Good afternoon. It’s a pleasure to be back in Minnesota. This is sort of home stomping grounds for me. I spent my summers as a youth, about 90 miles west of here in sort of an idyllic setting, running around the meadows around the lake and fishing and all those good things. So, it’s good to be back in Minnesota.

Title: Goals

- Review the history of protein C/APC
- Discuss the effect of therapy with regulatory proteins on severe sepsis
- Discuss non-anticoagulant activities of APC

Today we’re going on a trip, little bit down memory lane, perhaps appropriate that we’re sitting here in a science museum and next to the Mississippi River, which goes on a long journey from here all the way down to New Orleans.

I’d like to spend some time going through the history of the protein C system to give you background perspective on our knowledge about how the system works. I’m then going to switch topics a little bit and start talking about sepsis.

And you may wonder why the jump in midstream, so to speak, of topics but that will become readily apparent, and then we’ll close today’s journey with a talk about the non-anticoagulants of APC. Non-anticoagulant activities of APC.

Because it turns out that this molecule, activated protein C, is a very fascinating enzyme and it does a wide variety of things, as well as being a major controller of the coagulation cascade.
Now, the story begins 100 years ago. How many of you realize that this is really the centennial of modern hemostasis? Modern hemostasis started in 1904 when Morowitz published his findings, his thesis, on the theory of coagulation.

And there were several key observations that underpin our understanding coagulation to this day. First of all that in the healthy subject walking around, coagulation is not activated. Second, tissue damage initiates a coagulation process. This tissue is thromboplastin that we still used for our PTs.

Third, that it was a self-limited process. And he called that ability to inhibit the thrombin being generated by the coagulation cascade, the antithrombin. So he recognized that there was, it was an on-demand system, that tissue injury led to the generation of thrombin, and that there was an intrinsic mechanism to turn off the system. And that’s the basic coagulation system to this day.

Now, he described this principle of an antithrombin, the inhibition of thrombin, and over the next several decades, a lot of work went into describing these antithrombins, and we’ve had antithrombin I, II, III, IV, V, and VI, named.
I was going to give you a quiz and see how many of you could identify the various antithrombins here. We now know that what was originally called antithrombin I is really fibrin and the ability of fibrin to absorb thrombin and so remove it from solution.

Antithrombin II was the heparin cofactor in plasma. Antithrombin III was ascribed to the slowly progressive inhibitor activity present in plasma, now turns out that these are the same protein II and III. The progressive inhibitor is antithrombin in the absence of heparin, and the heparin cofactor is antithrombin as well.

This was antithrombin IV, it doesn’t exist. It was an experiment gone wrong. Antithrombin V was an anticoagulant found in plasma from patients with rheumatoid arthritis. Anybody want to guess what antithrombin V is? Probably not rheumatoid factor, could be, could be a lupus anticoagulant.

Again probably not a physiologic agent. And then, antithrombin VI is an anticoagulant activity of partially lysed fibrinogen. So of the antithrombin I, II, III, IV, V, and VI, really the only ones that correspond to the true antithrombin-specific proteins are II and III and that’s the same protein.

So antithrombin III is antithrombin II, which is heparin cofactor I. Confused? Yeah, so is everybody. And that’s one of the reasons why the nomenclature, people have recommended that we actually just talk about antithrombin rather than antithrombin III.

You’ll still hear antithrombin III referred to frequently but the appropriate term really now is antithrombin. If you slipped and said antithrombin II, that would also be correct, you may want to go back to your schooling days and get your teacher to change your grade for the wrong answer. Perfectly right.
There was another thing that happened, actually not too far from here. There was a disease in cattle called sweet clover disease. And that was a disease caused by, yeah, what was in the clover? Warfarin, coumarin. And with that really opened up something that was a fundamental different way of controlling coagulation.

The Morowitz theory speculated on something that would inhibit thrombin, the activity of thrombin. The coumarins opened up the possibility that you could inhibit the generation of thrombin. So we now have another class of anticoagulation, the inhibition of thrombin formation.

