ICA patients enrolled in Arm 3 of the project

	ICA patients (n = 17)
Age	59 years (range 37 – 83 years)
Gender	89% Female
ICA Location (20 Aneurysms)	10 IC, 2 MCA, 3 Basilar, 2 Acom, 1 Pcom, 1 PICA, 1 Vert
ICA Size	5.8 mm (range 1 – 20 mm)
Multiple ICA	18%
ICA Ruptured	12%
ICA Previously Treated	24%
HTN	53%
Smoking	35%
+ Family History of ICA	35%
Anti-Inflammatory Meds	47%

Acom = anterior communicating artery; HTN = hypertension; IC = internal carotid artery; MCA = middle cerebral artery; Pcom = posterior communicating artery; PICA = posterior inferior cerebellar artery; Vert = vertebral artery

Table 6. Nucleic acid concentration, purity, and integrity for peripheral blood samples analyzed for the signature mRNA aneurysm profile.

Sample ID	Nucleic Acid Concentration ng/ul	A260	A280	260/280	RIN
1	115	2.874	1.442	1.99	8.3
2	144.7	3.618	1.771	2.04	8.6
3	231.1	5.776	2.779	2.08	7.7
4	100.4	2.509	1.237	2.03	8.6
5	101.2	2.529	1.24	2.04	7.7
6	142	3.549	1.726	2.06	8.8
7	161.3	4.032	1.958	2.06	8.3
8	102.8	2.571	1.258	2.04	8.2
9	142.3	3.556	1.705	2.09	9.5
10	113.5	2.839	1.353	2.1	9.5
11	395.2	9.88	4.696	2.1	8.5
12	124.7	3.116	1.478	2.11	9.1
13	5.5	0.138	0.04	3.47	NA
14	152.3	3.806	1.838	2.07	9.7
15	74.8	1.871	0.905	2.07	9.1
16	148.3	3.708	1.774	2.09	8.9
17	148.9	3.722	1.778	2.09	8.6
18	75.7	1.892	0.92	2.06	9.5

Sample 13 was excluded from analysis prior to the cDNA synthesis stage due to inadequate RNA concentration. RIN was not calculated for this sample. RIN = RNA integrity number.