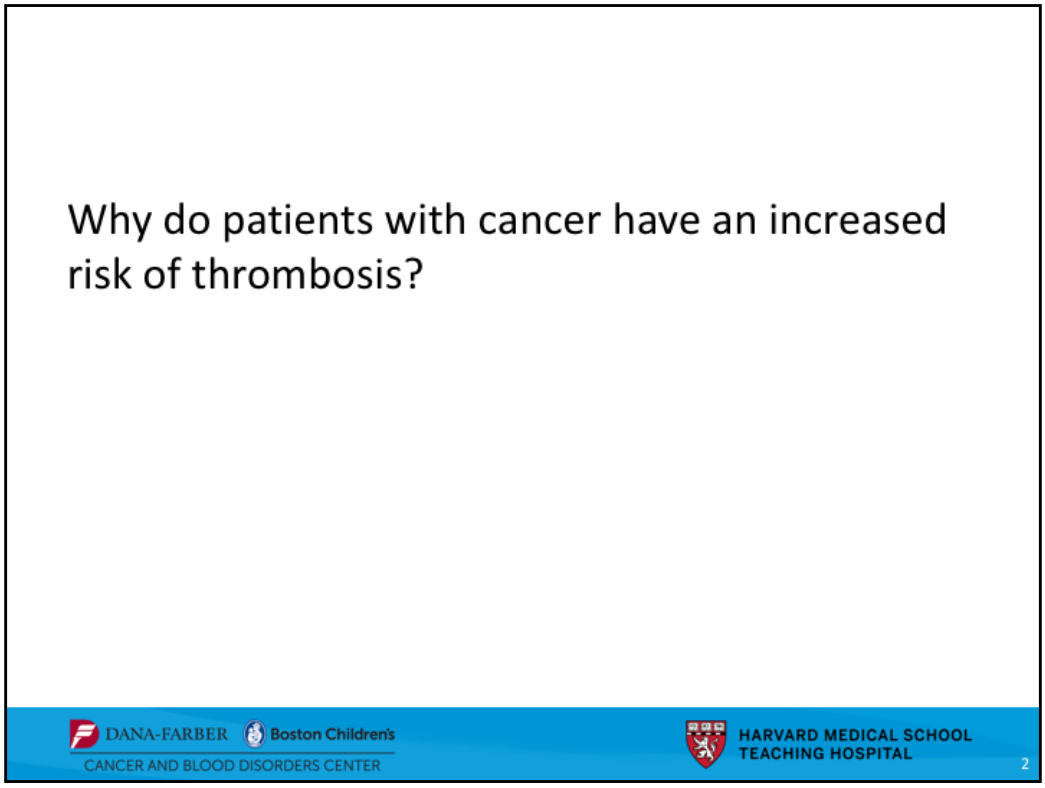
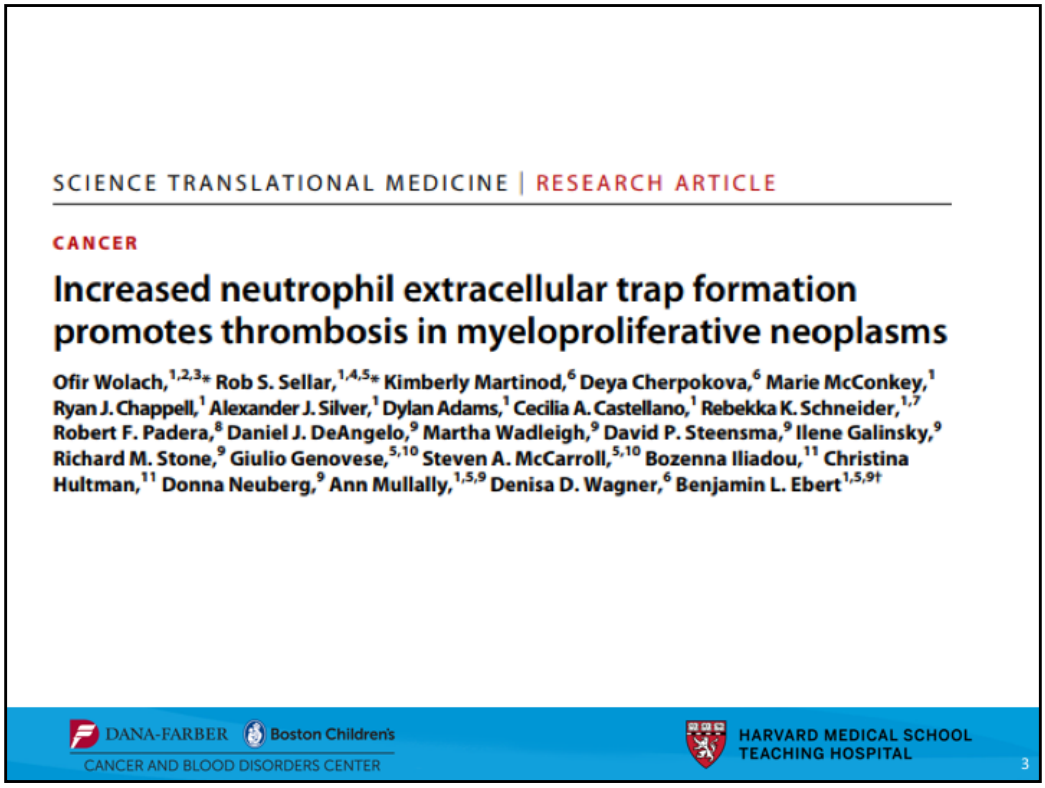
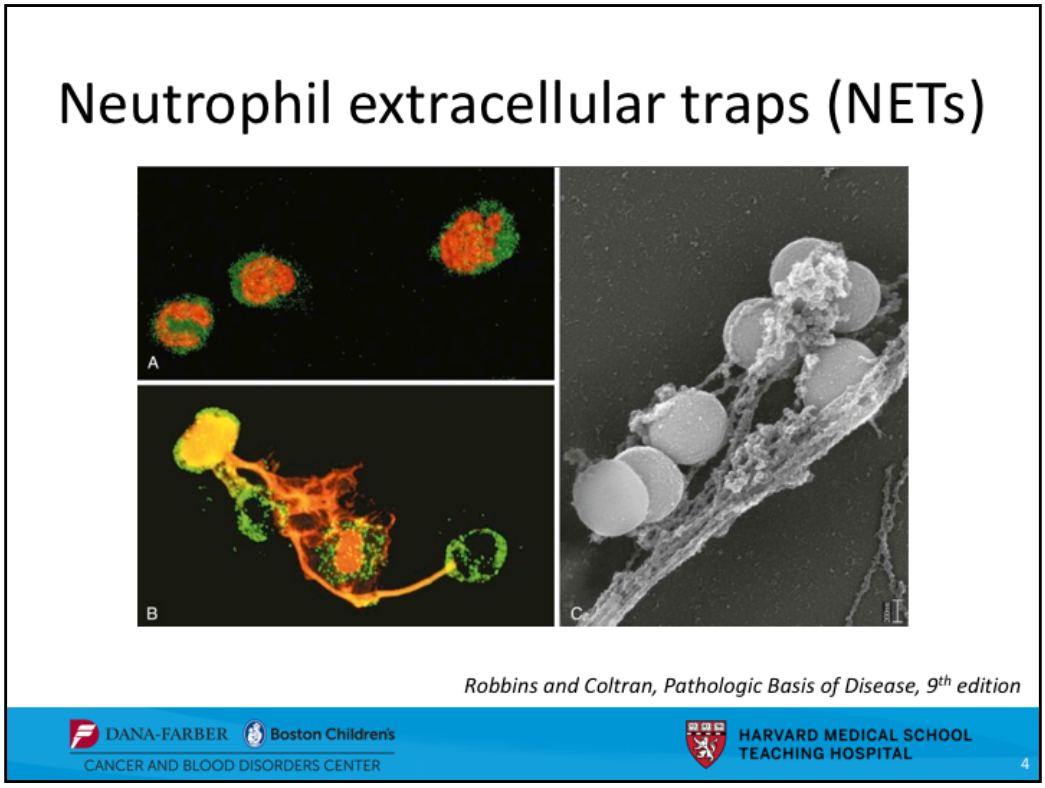


Seeing Mr. S led me to ask the question, why do patients with cancer have an increased risk of thrombosis?