Then in the 1960s, early 1960s, Seegers and his group described an inhibitor of coagulation that was identified in prothrombin complex preparations. Now, prothrombin complex preparations, we now know as the, what kind of proteins? Vitamin K dependent proteins.

And but they really at this point didn’t know what vitamin K did to these proteins. And it was still I think yet to be discovered. And what they found was that this inhibitor was formed by incubating small amounts of thrombin with this prothrombin complex.

The inhibitor was found to suppress thrombin formation by the prothrombinase complex Xa, Va, phospholipid, calcium, and that this inhibitor activity was unstable in plasma. Its activity deteriorated over time. And they called this autoprotrombin II-A.
Now, does anybody know what this A stands for? Anticoagulant, little bit of logic in there. So autoprothrombin II-A, the Seegers group had their whole own nomenclature for the coagulation cascade. The good thing it was not adopted by the Congress in Rome.

But autoprothrombin II, this is autoprothrombin II anticoagulant indicating that you had an inhibitor of thrombin formation in this complex.

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**Title: Identification of protein C**

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**Identification of Protein C**  
(Stenflo, 1976)

- Isolated a novel vitamin K dependent protein from plasma.
- Demonstrated that it was distinct from factors II, VII, IX and X.
- In contrast to prothrombin, it was capable of binding phospholipid in the absence of Ca²⁺.
- Speculated that it might be a precursor of an active enzyme.
- Designated protein C due to its position on chromatography.

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Then in 1976, Stenflo and his colleagues isolated a novel vitamin K dependent protein from plasma. By this time we knew a lot more what vitamin K did to the various proteins. And they demonstrated that it was a distinct protein from II, VII, IX, and X. The other vitamin K dependent proteins that were known at that time.

One of the interesting properties, and we will come back to that later in this talk, is adding contrast to prothrombin. This protein was capable of binding the phospholipid in the absence of calcium. So even in the absence of calcium, bind all the calcium, this protein could still associate with phospholipid membranes.

They speculated that there might be a precursor of an active enzyme. They had absolutely no idea what it did. It was called protein C because it was the third protein to elute off their column. Protein A was one of the known factors, B one of the known factors, and then C an unknown factor. And this name has stuck.

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**Title: Delineation of the protein C anticoagulant system - It was recognized that protein C was the same...**
Well, Seegers and his group looked at this and said, could it be? And so they did some experiments to look at this newly identified protein C and found that protein C was actually the protein that they had described almost 15 years earlier as autoprothrombin II-A.

Well, this gave people a great step forward in terms of understanding protein C, because in those early studies, remember, they had found that thrombin could generate this activity, this autoprothrombin II-A activity, so then it became clear very quickly that thrombin could convert protein C to APC.

It was also recognized that autoprothrombin II-A had anticoagulant activities, and so it was very quickly then found that the mechanism of that anticoagulant activity was through the degradation of factors Va and VIIIa by APC.

What is fascinating about this system is the selectivity. APC will but only very slowly degrade circulating V and circulating VIII. But it is very effective at degrading Va and VIIIa. In other words, the conversion of V to Va, the conversion of VIII to VIIIa make those substrates for APC.
And then, amidst all this, protein S was isolated from plasma and it was found to be a cofactor for APC. Now where does the S in protein S come from? Seattle. It’s nice that we follow uniform conventions in naming, isn’t it?

And so the last of these proteins to be isolated from plasma was called or the last so far that we know was protein Z and that was tongue-in-cheek. Because there, I think we’re an optimist thinking that this is going to be the last of the vitamin K proteins isolated from plasma. So, S is for Seattle.

Now, for a long time we have known that the protein C system is intimately involved with the vessel wall. There are significant interactions with the vessel wall.

And the first studies that really relate to this go back to the early 1970s when Wetmore and Gurewich injected small amounts of thrombin into experimental animals and what they found was that the aPTT was significantly prolonged. Now you might think that well, they injected thrombin, they induced DIC. They defibrinated the animals.

