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Discordance between Functional Screening and Genetic Confirmatory Diagnostic Tests:

Five over Five

Or,

Clinical Implications of APC-Resistance and Factor 5 Leiden Mutational Testing over a Five Year Period at Yale-New Haven Hospital

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Activated protein C (APC) resistance can be used as a screening test for patients with Factor 5 Leiden mutations. We evaluated 2104 patients tested for APC resistance and 4082 patients for factor 5 Leiden mutations between 2010 and 2015. Greater than 90% of all factor 5 Leiden mutation tests were negative, representing a potential annual savings of over $40,000. It was also noted that quantitative APC resistance values are directly related to the factor 5 Leiden genotype. Patients with non-Leiden APC resistance had significantly higher values for APC resistance (1.85 vs. 1.97 with p < 0.005). Discordance between APC resistance testing and factor 5 Leiden testing was also examined, and autoimmune disease and liver transplantation emerged as common associations. Based on these observations, we propose specific modifications to the thrombophilia diagnostic algorithm that we believe will decrease cost and increase diagnostic clarity.
INTRODUCTION

Patients with coagulation disorders are at risk for a variety of complications ranging from thrombosis to hemorrhage. Reliable laboratory testing is required to pinpoint the pathophysiologic defect underlying the patient's clinical syndrome. Some unfortunate individuals are born with a genetic tendency toward thrombosis, whereas others acquire risk factors over the course of a lifetime. The most common inherited risk factor for thrombosis is the factor 5 Leiden mutation, which is transmitted in an autosomal dominant fashion. Notably, 5.2% of white Americans carry a copy of the factor 5 Leiden mutation. Such carriers of the mutation have been estimated to have somewhere between a 10-40% risk of experiencing pathological thromboembolism during their lifetime. Such patients are often treated with lifelong anticoagulation, a fate laden with potential for unintended but common consequences of the concomitant bleeding diathesis.

The activated form of factor 5, also known as factor 5a, is normally inactivated by activated protein C, which proteolytically cleaves factor 5a at three distinct arginine residues, located at positions 306, 506, and 679. The factor 5 Leiden mutation causes production of a mutant factor 5 protein impervious to regulatory cleavage by activated protein C, a gamma-carboxylated endogenous anticoagulant protein also produced by the liver. The culprit mutation is a G->A transition mutation at position 1691 in the factor 5 gene, which results in a substitution of glutamine rather than arginine at the 506th amino acid. Thus, the factor 5 Leiden protein is resistant to activated protein C (APC).

Other, rarer mutations in the factor five gene have been shown to cause APC resistance by similar mechanisms. For example, a G to C mutation which alters arginine 306 to threonine, also attenuates APC cleavage of factor 5a, and has been dubbed as the Factor 5 Cambridge mutation. A mutation at precisely the same codon that converts arginine 306 to glycine has also been reported, and is referred to as the Factor 5 Hong Kong mutation.

The clinical evaluation of patients for the factor 5 Leiden mutation is typically a two-step process, beginning with a highly sensitive, rapid and inexpensive test for APC resistance. The patients baseline activated partial thromboplastin time is assayed by standard lab techniques, and a separate aliquot of the patient’s plasma is treated with activated protein C. In patients with a normal factor 5 gene, the addition of APC will result in cleavage of factors five and eight, leading to prolongation of the patients PTT, much the same as in a patient who had congenital deficiency of factor five (parahemophilia) or factor eight (hemophilia A). Hence the ratio of the patients PTT following APC administration divided by the baseline PTT is computed as an in vitro physiologic index for the extent to which APC is capable of cleaving factors 5 and 8. The higher the ratio, the more APC was able to prolong the PTT by cleaving factors five and 8. Patients possessing the factor 5 Leiden mutation synthesize a protein that is relatively impervious to cleavage by APC. The PTT(APC) / PTT ratio in such patients is abnormally low, with the clinical cutoff at Yale New Haven Hospital being 2.20. Thus patients with such a ratio below 2.20 are deemed to demonstrate APC resistance.

Naturally, this screening test should be followed by more specific, precise, and costly confirmatory genetic testing for the factor five Leiden gene mutation, which is conducted by PCR amplification of the factor five gene, followed by hybridization of an oligonucleotide probe with higher affinity for DNA containing the factor five Leiden mutation. If the Leiden mutation is present, the probe will adhere to the amplified DNA, leading to a measurable increase in the melting point of the resulting duplex piece of DNA. It is important to emphasize that the APC resistance assay will theoretically detect all the above described mutations in the factor 5 gene (Leiden, Hong Kong, Cambridge), as well as other acquired causes of APC resistance mentioned below. However, clearly, the molecular test for the factor five Leiden mutation will only detect the factor 5 Leiden mutation, and will certainly fail to detect factor 5 Cambridge, Hong Kong mutations, as well as the acquired causes for APC resistance.