I found a recent paper by Dr. Ebert that attempts to answer this question. This paper, published last month in Science Translational Medicine, implicates neutrophil extracellular traps, or NETs, as a potential mechanism through which cancer results in a hypercoagulable state. The rest of my presentation will address the key figures and experiments in his paper and attempt to tie this to our patient Mr. R.S.



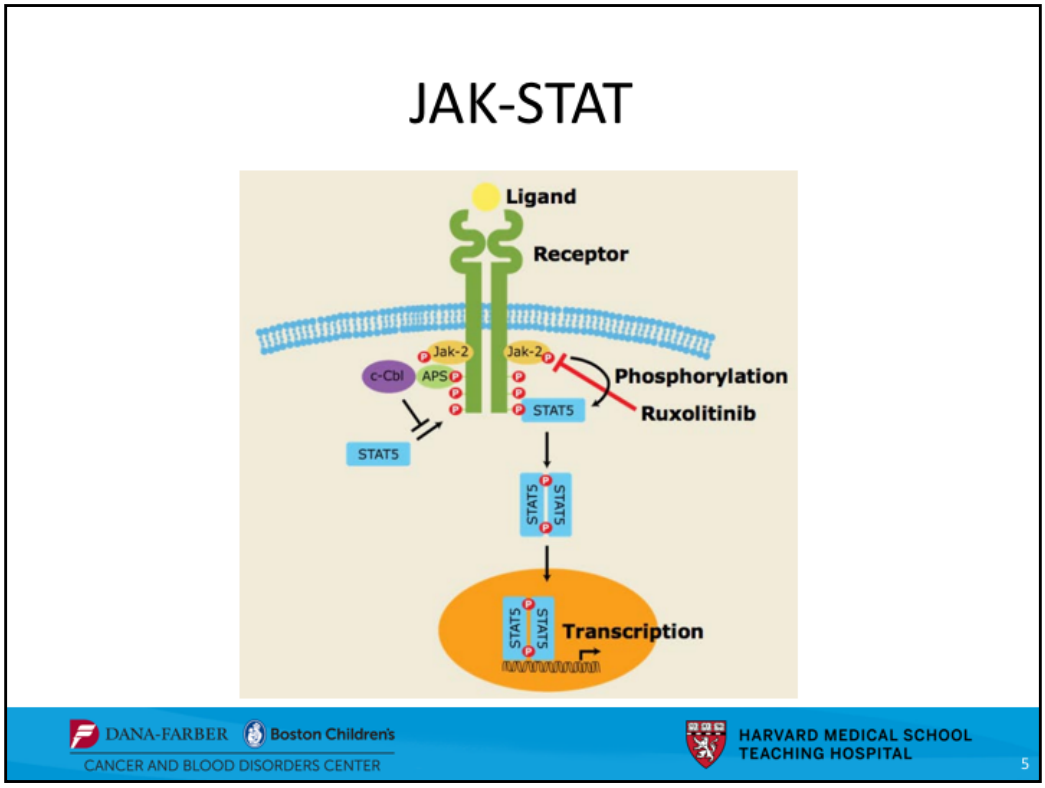
Before I begin with the paper's figures, I want to give a brief background of neutrophil extracellular traps. Here is a lovely figure from Robbins Pathology that helps to visualize what NETs look like.

In A, we see normal neutrophils where RED stains the nucleus, and GREEN stains the cytoplasm.

In B, we observe neutrophils extravasating their nuclear material to form an extracellular meshwork. Because they empty their nuclear material, this is actually a form of cell death.

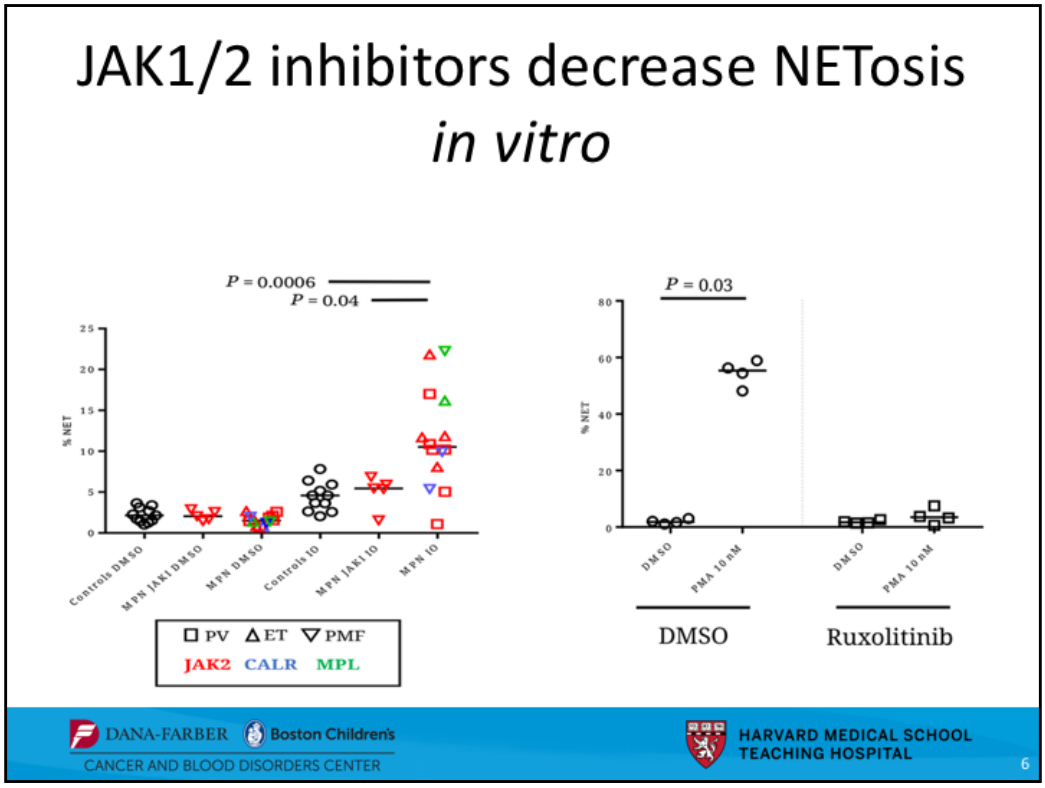
When NETs were first discovered, they were found to form in response to microbes. However, recent work has suggested that NETosis may form in autoimmune diseases as well. Dr. Denisa Wagner at the BCH played an important role in characterizing NETs.

In C, we see an electron micrograph that depicts staphylococci trapped in NETs.



Here is another background slide that will be important for understanding the paper I am about to describe. This is a depiction of the JAK-STAT pathway. In this pathway, a ligand, often a cytokine, binds to two transmembrane receptors, which dimerize and cause transphosphorylation of JAK. This in turn phosphorylates STAT, which eventually will localize to the nucleus to initiate transcription of specific genes.

A drug called Ruxolitinib inhibits the JAK protein which prevents the downstream activation of STAT.