Well, they looked at the fibrinogen levels, they were normal. They looked at the levels of split products, they were normal. There was no consumptive coagulopathy. But yet there was this anticoagulant activity that was present that again slowly disappeared over several hours. And they really did not know what that was due to.
Esmon and Owen working on APC recognized that the conversion of protein C to APC by thrombin was a relatively inefficient process when you tried to do it in the test tube.

So they seized on this previous work and developed an experimental model where they could add a small amount of thrombin to blood in the coronary arteries that was then perfused through the microvasculature of the heart and collected blood on the cardiac sinus end, on the outflow end.

So they added some thrombin to blood going through this microvasculature, collected blood coming out and what they found was that you formed a great deal of APC during that transit. So there was something in the microvasculature that was facilitating conversion of protein C to APC.

That something was thrombomodulin. And, thrombomodulin is a critical component of the system that does not normally circulate in any, to any extent but is rather found on the surface of endothelial cells.

And then more recently, an additional endothelial cell protein has been described. It was described as a receptor, which is basically a binding protein for protein C or APC on endothelial cells. And thus it was termed endothelial protein C receptor or EPCR.

Now it turns out that this is a bit of a misnomer because EPCR is present on other cells. It’s present on some of the circulating white cells and it’s also present in the brain and other sites.
So with that background let’s talk about a brief overview of how this system is thought to work. When you generate thrombin, it will bind to the endothelial cell surface to thrombomodulin on that.

One of the critical concepts here is that when thrombin binds to thrombomodulin, it undergoes a conformational change. Thrombin-thrombomodulin is no longer a procoagulant enzyme. It is an anticoagulant enzyme now. So just binding thrombin to thrombomodulin is in itself an anticoagulant property.

On that surface, the thrombin thrombomodulin complex will convert protein C to activated protein C. Endothelial protein C receptor facilitates this process such that if you block the binding of protein C to EPCR, you significantly decrease the amount of APC that you generate.

Now just how EPCR facilitates this conversion is still not entirely understood. But what is clear is that it does play a catalyst role, perhaps by presenting protein C to the thrombin thrombomodulin complex, to facilitate generation of APC. So the formation and the generation of APC is dependent on having thrombin around, protein C, thrombomodulin, and EPCR.

Now the activated protein C will interact with Va or VIIIa, could be either protein here, and degrade these proteins proteolytically slice them. Protein S serves as a cofactor helping to accelerate the grade of degradation of factor V and factor VIII.
So you end up with the degraded molecule that loses its procoagulant activity. This is proteolytic degradation. There is, this is a one-way street. Once you degrade that Va or VIIIa, it’s gone. You cannot have that reassociate. Now it turns out that the cleavage of factor V and factor VIII is not random.

There are very specific amino acid bonds in Va and VIIIa that are cleaved by APC. And one of the key bonds in factor Va that is cleaved is an arg-serine bond at arginine 506, and we’ll come back to that in a second.

**Title: Clinical significance of the protein C system - Heterozygous deficiency of protein C is...**

So we have a system that we know has anticoagulant activity. Does it have any clinical meaning? Is it an important system? And the answer to that obviously is yes. I probably wouldn’t be here talking about it if it didn’t.

But the first evidence for this came with the description of a heterozygous deficiency of protein C and its association with an increased risk of thrombosis, thrombophilia if you will, first described in 1981, five years after the initial description of protein C.

This came about a little bit by accident because when they were initially looking for the relationship between protein C deficiency and thrombosis, the laboratory phenotype they were looking for was a total deficiency of protein C.

And so when they first analyzed the samples and saw that the levels were 50%, they didn’t know whether they really had anything. It wasn’t until they matched the 50% level with the clinical history that the light went on and said oh, heterozygous deficiency causes thrombosis.
It wasn’t too long after that when they actually did find homozygous protein C deficiency. And homozygous protein C deficiency is associated with neonatal purpura fulminans. And until this time, this was a universally fatal disease. I don’t know if you have seen pictures of infants born with this, but it is an absolutely devastating disease.

Recognizing the pathophysiology of what was going on, they were able to intervene, and successfully in some of these infants so that they could actually survive.