It is currently unknown the current rate at which patients screened for APC resistance at our facility are ultimately confirmed to have the factor five Leiden mutation; there are rare reports in the literature from populations that are very different than that served by Yale-New Haven Hospital. Further, there are several notable causes of APC resistance that are not due to the factor five Leiden mutation. This list includes elevated factor 8 levels, which can occur in pregnancy, chronic inflammatory diseases, increased estrogen exposure, or the presence of antiphospholipid antibodies, including those targeting beta-2 glycoprotein 1, cardiolipin, and the lupus anticoagulant. It is also unknown the rate at which such ‘pseudo’ APC resistance occurs. Further, it is currently unknown how often clinicians evaluating hypercoagulable patients use the
sequential diagnostic scheme described above or directly order the less physiologic but more specific, expensive genetic test for factor five Leiden.

We hypothesize that patients who demonstrate discordance between APC resistance and factor five Leiden mutation testing represent a group of patients that are frequently inadequately diagnosed and either undertreated or overtreated as a result. We hypothesize that systematic examination of all patients tested for APC resistance and the factor five Leiden mutation over the past 5 years at Yale New Haven Hospital will reveal many patients with APC resistance but no factor five Leiden mutation. We believe a significant percentage of these patients may have a clinical history of liver transplantation, leading to confounding of their hypercoagulability testing. As chimeras, their liver graft produces the factor five Leiden protein, which is not detected with current testing for factor 5 Leiden which analyzes the DNA from peripheral leukocytes, not the patient’s liver cells. If they’ve experienced a thrombotic event, these patients should theoretically receive lifelong anticoagulation despite negative lab testing for the factor five Leiden mutation. Conversely, we hypothesize that the ostensibly paradoxical finding of negative APC-resistance testing along with a factor 5 Leiden mutation represents a cohort of patients who have undergone allogeneic bone marrow stem cell transplantation. Their graft, from a donor carrying the factor five Leiden mutation, causes the patient’s peripheral leukocytes to carry the mutation, which causes positive factor five Leiden mutation testing – which is clinically inconsequential because the patient’s liver is producing wild type factor five protein. Thus, we aim to perform a comprehensive analysis of APC resistance and factor 5 Leiden genetic testing done at Yale New Haven Hospital over the past 5 years. Specifically, we aim to answer the following questions.
1] How many patients were tested for APC resistance and for the factor five Leiden mutation over the past 5 years at YNHH? What percentage of APC resistance and factor five Leiden mutation tests were positive and negative?

This will enable statistical analysis of hypercoagulability testing, which has implications for how to best optimize testing from diagnostic and financial standpoints.

2] What percentage of patients with positive APC resistance were subsequently tested for factor 5 Leiden? Of these patients, what percentage were ultimate positive and negative for the factor 5 Leiden mutation?

This will assess the current clinical workflow for following up APC resistance with the appropriate diagnostic test

3] How many patients were tested for factor five Leiden mutations but not APC resistance? How many of these patients were negative for factor five Leiden?

This subgroup would be ideal for conducting further diagnostic testing for other factor five mutations that are currently missed because clinicians fail to pursue further diagnostic workup. We also hypothesize that if factor 5 Leiden testing was negative, patients should be still tested for APC-Resistance, and if positive, should be tested for
other more rare factor five mutations (Cambridge or Hong-Kong mutations), depending on their clinical history.

4] Of the patients who tested positive for APC-resistance but negative for factor 5 Leiden, what are the most likely clinical risk factors for acquired APC resistance?

Again, these patients with discordance between APC and factor 5 Leiden testing are ideal candidates for more advanced testing for rarer factor 5 mutations, or may be transplant recipients with a mutant graft but wild type germline factor five.

5] Is the quantitative value for APC-resistance predictive of the ultimate coagulation defect? What are the average levels of APC resistance of factor 5 Leiden homozygotes, heterozygotes, and those who lack the factor 5 Leiden mutation?

This could also enable clinicians to select the proper testing depending on the clinical scenario.

6] How many patients tested positive for factor 5 Leiden but negative for APC-resistance? Are we missing patients with factor 5 Leiden by relying on APC-resistance as a screening test? Are there environmental factors like anticoagulation that are concealing APC-resistance and interfering with the diagnostic process for factor 5 Leiden?
This will inform us of the actual clinical sensitivity of APC resistance as a screening test for factor 5 Leiden. This will help us produce evidence based recommendations for when patients should be tested directly for factor 5 Leiden, rather than relying on the traditional sequence of APC-resistance followed by factor 5 Leiden testing.