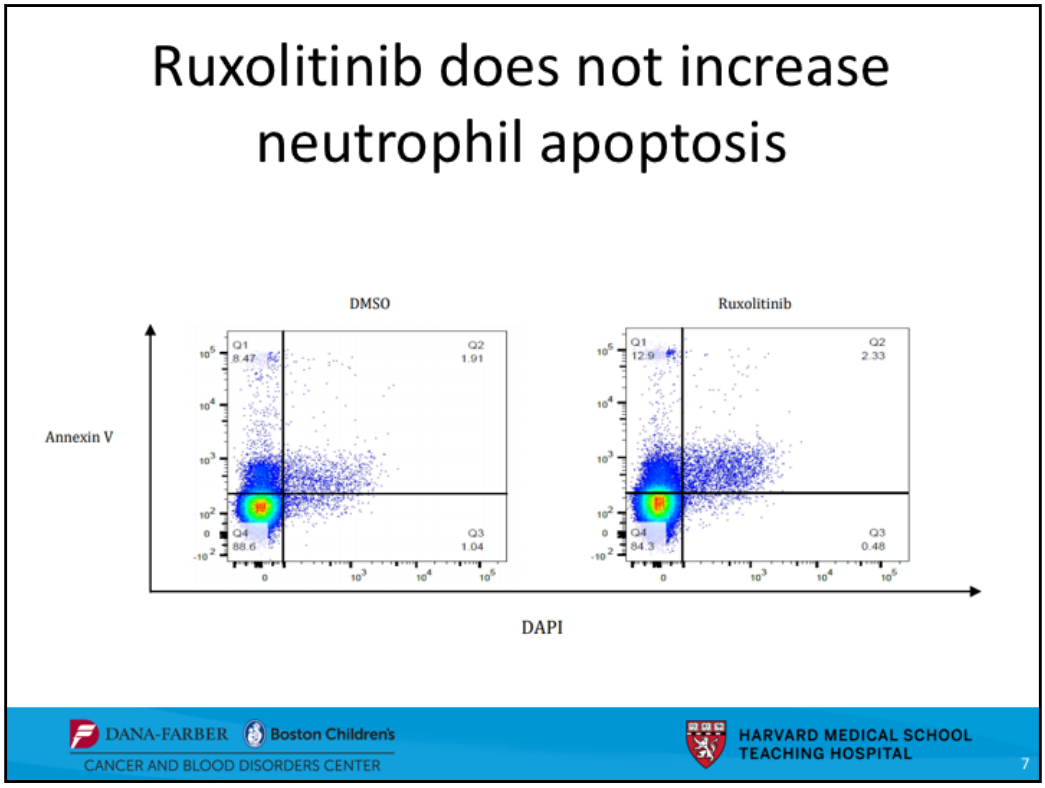
To begin our story, Ben Ebert's lab ran an unbiased screen to ask the question: Are there any differences in neutrophil functions in patients with myeloproliferative neoplasms, MDS, and controls? They found an increase in NET formation in MPN patients compared to the other groups. This was assessed by NET activation ratio, defined by NET-associated elastase before and after stimulation with PMA.

After this finding they posed several questions. First, do patients with MPNs have neutrophils that are more predisposed to NETosis?

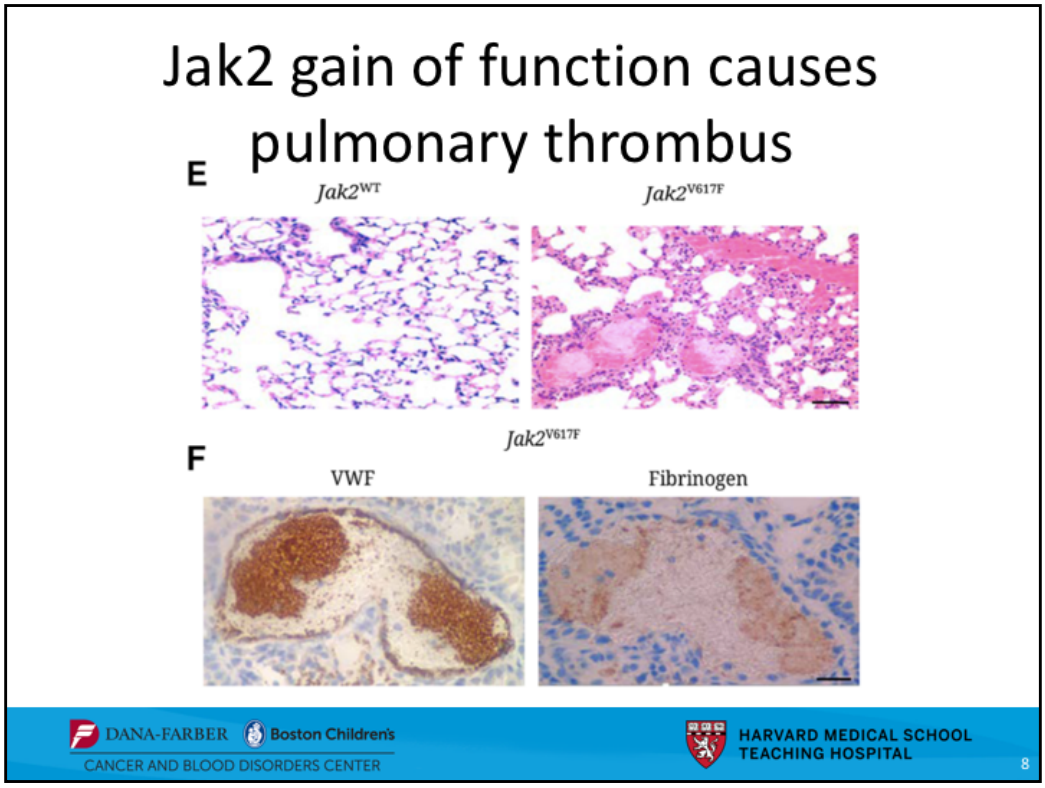
To answer this question, the scientists took peripheral neutrophils from three groups of patients: 1) healthy controls, 2) patients with MPN, and 3) patients with MPN who are taking a JAK inhibitor.

On the LEFT, you can see the results. When the neutrophils from all 3 groups are incubated in DMSO, there was low baseline NETosis. However, when stimulated with ionomycin – a substance previously shown to stimulate NETosis – the MPN group had a marked increase in NETosis. In the MPN + JAK inhibitor group, the amount of NETosis was as low as in the control + ionomycin group. (In the legend, the above row is the myeloproliferative neoplasm, and the row below characterizes the gene whose mutation explains the MPN).

On the RIGHT, peripheral neutrophils were collected from HEALTHY patients and stimulated with PMA. The left half of the graph demonstrates a proper NETosis response with PMA; the right half of the graph demonstrates that, when treated with ruxolitinib, the amount of NETosis decreases to similar levels compared to neutrophils + DMSO.



NETosis is a form of cell death. The authors considered the possibility that perhaps ruxolitinib is not inhibiting NETosis, but rather inducing apoptosis in the neutrophils that would have otherwise undergone NETosis. To prove that this wasn't the case, they performed an Aneexin V assay which demonstrated no increase in early apoptosis after treatment with ruxolitinib. To see this, look in Q2 and note that there was no significant increase in the percentage of cells.