The next was description of protein S deficiency. And again heterozygous deficiency was associated with thrombophilia. The clinical manifestations indistinguishable from heterozygous protein C deficiency. And then shortly after that, homozygous protein S deficiency was described and there the clinical manifestations are identical to homozygous protein C deficiency.
The final part for this story was the description of this curious resistance to activated protein C that Dr. Dahlback made in 1993 and the race was on to understand what the pathophysiology of this APC resistance was. Dahlback was able to show a reproducible resistance to the enzymatic or the anticoagulant activity of protein C and that it was related to thrombosis.

But he didn’t know why. Bertina’s group was the first to demonstrate that the cause of this was actually a mutation in factor V. Protein C was perfectly fine but there was a mutation in factor V at that arginine 506. So that APC could no longer cleave the Va. And thus the Va remains intact. You can continue to generate thrombin and you have excess clotting.

Now the interesting thing about factor V Leiden, and I think we heard that earlier this morning, is that it is present in about 4% to 6% of the Caucasian population. That’s extremely high for mutation that causes problems like venous thrombosis.

**Title: Sepsis**

So that’s the protein C system. Let’s switch gears here for a moment and talk about sepsis, and then we will integrate what we know about the APC system into this. Sepsis is any systemic inflammatory response that occurs in the setting of infection. It can be bacterial, viral, fungal.

It can result in multiple organ dysfunction, shock, and death. The combination of the systemic inflammatory response and organ failure is what is referred to as severe sepsis. Now, what do you think the mortality rate is for severe sepsis? Any guesses? Well, high, oh you are a confident group, I can tell.
The mortality rate still, despite best medical support is 30% to 50%. That’s high. That’s high. Though it occurs more frequently in people who are over the age of 55-60, it can occur at any age, and it would surprise me if there was not someone in this room who had a family member or knew of somebody who died of this disease.

It is actually a fairly common cause of death. The pathophysiology is really related to the activation of both pro-inflammatory and anti-inflammatory networks. Now that’s a fancy way of saying it isn’t the infection that kills you, it’s the body’s response to the infection that kills you.

Title: Sepsis cascade

![Sepsis Cascade Diagram](image)

And what’s thought to happen is that there is an activation event in the case of infection, this would be an infection. This leads to some initial cytokine activation, which will lead to further recruitment and amplification, something I call a cytokine surge, and that then has a variety of downstream effects and a variety of functions and tissues.

Title: Sepsis cascade - Downstream effects of cytokine activation

![Sepsis Cascade - Downstream effects](image)

It includes continuing release of inflammatory mediators, altered cell functions, the endothelial cells go haywire, the leukocytes go haywire. There are effects on the liver, heart, on its contractility. The lung is commonly affected.

You can get widespread apoptosis, that’s cell death, both in organs and in endothelial cells, almost invariably there is activation of the coagulation system associated with this. And ultimately you get activation of anti-inflammatory pathways and so the patient who has severe sepsis may be more susceptible to getting a second infection because their defenses are now being knocked down.
The general hypothesis that is now I think gaining pretty much uniform acceptance, is that the clinical manifestations are mediated by the host response rather than the infectious agent.

There are notable exceptions to this where toxins derived from bacteria cause specific effects. But in general, sepsis, the problem is the host response to what’s going on.

Title: Rationale for the use of anti-inflammatory agents in sepsis

There have been a large number of clinical trials aimed at finding effective treatments for this. I mean if you go in and say you’ve a 50%-50% chance of dying, think you’d be looking for something that could change that risk. Many of these therapies, experimental therapies have been directed at trying to control the initial inflammatory response.
I’m not going to spend a lot of time on this slide, what this just lists are some of the many clinical trials. There have been over 30 major clinical trials looking at anti-inflammatory agents trying to alter the course of sepsis. They all failed. None of them worked.