7] Of patients who were tested for factor 5 Leiden but not APC-resistance, what percentage were positive and negative for factor 5 Leiden?

This is a very important healthcare utilization question. If factor 5 Leiden testing is being ordered excessively in clinical scenarios where the pre-test probability is low, (ie the patients were not tested or tested negative for APC-resistance) many of these patients will test negative. This information could be used to guide provider decision-making about hypercoagulability diagnostics.

METHODS

Retrospective Analysis of Patient Diagnostic Data

Factor 5 Leiden and APC resistance data from the past 5 years were queried and collected using the SoftLabMic program in the department of Lab Medicine at Yale.
Data regarding clinical history and specific diagnostic testing were collected using EPIC EMR system, and analyzed statistically using Microsoft Excel.

Patient samples that are deemed appropriate may be sent for clinical testing for the factor 5 Leiden mutation of other less common mutations in the factor five gene.

Factor 5 Leiden mutation Testing

DNA was isolated from the patient samples and tested for the Factor V Leiden mutation. Regions of the Factor V gene and for wild type Factor V were amplified enzymatically, then characterized as positive or negative for the Factor V Leiden mutation on agarose gels. The Factor V primers FVNOR and FVMUT (Thromb Haemost 75:520,1996) and FVFOR2 (Thromb Haemost 74:874,1995) were used. Beta-actin primer were used to test integrity of the sample DNA. Positive controls were detected and negative controls appropriate. Control PCR indicated the DNA preparation was adequate.

LIMITATIONS

As with any clinical study, confidence in our results can be limited by the size of the study. We aim to study as many patient samples as needed to achieve statistical significance as well as confidence that our results will apply to future patients. As such, after conducting initial analysis of patients from the past five years, it is possible that more samples from previous years will need to be integrated and analyzed to strengthen
our measures of association. It is also possible to integrate new results from the clinical lab as they occur in real time to the study.

Another potential limitation of our study is its external validity or generalizability. Since we will be studying patient samples from Yale New Haven Hospital, naturally our results will be most applicable to lab practice here. Since not all laboratories serve the same ethnically diverse population, use the same reagents for APC-R and factor 5L testing, or clinical diagnostic algorithms, it is possible that even if our results our internally valid, they simply may not be applicable to coagulation testing elsewhere. Regardless, the opportunity to potentially improve the laboratory practice at YNHH alone is more than sufficient justification to study this common but unexplained phenomenon.

RESULTS

Between January of 2010 and September of 2015, 2104 patients were tested for APC resistance at the Yale New Haven Hospital coagulation laboratory. Of these, 8.9% of them demonstrated APC resistance. In other words, 188 patients demonstrated APC resistance during this time period (table 1).

Over the same time period, in the molecular laboratory of Yale New Haven Hospital, 4082 patients were tested for the factor 5 Leiden gene mutation, and 381 or 9.3% of these patients had a positive test, demonstrating either one or two copies of the factor 5 Leiden mutation. Of these, 379 patients or 99.5% were heterozygous, and the
remaining 2 patients (0.5%) were found to have two copies of the factor 5 Leiden mutation. The remaining 90.7% of patients tested were found to have no evidence for the factor 5 Leiden mutation. In absolute terms, 3701 patients were found to lack the factor five Leiden mutation over the relevant five year period.

2]
These initial results prompted further subset analysis within these two groups. Of the 188 patients that demonstrated APC resistance, 63.3% (119 patients) were also tested for the factor 5 Leiden gene mutation, while the remaining 36.7% (69 patients) were not tested (table 3). Of those with APC-resistance who were tested for the factor five Leiden mutation, 50.5% (95 patients) were confirmed to have either one or two copies of the factor five Leiden gene mutation. Therefore, of those patients demonstrating APC resistance with factor 5 Leiden gene testing on record, 12.8% (24 patients) were negative for the specific mutation.

3]
We next determined how many patients were tested for APC-R only (and not for the factor five Leiden gene mutation), how many patients were tested for both APC-R and the factor five Leiden gene mutation, and how many patients were tested for the factor five Leiden gene mutation only (and not APC-R).
We found that 991 patients were tested for APC-R only, 1113 patients were tested for both APC-R and factor five Leiden gene mutation, and 2969 patients were tested for the factor five Leiden gene mutation but not for APC resistance.