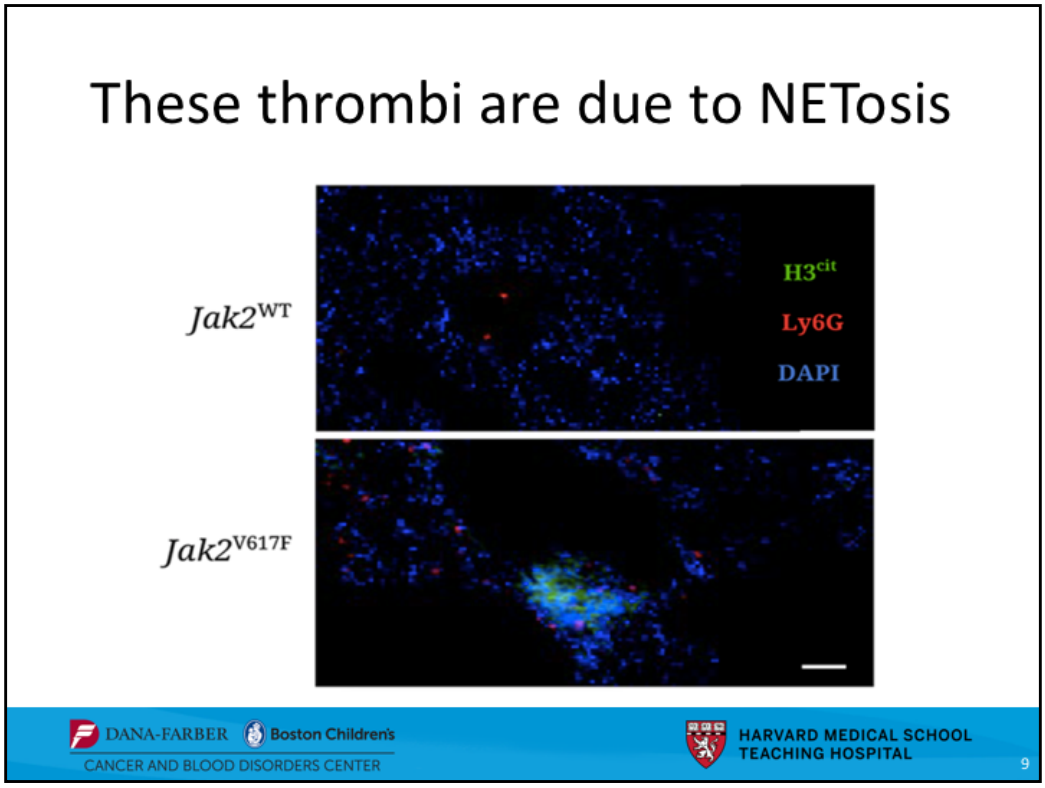


Now that the authors have established some *in vitro* evidence that MPNs are associated with an increased level of NETosis, they wanted to see if they could recreate their findings in an *in vivo* model.

To do this, they used an established strain of mice that was heterozygous for Jak2 V617F, a gain-of-function mutation in the Jak2 gene that causes constitutive activation of the JAK-STAT pathway. These mice developed a myeloproliferative neoplasm phenotype similar to polycythemia vera, and had a shortened lifespan.

They found that, compared to Jak2 wild-type mice, these Jak2 mutant mice had an increase in pulmonary thrombus formation.

When they stained the thrombi for von Willebrand factor and fibrinogen, they found the stains to be positive, showing involvement of platelets.

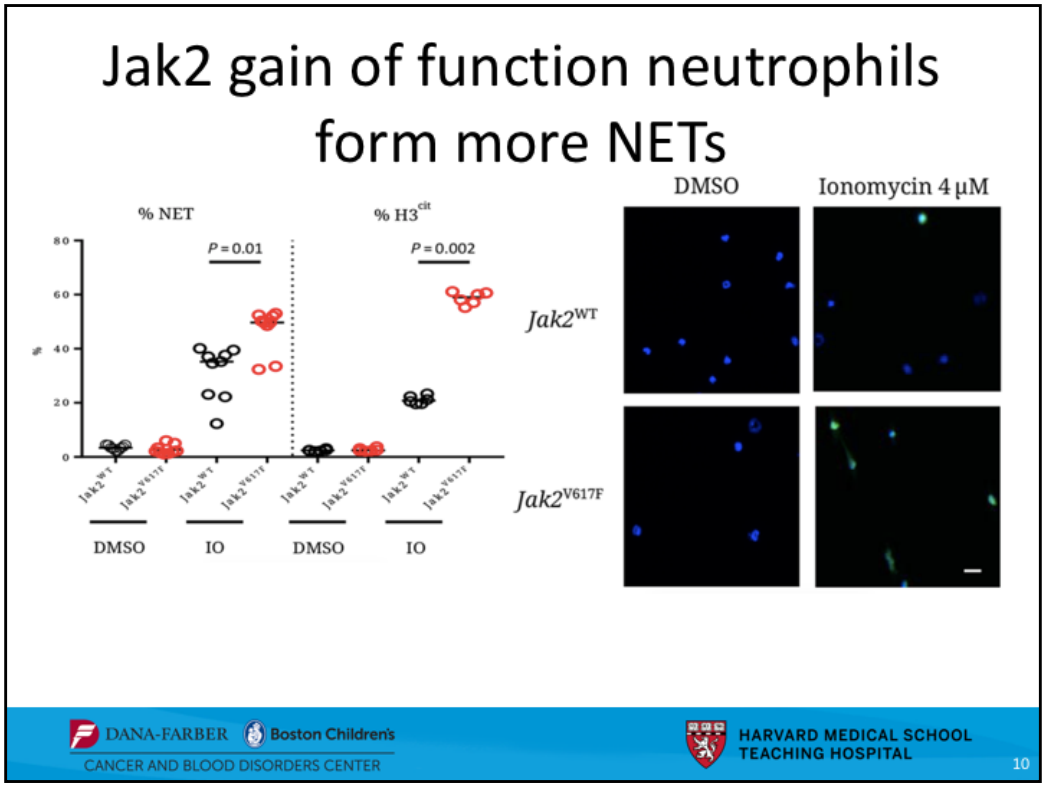


They took lung tissue from both the wild-type and Jak2 mutant mice and stained them.

They stained for H3cit, a histone that is citrullinated and previously established as a good marker for NETosis. Citrullinated histones are indicative of the decondensed nucleosomes that are found in the extracellular milieu in NETosis.

They also stained for Ly6G, which stains for neutrophils.

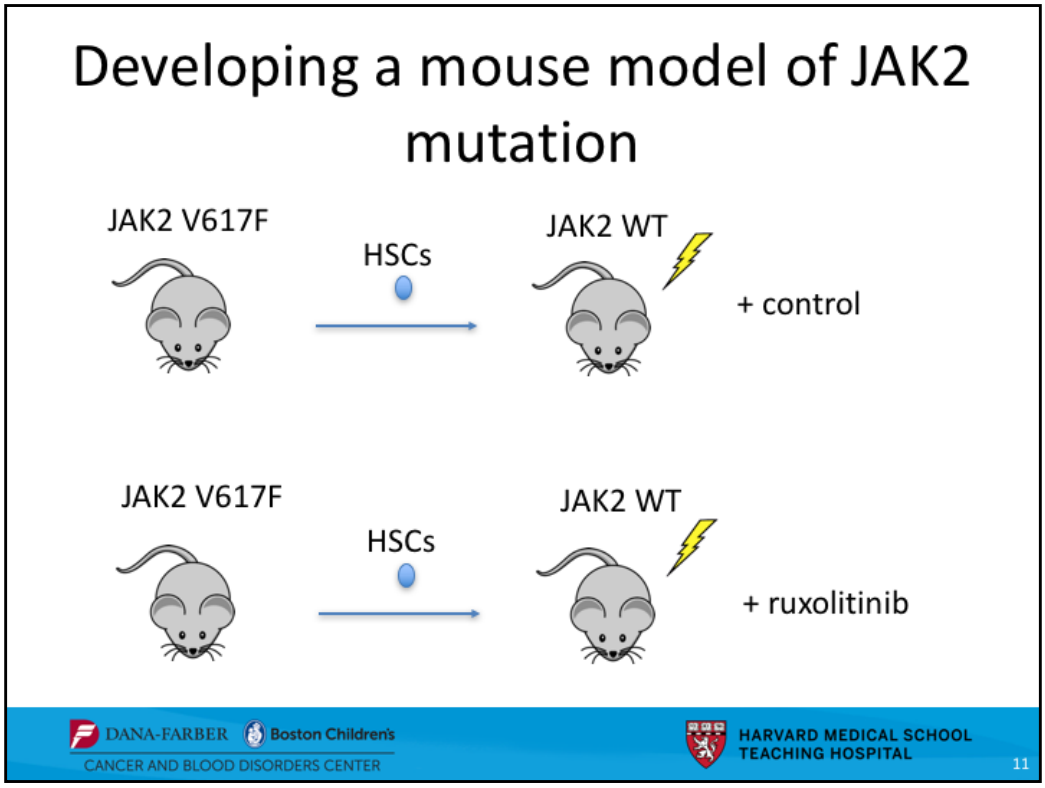
As you can see above, there is an increase in green staining in the heterozygous mice, indicating an increase in H3cit. This suggests that the thrombi indeed involve NETosis.