Title: Sepsis cascade - Downstream effects of cytokine activation - Activation of coagulation

That caused people to scratch their head and say, well is there anything we can do? So one of the things people looked at, well what are the other downstream effects of cytokine activation? Activation of the coagulation cascade. Is there anything we can do in terms of controlling the coagulation cascade that might be beneficial in these patients?
And there was actually some rationale for this. It had been recognized for a long time that sepsis was associated with an increased risk of DIC. More recent data indicated that the coagulation cascade was activated early in the course of sepsis, and we’ll see a slide indicating just how early.

And finally, it was hypothesized that microvascular thrombosis, sort of a DIC-type process, might actually be contributing to the tissue injury associated with severe sepsis, the organ failure, and subsequent death.

Title: Effect of septic shock on antithrombin and protein C levels

This was an interesting experiment conducted by Mesters and his colleagues. What they did was to take a group of patients with leukemia who were to undergo ablative chemotherapy. Now as you know, what happens in those patients is their white count drops way down and they become quite susceptible to infection.

And what their plan was, was to take blood samples before they started chemotherapy, the nadir of the white count as soon as they spiked a fever and then at periods, defined periods after they spiked a fever. So, here we have the antithrombin and protein C antigen in these patients.

Here’s pre-chemotherapy. Here’s at the nadir of their white count. Here’s at the onset of fever. The first time, first manifestation, onset of fever, protein C and antithrombin are reduced down to about 40%. Now, contrast to that in the patients who actually had shock, the shock itself, the drop in blood pressure, did not become manifest here for a number of 12 hours.
So you had dropped your protein C and antithrombin even before you dropped your blood pressure. So the changes that occur in coagulation occur very early in this process of the host response.

**Title: Activation of coagulation in sepsis**

![Activation of Coagulation in Sepsis](image)

Now how do you activate the coagulation cascade in sepsis? It really begins with the cytokines, which interact with both endothelial cells and mononuclear cells to release tissue factor or expose tissue factor to blood. So this is a tissue factor-driven process. The exposure of tissue factor will then lead to generation of VIIa and the whole cascade. The generation of thrombin will lead to the fibrin formation. Remember that thrombin has a variety of other activities including platelet activation and it is a very pro-inflammatory protein.

It activates endothelial cells to set up if you will, a vicious cycle of activation. So generation of thrombin was thought to be a key component in accelerating this cascade of events that led to organ failure and bad outcome.

**Title: Regulation of TF pathway of coagulation**

![Regulation of the TF Pathway of Coagulation](image)

There are three major endogenous pathways involved in controlling thrombin generation. Antithrombin, activated protein C, and tissue factor pathway inhibitor. Antithrombin works by inhibiting thrombin and Xa principally, activated protein C as we heard earlier, inhibits Va and VIIIa, and tissue factor pathway inhibitor inhibits VIIa and tissue factor, the initiation pathway.

It’s not by accident that there have been development programs for each of these proteins for use in sepsis and in fact all three proteins have been tested in large phase III trials for the treatment of sepsis, trying to control thrombin generation.
The first one to undergo a phase III trial was antithrombin. There was actually a fairly strong rationale for this. Antithrombin, as we could see from that Mesters study is frequently decreased in sepsis, and the degree of decrease is predictive of mortality. The lower your antithrombin, the higher your risk of mortality. The decrease is probably due to a combination of consumption, decreased synthesis by the liver, and perhaps destruction by enzymes such as elastase released from the responding neutrophils. Animal models of sepsis would suggest that replacement with antithrombin could reduce the mortality. So in animal models it looked like it was a very successful agent. And there was some thought that administration of antithrombin could alter prostacyclin production, and an increase in prostacyclin production was thought to be protective of the vasculature, if you will.

It went through a number of phase II trials, and in a meta-analysis of these phase II trials, it looked like antithrombin treatment was associated with about a 20% reduction in mortality. So it looked very promising. I would point out, however, though that these estimates including in the meta-analysis all cross zero. So it was not clear-cut that it was better but there was a suggestion. So based on this, a large phase III trial was undertaken.
This was known as the Kyber-Sept study. It was multinational double-blind phase-III type trial, primary objective to demonstrate a reduction in mortality at 28 days, among patients treated with antithrombin. The regimen was to give them a large bolus over 30 minutes and then 6,000 units a day by continuous IV infusion for 4 days.