Of those who had only been tested for APC-R, 7.0% (69 patients) demonstrated APC-R, indicating that this positive finding was not further investigated with a factor five Leiden gene mutation test. In other words, we identified 69 patients who demonstrated APC resistance, but who did not have factor five Leiden mutation test results on record. Of those who had only been tested for the factor five Leiden gene mutation, 9.5% (283 patients) were positive. In other words, of the 2969 patients who were tested for the factor five Leiden gene mutation but not APC resistance, 90.5% of them (2686 patients) tested negative. Since the factor five Leiden gene mutation test costs $83.37 as of November 2015, this represents $223,931 of healthcare expenditures over a five year period, used to determine that patients who had not undergone APC-R screening were negative for the factor 5 Leiden mutation. This averages to $44,786 per year in testing for the factor 5 Leiden mutation in patients who have not been tested for APC-resistance.

We carefully reviewed the medical charts of the patients who demonstrated APC resistance but tested negative for the factor five Leiden gene mutation. Of these 24 patients examined, by far the most common associated pathologic condition was systemic lupus erythematosus or antiphospholipid antibody syndrome. Eight patients or 33% of non-Leiden APC resistance demonstrated specific immunologic evidence for lupus or for
antiphospholipid antibodies, as indicated either by a positive dilute Russell viper venom time, anti beta-2 glycoprotein 1 antibodies, anticardiolipin antibodies, or anti double stranded DNA antibodies.

Two patients had undergone liver transplantation, prior to testing positive for APC resistance. The first is a 23 year old female born with biliary atresia, who consequently received a liver transplant at age 3 in 1996. In 2015, she was diagnosed with hepatic artery thrombosis, and was noted to have abnormal APC resistance of 1.83. However, her factor five Leiden gene mutation testing was negative. A variety of hypercoagulability tests yielded negative results. Ultimately, tissue from her liver biopsy was sent for the factor five Leiden gene mutation, and was found to be heterozygous. In light of these findings, she was started on warfarin for a 3 month period, with plans to use Coumadin only post-operatively for thrombosis prophylaxis.

The second patient is a 5 year old male with a history of familial hypercholesterolemia, who underwent liver transplantation in April of 2013. He was subsequently tested for APC resistance, which was found to be abnormal, at 2.14. A subsequent factor five Leiden gene mutation test was negative. In light of these discordant findings, a strong possibility is that the patient was transplanted with a liver containing either a factor five Leiden mutation or a non-Leiden mutation in the factor five gene, leading to APC resistance due to mutant protein production, but negative genetic studies, because the patient’s leukocytes contain wild type copies of the factor five gene.
Considering the patients who demonstrated APC-resistance, we honed in further on the relationship between etiology and the quantitative value for APC resistance. We found that patients with APC-resistance who were confirmed to be heterozygous for the factor five Leiden gene mutation had an average APC-R of 1.85, with a standard deviation of 0.14 (95 patients) (see figure 1).

Of the patients with documented APC resistance but a negative factor five Leiden mutation test, the average APC resistance value was 1.97, with a standard deviation of 0.29 (24 patients) (Figure 2). Using unpaired (student’s) T Tests, P values were calculated to compare the populations of patients with 0 and 1 copy (heterozygotes) of the factor five Leiden gene mutation. The P value was 0.0023.

Of the patients who had APC resistance, but no testing on record for the factor five Leiden gene mutation, the average value for APC-resistance was 1.87 (69 patients), with a standard deviation of 0.14.

We were not only interested in identifying those patients with positive APC-resistance testing but a negative factor five Leiden test, but also in those patients with the converse. Therefore, we queried our dataset for patients that tested positive for the factor 5 Leiden gene mutation, but negative for APC resistance. In the 5 years we studied, three
such patients were found. One of these patients was a 30 year old female with a history of celiac disease, factor 5 Leiden heterozygosity, and subsequently presented with cirrhosis of unclear etiology, but likely due to a combination of Budd-Chiari syndrome and autoimmune hepatitis (negative ANA, anti F-actin IgA 66.1, anti-liver-kidney microsomal Ig 2.8, Total IgG 1426). She received a liver transplant in early May, 2012, and unfortunately her postoperative course was complicated by an apparent left posterior cerebral artery infarction (which was subsequently found to be a fungal brain abscess). A hypercoagulable panel was sent as part of a neurologic workup, which indicated APC-resistance of 3.5. As such the lab medicine physician entered the following interpretation into the chart. “No deficiency in natural anticoagulants (protein C, protein S and antithrombin). No evidence for APC resistance or lupus anticoagulant.”