Additionally, the authors isolated neutrophils from the peripheral blood of the mice, and subjected them to stimulation with ionomycin. This led to two important results.

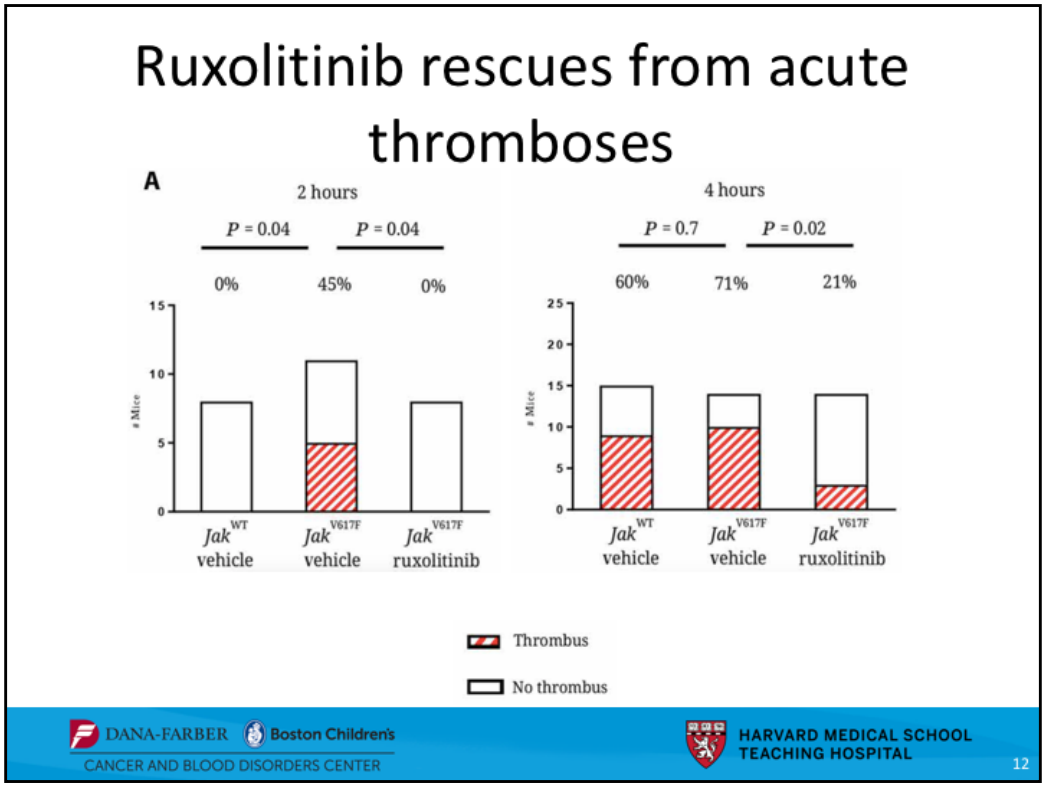
First, they found that while Ionomycin did increase the NETosis in both wild-type and Jak2 mutant mice, NETosis increased to a greater degree in Jak2 mutant mice. This was evaluated both visually (by looking for morphologic changes consistent with NETosis), and by staining for H3cit.

On the right, you can see representative immunofluorescence images that reflect the increase in H3cit staining particularly in Jak2 heterozygous mice.



Next, the authors wondered whether Jak2 gain-of-function mutation could confer an increase in propensity to form acute thromboses.

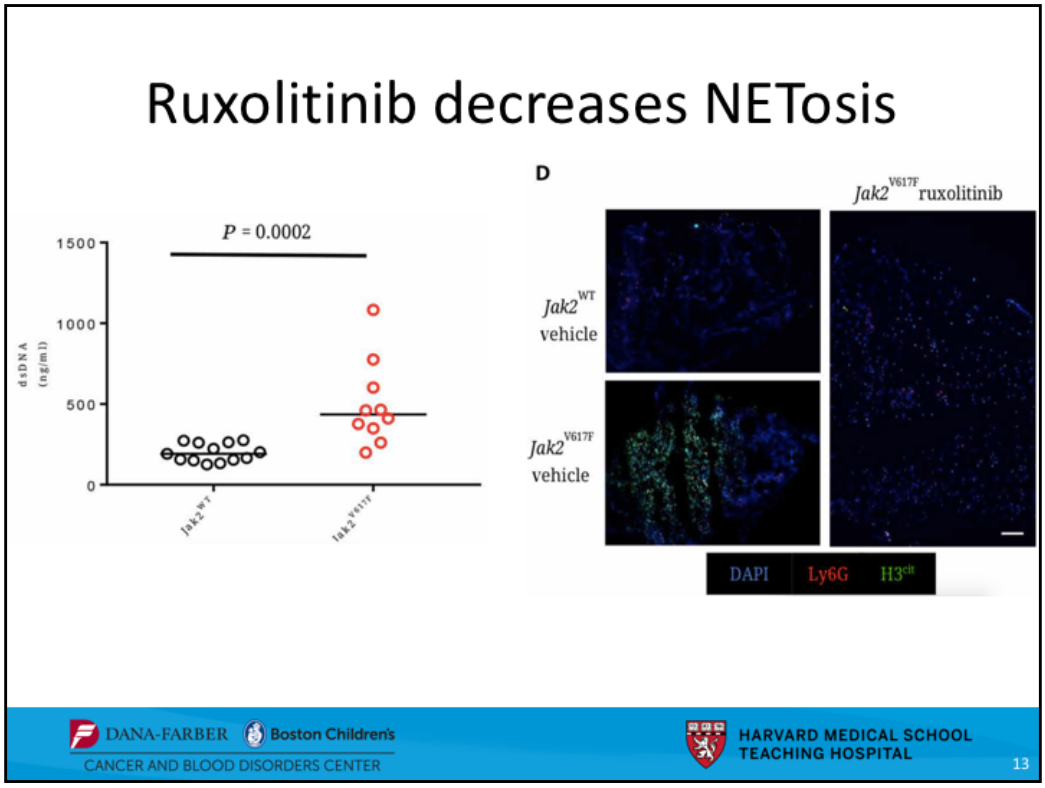
To do this, they isolated c-Kit positive cells from Jak2 mutant mice, then transplanted them into lethally irradiated Jak2 WT recipient mice. They found that these mice then subsequently developed polycythemia vera-like symptoms as expected. Afterward, the mice were treated with ruxolitinib or control.



To cause acute thromboses, the authors partially stenosed their inferior vena cavas. This has been shown to cause a NET-dependent thrombosis in prior literature.

After 2 hours, they found that only the mice with Jak2 gain-of-function neutrophils had any thromboses. However, by 4 hours, all groups had mice that developed thromboses. Interestingly, at the 4-hour mark there was no difference between the Jak2 wild-type and Jak2 gain of function in terms of number of thromboses. However, in the group that was treated with ruxolitinib, there was a significant decrease in the number of mice that developed thrombus.