They enrolled over 2,300 patients in the trial. They were evenly distributed. The therapy with antithrombin met their objectives. The level of antithrombin increased promptly from the reduced levels of 50% to 60% to around 200% of normal in the patients receiving antithrombin.

However, it had absolutely no effect on outcome. Mortality was the same in the two. As you might expect, there was a mild increase in bleeding, especially in those patients who also received heparin. I wonder why. So it didn’t work, everybody’s going.
Next was the activated protein C rationale, again protein C levels are frequently reduced. In addition, the thrombomodulin concentration on endothelial cells is decreased in sepsis.

One of the changes that occurs in the endothelium in response to the release of cytokines is to decrease expression of thrombomodulin. So not only is your protein C level reduced, but thrombomodulin which you need to activate protein C, is also reduced.

Again animal models suggested that APC could decrease the coagulopathy tissue damage and mortality looked good. And, at this point in our understanding of APC, there was a scattering of data to suggest that APC might have anti-inflammatory properties. Not a lot known about that.

This went into a large phase III trial. It was called the PROWESS trial. Primary objective again was to determine the effect on 28 day all-cause mortality. The regimen was 24 mcg/kg/hour; let’s not worry about how we got to that dose, continuous IV infusion for 96 hours.
This trial was stopped early and was stopped early because of a very strong efficacy signal. There were total of about 1,700 patients who were evaluated. The mortality rate was decreased from around 31% down to about 25%, with a highly significant p value. There was a higher incidence in the rate of serious bleeding in the APC group. Placebo was 2% and the APC group was 3.5%.

Title: Kaplan-Meier survival curves

Here are the survival curves and what you, one of the key things about these survival curves is that they separate very early and then they continue to separate over time.

Now, and that’s actually very interesting because the infusion actually stops here. But the effect continues to be felt as time goes by. Now there were questions as to whether or not this was just a fluke or not. Right on the heels of this there was an open label trial, same dose regimen, same condition, just no placebo.
It wasn’t really ethical to continue to use the placebo and the survival curve in that followup study called ENHANCE was virtually superimposable with the phase III study. So this looked to be a real phenomenon in terms of reducing mortality.

And as you might expect, there were changes in D-dimer F1.2, TAT, that would indicate that APC had anticoagulant activity in this setting. So it was working as we thought it might.
How about TFPI? This was the third agent to go into phase III studies. It inhibits the tissue factor mediated activation coagulation; administration of rTFPI inhibits the activation of coagulation observed following administration of endotoxin to healthy human subjects. How many of you have volunteered for that study?

This is an interesting model of human, the human response, where volunteers are given a small dose of endotoxin. They don’t feel too well for about six to eight hours. But then, it quickly reverses. And what you find in that model are changes in cytokine and coagulation that mimic what you see in sepsis.

So it’s been used to study the initial mechanisms of sepsis in humans. And TFPI shut down the coagulation activation in healthy humans. As an aside, a subsequent study doing, looking at APC in healthy subjects with endotoxin showed that APC had no effect on coagulation activation. Go figure. The administration of rTFPI was effective in animal models of sepsis and DIC.

So looked like things were going well. A phase II trial was done and it looked like there was a trend towards a decrease in 28 day all-cause mortality 20%. But this was a small trial to be able to really say anything. And it looked like there was a more rapid decrease in the thrombin-antithrombin complex, in IL6, so both anticoagulant anti-inflammatory properties.
The phase III trial was called the OPTIMIST Study. They looked at almost 2,000 patients. Their primary group that they wanted to look at were patients who had evidence of a coagulopathy, designated by an INR greater than 1.2 on the PT.

The 28 day all-cause mortality virtually identical. Slight increase in bleeding associated with the administration of rTFPI.
So, here’s a paradox for you. Why did APC work in two trials and antithrombin and tissue factor pathway inhibitor not? Interesting question. During the last several years, it’s become apparent that APC has a variety of other activities besides its ability to inhibit thrombin generation.