Blood collected on the same day was sent for the factor five Leiden gene mutation test, which came back with the standard pathologist interpretation (figure 3). The most likely reason for this testing discrepancy is as follows. The patient was born with one copy of the factor five gene containing the Leiden mutation, and one wild type factor five gene. Thus every cell in her body contained one wild type copy of factor five and one copy with the Leiden mutation. The liver allograft she received came from a patient with two wild type copies of the factor five gene, which following transplantation, proceeded to synthesize completely wild type factor five. As long as 3-4 half-lives of factor five were allowed to elapse (the plasma half-life of factor five is between 12 and 36 hours), then the vast majority of the factor five protein in her plasma would have been wild type factor five, fully sensitive to cleavage by activated protein C. This was indeed
the case, as the stroke she sustained, as well as the subsequent thrombophilia workup both occurred over 1 week after the transplant was completed.

Since the factor five circulating in her blood stream would have been wild type by that point, it is not surprising that her APC resistance testing returned negative. Her factor five Leiden testing remained heterozygous, simply because the tissue obtained for the mutation analysis was the peripheral leukocytes, which were generated from her bone marrow which was genetically her own, and as such these cells continued to carry one copy of the factor five Leiden mutation, which is now of no physiologic consequence. In short, following transplantation with a liver allograft, the patient became a chimera – with the allogeneic liver generating wild type factor five protein causing normal APC resistance testing, and her peripheral leukocytes faithfully carrying one copy of the Leiden mutation, which continued to be detected by the molecular assay. Of note, this patients germ cells also continue to carry the factor five Leiden mutation. Therefore, although the patient herself has been alleviated of her hypercoagulable state by the liver transplant, her children are still at a 50% risk of inheriting one copy of the factor 5 Leiden mutation from their mother.

Interestingly, at her most recent follow up appointment, the liver transplant specialist noted that “due to issues with thrombosis, she is not a good candidate for oral contraceptives,” seemingly due to the consistent mentioning of her heterozygosity for the factor five Leiden gene mutation throughout her medical chart, despite her successful liver transplant and subsequent normalization of her APC resistance testing.
The second patient who had a positive factor five Leiden gene mutation test but negative APC-resistance is a 35 year old female with no personal history of thrombosis but diagnosed with compound heterozygosity for the MTHFR C677T and A1298C mutations (with normal homocysteine levels). As part of further coagulation workup, she was determined to be heterozygous for the factor five Leiden gene mutation. Unfortunately, her written electronic medical record does not extend back to this time period, but the coagulation diagnostics are quite informative. She had a history of being treated with post-partum lovenox due to the aforementioned genetic findings, and within one week of her positive APC resistance testing, had a PTT of 31.3 seconds (PT/INR 11.4 seconds/1.00). Thus, it is quite possible that at baseline, this patient would demonstrate APC resistance. However, due to anticoagulation, her elevated baseline PTT may very well explain the negative APC resistance testing, and its discordance with the positive genetic test for the factor five Leiden gene mutation.

The third and final patient is a 54 year old male who suffered his second myocardial infarction in 2010. He subsequently had a coronary artery bypass graft, and he was diagnosed post-operatively with a massive left thigh deep vein thrombosis as well as with bilateral pulmonary emboli. He was subsequently diagnosed with heterozygosity for the factor five Leiden gene mutation and noted to have an APC resistance of 2.35 (which was regarded as unremarkable) in the immediate post-operative period during a thrombophilia workup. Although unfortunately, the written electronic medical record does not extend back to this time period, it is clearly stated in subsequent documentation
that he received heparin in the Hospital as treatment for the aforementioned thromboemboli, which very likely explains his normal APC resistance testing despite a positive genetic assay for the factor five Leiden gene mutation.

In summary, three patients over the past five years exhibited negative APC resistance despite a positive factor five Leiden gene mutation test. Two of these patients were likely actively receiving anticoagulation during the testing, and the third had previously received a liver transplant, indicating that far greater than 99% of patients with at least one germline copy of the factor five Leiden gene will be detected by APC resistance testing.

7] We also sought to determine the diagnostic routes that are typically used to arrive at a diagnosis of the factor five Leiden gene mutation. Of the 4082 patients tested for the factor five Leiden gene mutation over the past five years, 381 were positive for at least one copy of the mutation. Strikingly, of the 381 total patients testing positive for the factor five Leiden gene mutation, only 98 of them, or 25.7% had been tested for APC resistance by the coagulation lab at Yale New Haven Hospital over the same time period (table 4). Therefore, 283 or 74.3% of the 381 patients with factor five Leiden mutations did not have APC resistance testing on record over the five year time period mentioned.

Of the 1113 patients who were tested for both APC resistance and the factor five Leiden gene mutation, 8.8%, or 98 patients tested positive for the factor five Leiden gene
mutation. Finally, of the 119 patients who demonstrated APC resistance and also had factor five Leiden testing on record, 79.8% (95 patients) were confirmed to have the factor five Leiden gene mutation.