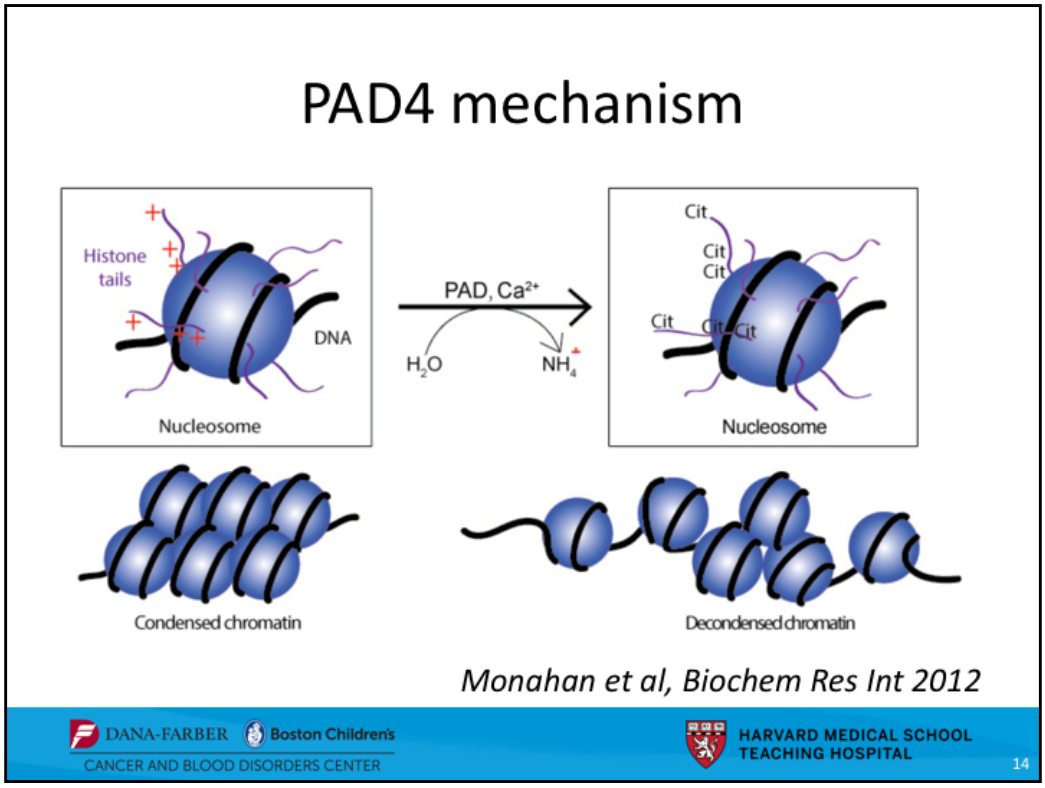
They did not report doing this experiment for longer than 4 hours.



Plasma double-stranded DNA is a good marker for NET activity since NETs are composed of extravasated nuclear material. They found that in the plasma of Jak2 mutant mice, there was an increase in dsDNA, indicating increased NET activity.

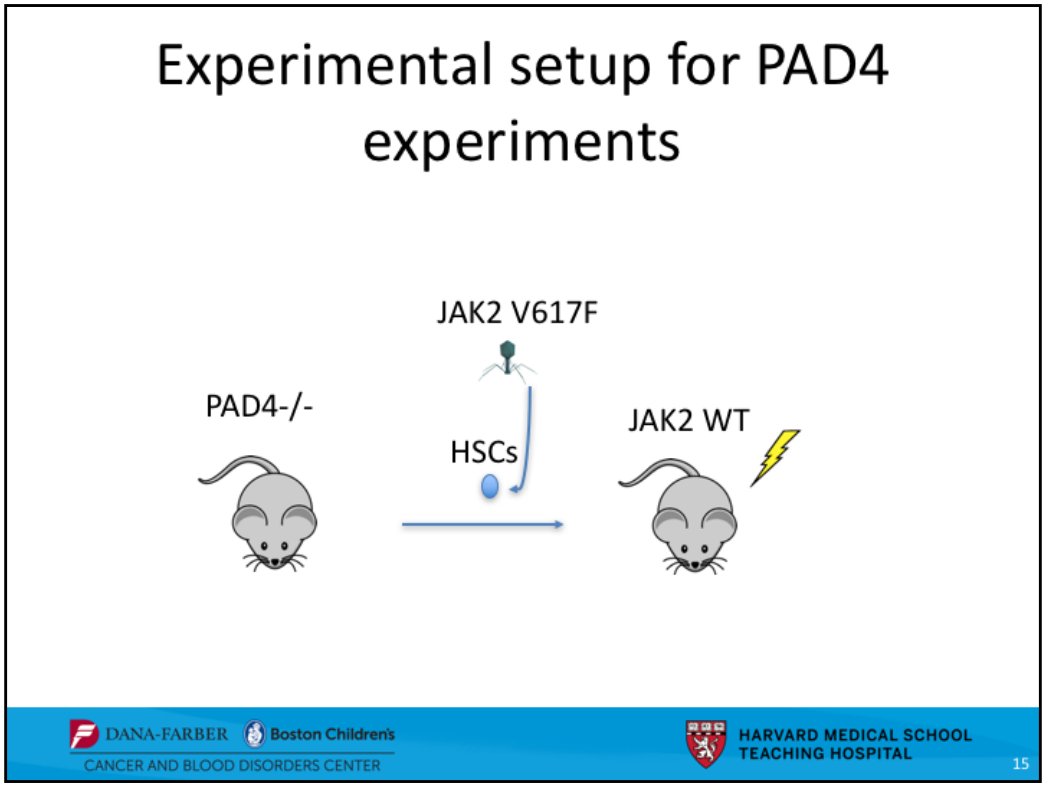
They took the thrombi from the previous experiment (where they had stenosed the IVCs of the mice), and stained the thrombi. In the thrombi from the mice with Jak2 gain-of-function neutrophils, there was a marked increase in citrullinated H3, which I previously mentioned was a marker for NETosis. In the thrombi from the mice with Jak2 gain-of-function AND treated with ruxolitinib, the staining for citrullinated H3 has decreased, suggesting that ruxolitinib has indeed yielded a decrease in NET formation.

In an experiment that is not shown here, they saw that there was no significant difference in HCT, PLT, and ANC between (Jak2 mutant + vehicle) and (Jak2 mutant + ruxolitinib).

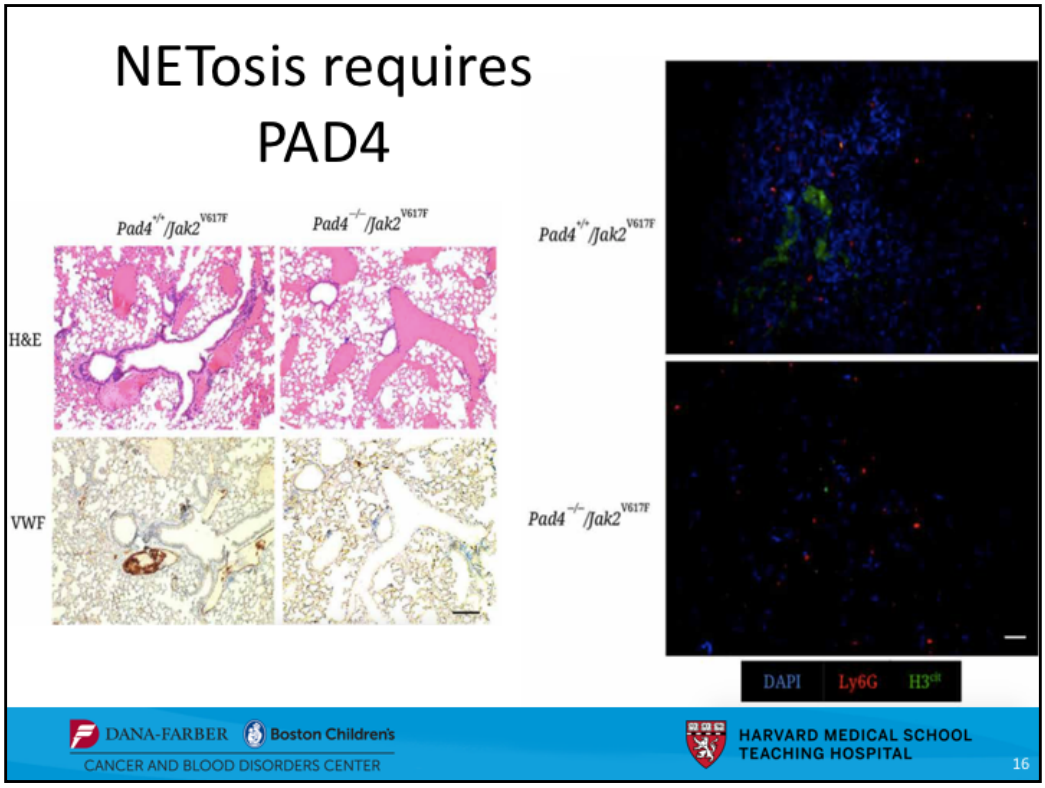


One known mechanism of NET formation involves a mediator called PAD4, or peptidyl arginine deiminase. This enzyme is known to lead to the decondensation of chromatin, through the citrullination of histones. H3 is one of these histones that was previously used as a marker for NETosis.