DISCUSSION

In the current study, we sought to examine thrombophilia testing at Yale New Haven Hospital over the past five years. Specifically we focused on the diagnostic process for the most common inherited thrombophilia, the factor five Leiden gene mutation, for which two primary lab tests are available: APC-resistance, a phenotypic test that measures the ability of APC, an endogenous anticoagulant to prolong the PTT of a patient’s plasma in vitro, and the factor five Leiden molecular test, a genotypic assay that simply probes for the single nucleotide change from the DNA of a patient’s leukocytes that ultimately makes the factor five protein impervious to cleavage by APC. Since it is sensitive and inexpensive, the APC resistance assay is meant to serve as a screening test for the factor five Leiden mutation. Since it is relatively expensive and highly specific, the factor five Leiden mutation test is designed to confirm the diagnosis in a patient previously flagged with a positive APC resistance.

Upon analyzing our data, we noted several immediately striking trends, hinting at the lack of execution of the above “screen and confirm” paradigm emphasized for special coagulation testing at Yale-New Haven Hospital. First, there were nearly twice as many factor five Leiden gene mutation tests (4220) conducted as there were APC resistance
assays (2201) (table 2). Clearly, if APC resistance testing were truly used as a screening test for the factor five Leiden gene mutation, one would expect the lab to have conducted far more APC resistance assays than factor five Leiden mutation tests. This implies that nearly half of patients suspected to have the factor five Leiden mutation are being assayed upfront with the relatively expensive molecular test, which has a relatively low pre-test probability. Most likely for many of these patients, a rapid and inexpensive APC resistance test would have been negative, which could have saved the healthcare system a significant amount of money if this paradigm were applied consistently to the thousands of patients in question.

Unsurprisingly, we found that the vast majority (90.8%) of all factor five Leiden gene mutation tests over the past five years were negative, representing a significant opportunity for reducing healthcare costs if a large number of these patients would have simply been tested for APC resistance beforehand, thereby obviating the additional and unnecessary cost of the molecular assay. In light of the sensitivity of APC-R testing for identifying factor five Leiden mutations demonstrated by our study, this would be a much more cost-effective approach.

We reasoned that perhaps patients who were tested directly for the factor five Leiden mutation without prior APC resistance testing were treated as such due to a higher pre-test probability. For example, perhaps this group was comprised of patients with a notable family history of confirmed factor five Leiden mutations. However, we noted that the patients who had only had factor five Leiden testing on record without APC
resistance testing, had a nearly identical rate of positivity for the mutation. Specifically, we found that 90.5% of these 2969 mono-tested patients were found to have no evidence for the factor five Leiden gene mutation. Thus, it was readily apparent from our initial analysis, that the factor five Leiden gene mutation test has been over-utilized over the past five years at Yale-New Haven Hospital.

After observing the over-abundance of factor five Leiden gene mutation tests, we also wondered if in certain situations, the test was being order sufficiently. Specifically, we wanted to know if all patients with positive APC resistance testing were followed up with molecular testing for the factor five Leiden gene mutation test. We found that 36.7% of APC resistant patients had no factor five Leiden testing on record. Of course, this could be due to gaps in the electronic medical record. For example, in a patient’s provider pursued external testing with an outside lab that wasn’t entered into the patients’ electronic medical record. We also considered the possibility that clinicians were less likely to pursue molecular testing if the patient’s APC resistance was less dramatic, as the assay is quantitative. However, we noted that patients without molecular testing on record had indistinguishable APC resistance values from those confirmed to be heterozygous for the factor five Leiden mutation. Specifically, the 69 patients with positive APC resistance but no factor five Leiden testing on record, the average APC resistance was 1.86 (figure 4). The 95 patients with positive APC resistance who were found to be heterozygous for the factor five Leiden mutation had an average APC resistance value of 1.85.
It is well known that patients with factor five Leiden mutations are not the only patients expected to test positive for APC resistance. Among our patient population, we found that the most common association with non-Leiden APC resistance was serologic evidence for the lupus anticoagulant or the anti-phospholipid antibody syndrome. We also noted a previously unheralded risk factor for acquired APC resistance – liver transplantation. We described two cases of patients with abnormal APC resistance testing which was proceeded by a liver transplant. Since factor five is produced by the hepatocytes, these patients’ circulating factor five is derived from the donor liver, whose genotype is not their own. In short, these patients are genotypic chimeras, with their own bone marrow, and the liver of another.