The authors wondered whether NETosis in their experiments were dependent on this PAD4 pathway.

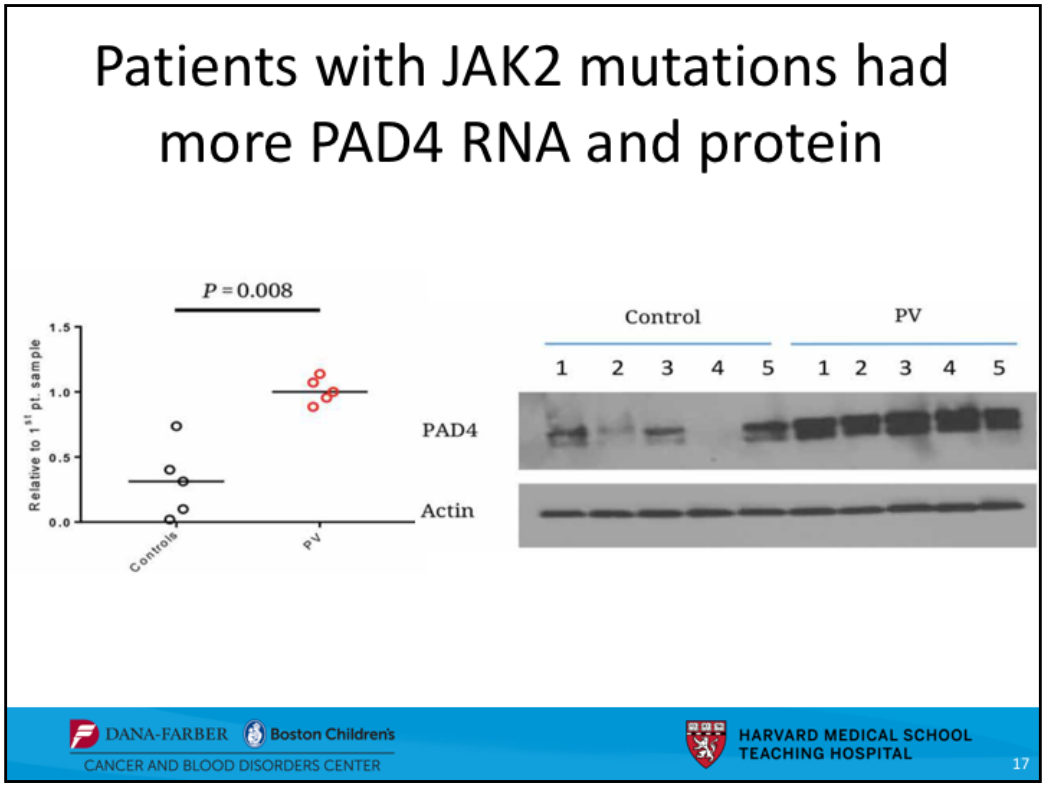


To do this, they took c-Kit positive bone marrow cells from PAD4-/- mice, transduced them with a retrovirus containing the gene for the Jak2 gain-of-function mutation, and transplanted these cells into lethally irradiated Jak2 WT recipients. They also did this with PAD4+/+ mice. Both groups subsequently developed polycythemia vera-like symptoms as expected.



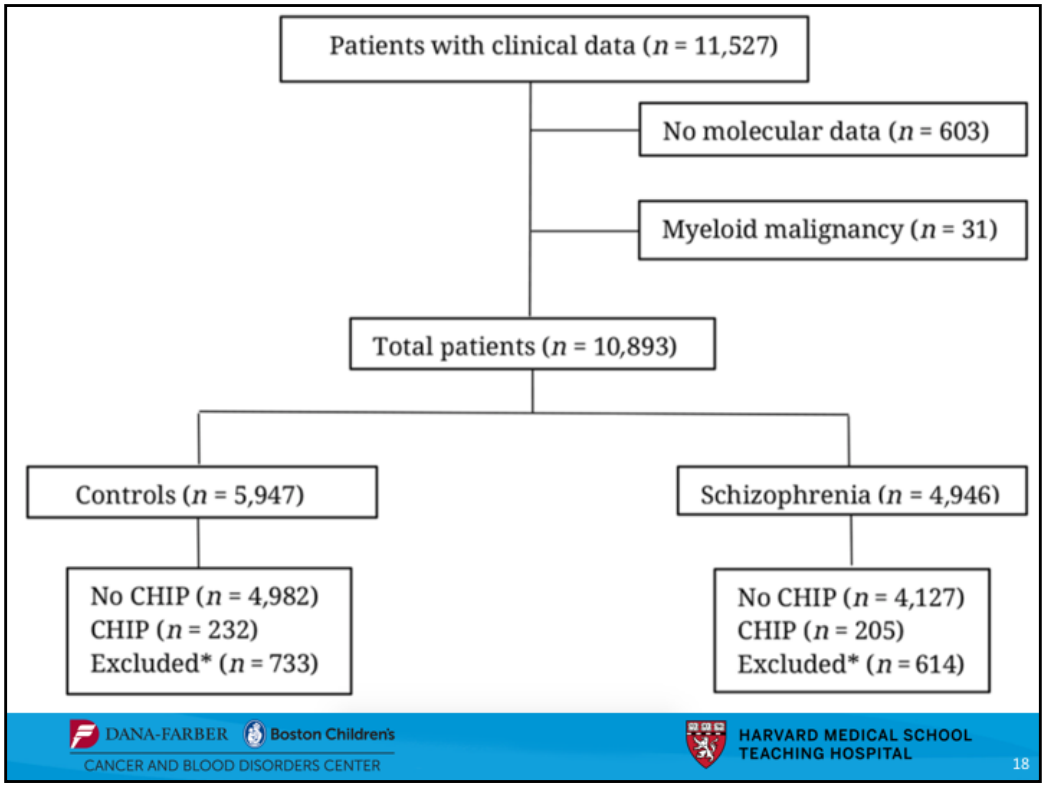
The PAD4-/- mice did not form any thrombi. However, the PAD4+/+ mice did. In these two images you can see the absence of thrombi in the PAD4-/- lung tissue. There was also minimal citrullinated H3 staining in the PAD4-/- group.

These results suggested that NETosis in the Jak2 gain-of-function mutation requires PAD4.