We were also interested in discordance in the opposite direction – in other words patients with positive factor five Leiden genetic testing, but negative APC resistance. Overall, we found this to be a far rarer occurrence – only three patients were identified over a five year period. This reinforces the notion that APC resistance is quite sensitive as a screening test for the factor five Leiden mutation; of 1113 patients tested for both factor five Leiden and APC resistance, only three of them had positive factor five Leiden mutation and negative APC resistance – and all three had plausible explanations. Chart review indicated that one of them was actively receiving intravenous heparin, and the other was receiving enoxaparin and had an elevated PTT around the time of APC resistance testing. In addition, we once again found that liver transplantation was an important differential to consider when considering discordance between APC resistance and factor five Leiden testing, as indicated by a patient who seems to have been
alleviated of her APC resistance by a liver transplant, despite consistently testing positive for the factor five Leiden gene mutation, when her peripheral leukocytes were accurately reported to contain the factor five Leiden gene mutation.

Finally, seeking to uncover a clear genotype-phenotype correlation, we calculated the average APC resistance value for patients depending on their factor five Leiden genotype. Heterozygotes were typically clustered around APC resistance values of 1.85, whereas patients with a negative factor five Leiden assay had an average APC resistance of 1.97. Therefore, examination of this data revealed an important trend that we can use as a predictor of factor five Leiden status based on APC resistance alone. No APC resistant patient ultimately found to have a factor five Leiden mutation had an APC resistance value greater than 2.16 (a total of 95 patients over the past five years). Further, 84% of these Leiden positive patients demonstrated APC resistance values of 2.00 or lower.

On the other hand, of the 24 patients with positive APC resistance but a negative factor five Leiden test, 16 of them (67%) had APC resistance values of 2.01 or greater. Therefore, we can expect that in the vast majority of cases those patients with APC resistance of 2.01 or higher are much more likely to test negative for the factor five Leiden gene mutation. On the other hand, patients with APC resistance values of 2.00 or lower, are far more likely to ultimately test positive for the factor five Leiden mutation.

CONCLUSIONS
In the present study, our findings suggest several opportunities for improvement in the diagnosis and management of patients suspected to have a hypercoagulable state. Due to the overabundance of factor five Leiden gene mutation tests conducted in the absence of prior APC resistance testing, (and in light of the extremely high sensitivity of APC resistance testing for the factor five Leiden mutation) it would seem to be a reasonable lab policy to require documented APC-R testing before accepting specimens for factor five Leiden testing. If the APC resistance is negative, then an appropriate interpretation could be sent back to the clinician indicating that the factor five Leiden gene mutation has been effectively ruled out by negative APC resistance testing. If the APC resistance is positive, but above 2.01, an intermediate sign-out could be offered, suggesting that “low level” APC resistance is present, and hence the factor five Leiden mutation is possible, but less likely. Specifically, if the APC resistance is between 2.01 and 2.19, there is approximately a 51.6 % chance the patient will test negative for the factor five Leiden mutation, and a 48.3% chance the test will reveal a factor five Leiden mutation. Further workup for antiphospholipid antibodies, lupus anticoagulant, and other associated immunologic markers for SLE, like ANA or anti dsDNA antibodies could also be recommended.

On the other hand, if the APC resistance value is 2.00 or lower, there is a 90.9% chance that the factor five Leiden test will be positive, and only a 9.1% chance the patient will test negative for a factor five Leiden mutation.
As a result, APC resistance values 2.00 or below should automatically reflex to the factor five Leiden gene mutation test. This adjusted paradigm would undoubtedly accomplish two major goals. First, it would reduce the overall cost associated with APC resistance and factor five Leiden gene mutation testing, by massively reducing the number of factor five Leiden gene mutation tests that are negative. If all factor five Leiden gene mutation tests were only conducted on APC resistant patients, we can reasonably expect approximately 79% of them to be positive. This would likely lead to higher clinician satisfaction with the lab workup of thrombophilias, as currently over 90% of factor five Leiden mutation tests return negative. This would also clearly reduce overall healthcare costs for the Hospital system, saving tens of thousands of dollars each year simply by instituting a modified utilization scheme while using the same individual diagnostic tests.

Second, we propose that when signing out factor five Leiden mutation testing into the electronic medical record, the pathologists should always refer to the results of the APC resistance testing. This would increase clinician awareness about the utility of the APC resistance test, and reinforce its role at the very top of the thrombophilia workup, as a screening test for the factor five Leiden mutation.

Finally, when entering a signout interpretation into the electronic medical record for patients with APC resistance testing but a negative factor five Leiden gene mutation test, the most common differential diagnoses for this situation should be mentioned –
including non-Leiden factor five mutations including factor five Cambridge, liver transplantation and antiphospholipid antibodies, and the lupus anticoagulant.

In summary, an evaluation of the APC resistance and factor five Leiden testing at Yale-New Haven Hospital between 2010 and 2015 was undertaken. An overabundance of factor five Leiden gene mutation testing and corresponding financial ramifications were considered. It was also noted that quantitative APC resistance values are directly related to the factor five Leiden genotype. Discordance between APC resistance testing and factor five Leiden testing was also examined, and autoimmune disease and liver transplantation were identified as common associations. In light of these observations, specific modifications to the thrombophilia diagnostic scheme have been proposed.
Figure 1. Distribution of APC-R Values for All APC-Resistant Patients found to be Factor 5 Leiden Heterozygotes Over a 5 year Period
Figure 2. Distribution of APC-R Values for All APC-Resistant Patients found to be negative for the Factor 5 Leiden Mutation Over a 5 year Period
Figure 3. Laboratory Medicine Signout for a Positive Factor 5 Leiden Gene Mutation Test

INTERPRETATION:

The Factor V Leiden mutation WAS demonstrated; this patient has one normal Factor V gene and one gene with the Leiden mutation (HETEROZYGOUS for Factor V Leiden). This heterozygous state is significantly associated with venous thromboembolism. The prevalence of this mutation in a healthy, asymptomatic European-American population is 3-7%, while the prevalence of the Leiden mutation in subjects with a history of venous thrombosis ranges from 15-40%. There is a 3- to 10- fold increased risk of venous thrombosis with the isolated presence of the heterozygous Leiden mutation. Interpretation of the results of genetic testing may require consultation with a clinical geneticist, pathologist or other expert in the field to determine the risk of developing overt disease, the need for specific therapy, or the possible reproductive consequences for this individual.
Figure 4. Distribution of APC-R Values for All APC-Resistant Patients without Factor 5 Leiden Mutation Testing on Record Over a 5 year Period
Table 1: Breakdown of All Patients Tested for APC-R and the Factor V Leiden Mutation Over a Five-Year Period

<table>
<thead>
<tr>
<th>Patients Tested for APC-R only</th>
<th>Total APC-R</th>
<th>Patients Tested for Both Factor V Leiden Only &amp; Total FV Leiden</th>
<th>Factor V Leiden Mutation (%)</th>
<th>APC-R (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3701</td>
<td></td>
<td>2.0686</td>
<td>313</td>
<td>-</td>
</tr>
<tr>
<td>381</td>
<td></td>
<td>2.83</td>
<td>88</td>
<td>-</td>
</tr>
<tr>
<td>1916</td>
<td></td>
<td>2.0686</td>
<td>313</td>
<td>-</td>
</tr>
<tr>
<td>131</td>
<td></td>
<td>2.0686</td>
<td>313</td>
<td>-</td>
</tr>
<tr>
<td>402</td>
<td></td>
<td>2.0686</td>
<td>313</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2. Breakdown of Total Number of APC-R and Factor Five Leiden Tests Conducted at Yale-New Haven Hospital over a Five Year Period

<table>
<thead>
<tr>
<th></th>
<th>Factor Five Leiden</th>
<th>APC-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total # Tests</td>
<td>4220</td>
<td>2201</td>
</tr>
<tr>
<td>Positive</td>
<td>388</td>
<td>197</td>
</tr>
<tr>
<td>Negative</td>
<td>3832</td>
<td>2004</td>
</tr>
<tr>
<td>% Positive</td>
<td>9.19</td>
<td>8.95</td>
</tr>
<tr>
<td>% Negative</td>
<td>90.81</td>
<td>91.05</td>
</tr>
</tbody>
</table>
Table 3. Breakdown of Patients with Positive APC-R Testing at Yale-New Haven Hospital Over a Five Year Period

<table>
<thead>
<tr>
<th></th>
<th># Patients</th>
<th>% Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC resistant</td>
<td>188</td>
<td></td>
</tr>
<tr>
<td>hetero</td>
<td>95</td>
<td>50.53</td>
</tr>
<tr>
<td>negative</td>
<td>24</td>
<td>12.77</td>
</tr>
<tr>
<td>?</td>
<td>69</td>
<td>36.70</td>
</tr>
</tbody>
</table>
Table 4. Breakdown of Patients with Positive Factor Five Leiden Mutation Testing at Yale-New Haven Hospital Over a Five Year Period

<table>
<thead>
<tr>
<th></th>
<th># Patients</th>
<th>% Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Factor 5 Leiden</td>
<td>381</td>
<td></td>
</tr>
<tr>
<td>Tested for APC</td>
<td>98</td>
<td>25.72</td>
</tr>
<tr>
<td>Not Tested for APC</td>
<td>283</td>
<td>74.28</td>
</tr>
</tbody>
</table>