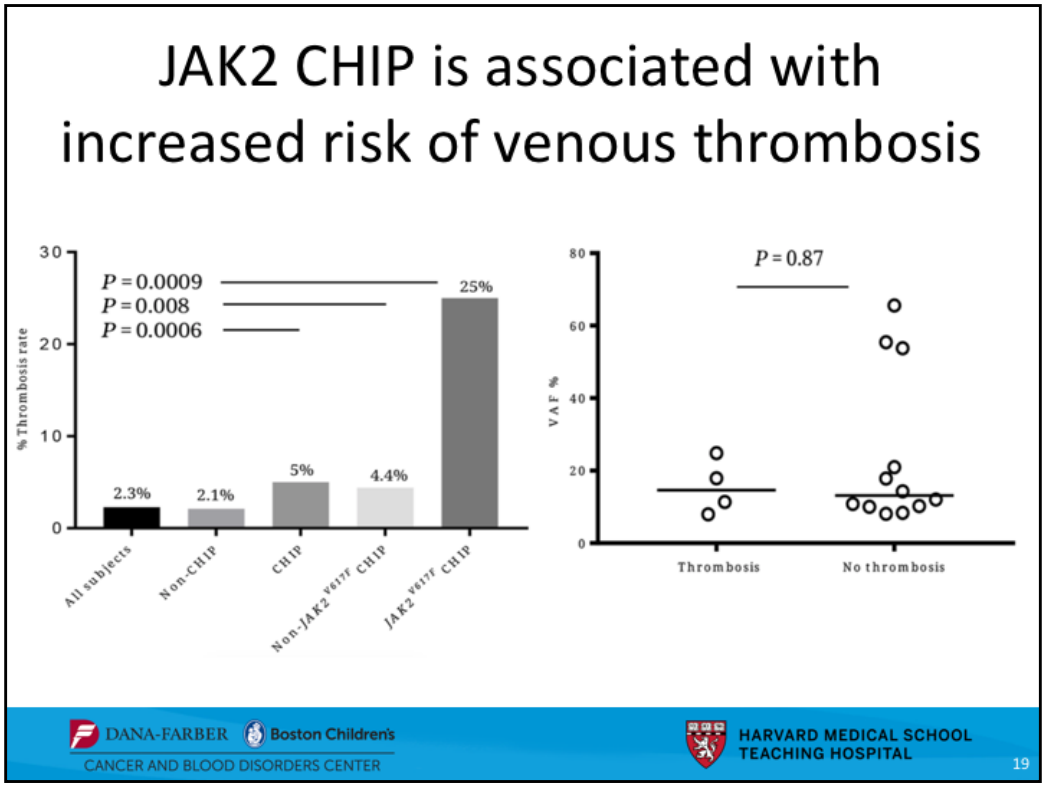
To follow up on this finding, the authors wondered whether there was any data on the correlation between this particular Jak2 gain-of-function mutation and PAD4 in human patients. They analyzed published gene expression profiling data from neutrophils derived from patients with myeloproliferative patients and healthy controls, and found that neutrophils from patients with homozygous Jak2 mutations had 2.4-fold higher PAD4 RNA expression.

They also found that neutrophils taken from Jak2 mutant polycythemia vera patients expressed more PAD4 protein.



In the next part of their paper, the authors looked into otherwise healthy people who may carry clonal JAK2 mutations. This is known as Clonal Hematopoesis of Indeterminate Potential, or CHIP. The main question was whether CHIP with JAK2 mutations might be predisposed to venous thrombosis.

To do this, they accessed the clinical data and exome-sequencing data of a large collection of patients from a recent case-control study evaluating incidence of venous thrombosis in the schizophrenic population compared to healthy controls. They excluded people with myeloid malignancies and ended up with over 10,000 patients.



They found that there was a marked association between JAK2 CHIP and venous thrombotic events such as DVT and PE. If you look at the left, having CHIP causes a significant increase in the risk for venous thrombus formation. Having specifically JAK2 gain-of-function CHIP caused an enormous increase in the risk.

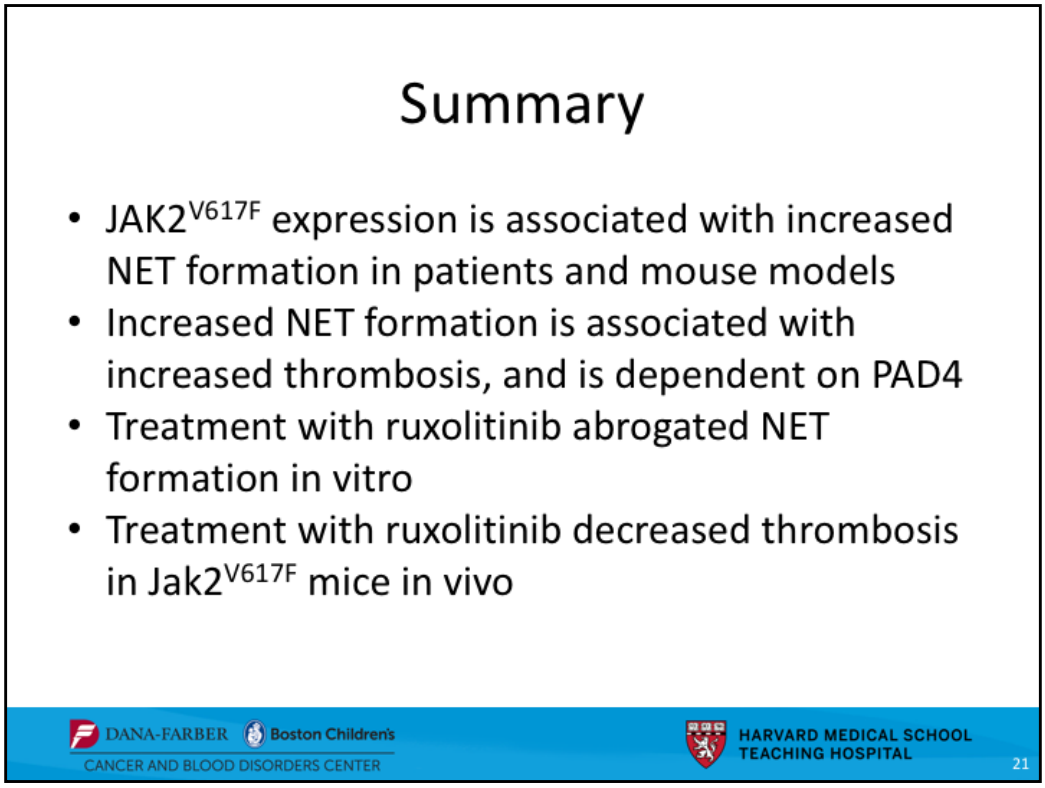
They also looked into whether thrombosis formation was associated with the variant allele frequency of the CHIP. There did not appear to be any association.

Table 1. Comparison of incidence of venous thrombosis between groups according to the presence of CHIP and *JAK2*^{V617F} mutation.

Analysis group*†	Control *P* value‡	Schizophrenia *P* value‡	All *P* value‡
Non-CHIP versus CHIP	0.0006 (0.003)	0.11	0.0004 (0.002)
Non-CHIP versus non-*JAK2*^{V617F} CHIP	0.008 (0.04)	0.57	0.025 (0.125)
Non-CHIP versus *JAK2*^{V617F} CHIP	0.0009 (0.0045)	0.12	0.0003 (0.0015)

Here's a table that summarizes the previous data. Interestingly, when they only looked at the schizophrenia group, there was no statistically significant difference between any of these comparisons. They attributed this to the fact that the study found a higher incidence of venous thrombosis in patients with schizophrenia which may have masked the effect of the CHIP, though I suspect that looking at the sample sizes of the group of schizophrenics it may be slightly underpowered as well.

I would have liked them to compare between non-JAK2 CHIP and JAK2 CHIP here to really emphasize the impact that JAK2 has, but they did not report the p-value of this comparison.



In summary, there are 4 main takeaways from this paper that I think are valuable.

1) The gain-of-function mutation in JAK2 that they investigated is associated with increased INDUCIBLE NETosis in both patients and mouse models.
2) Increased NETosis is associated with increased thrombosis and is dependent on PAD4, in the context of this specific mutation.
3) Treatment with ruxolitinib abrogated NET formation in vitro
4) Treatment with ruxolitinib decreased thrombosis in JAK2 gain-of-function in vivo models

In terms of whether this is directly applicable to clinical care at the moment, I think that there are several questions that remain unanswered.

1. The authors only assessed one particular JAK mutation and it is difficult to say this is applicable to other genetic causes of myeloproliferative neoplasms, though this is a good start.

2. They used ionomycin and PMA to induce NETosis, which may not be representative of what happens physiologically.

3. The models that they used were heterozygous for JAK2 whereas in patients the clonal burden is unlikely to be this high.

4. PEs in patients are embolic from other sites, and could very well be tumor emboli as well. It is hard to directly compare PEs with pulmonary thrombi in these experiments.

5. IVC stenosis is shown to be NET-dependent, but IVC occlusion is known to be NET-independent. This suggests that acute thrombus formation is tricky to model, and there are potentially many more explanations and contributors for thrombus formation than NETosis that could explain hypercoagulability in patients with MPN.

However, given that patients treated with chemotherapy for cancer get clonal MPN-like hematopoiesis, I think this paper may be more broadly applicable than it first appears.