**Title: Non-anticoagulant activities of APC**

- Inhibition of NFκB pathway
- Inhibition of cytokine synthesis and release
- Inhibition of leukocyte adhesion
- Inhibition of neutrophil migration
- Inhibition of apoptosis
- Prevention of hypotensive response to endotoxin
- Diminution of pulmonary fibrosis
- Diminution of CNS injury

Many of these have been shown in cell-based models, experimental animal models. Very few have been shown with human data. So a lot of this is, we think that these pathways might be important, might be relevant, here’s a model for this, inhibition of the NFκB pathway.

What is this? This is one of the master switches that turns on the inflammatory response. Endotoxin and tumor necrosis factor are strong signals to turn on the NFκB pathways, which lead to the inflammatory response. And APC can inhibit that pathway, can turn off that pathway if you will.

Associated with that you can inhibit cytokine synthesis and release, you can change the surface of the endothelial cells so that neutrophils, monocytes don’t adhere as well.

One of the changes that occurs in the setting of sepsis is that the endothelial cells get very sticky, making it easy for inflammatory cells to bind and in that setting those turned down white cells are often quite toxic to the surrounding cells.
By inhibiting an adhesion, you will be protecting the endothelial cells. You can inhibit neutrophil migration, you can inhibit apoptosis. Fascinating, and this is actually in the human endotoxin model, you can prevent hypotension that is caused by administration of endotoxin. You can decrease pulmonary fibrosis. In an experimental model where you administer bleomycin to induce pulmonary fibrosis, a single intrapulmonary dose of APC will prevent pulmonary fibrosis in those animals.

How? That’s a fascinating question still. And in animal models of stroke, administration of APC can reduce the injury and in a spinal cord crush model of injury, the protein C system has been shown to decrease the injury there. So there are a variety of these activities but are they involved in sepsis. I mean is this the answer?

Title: Factor V Leiden in severe sepsis

I’m going to try and convince you yes. And some of this comes from that PROWESS trial. As a part of that trial, we tested to see if this, if the patients had factor V Leiden, and the original hypothesis going in is that patients who have factor V Leiden may actually have increased mortality.

Because with factor V Leiden, you would have increased thrombin generation, which would potentiate its effect on sepsis leading to worse outcomes, and the patients may not even respond to the drug because of the factor V Leiden. And so we thought this is what we might find.

Title: Factor V Leiden in the prowess study - FV Leiden

<table>
<thead>
<tr>
<th></th>
<th>FV Leiden</th>
<th>FV Cambridge</th>
<th>FV Hong Kong</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Patients</td>
<td>4.1% (65/1601)</td>
<td>0.13% (2/1582)</td>
<td>0.06% (1/1582)</td>
</tr>
<tr>
<td>Caucasians</td>
<td>4.6% (61/1315)</td>
<td>0.15% (2/1301)</td>
<td>0% (0/1301)</td>
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<td>9.1% (1/11)</td>
<td>0% (0/10)</td>
<td>0% (0/10)</td>
</tr>
<tr>
<td>East/SE Asians</td>
<td>0% (0/18)</td>
<td>0% (0/17)</td>
<td>5.9% (1/17)</td>
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The incidence of factor V Leiden and don’t worry about these two over here, was 4.1% overall, in Caucasians 4.6%. Dead on with what the general population is. Now that’s important because it tells you that people with factor V Leiden are neither more likely or less likely to develop severe sepsis. They get it same rate as everybody else.

**Title: Factor V Leiden in the prowess study - 28-day all cause mortality**

![Factor V Leiden in the PROWESS Study - 28-Day All Cause Mortality](image)

However, when we looked at the mortality, if you looked among patients who were negative for factor V Leiden quote unquote, wild type or normal, the mortality in the placebo group was 31%, treated group 24.7%.

If you looked at V Leiden positive, there were no homozygous, just heterozygous. Mortality rate in the placebo group 15.6%, the APC group 12.1%. Mortality rate was half that in the non-Leiden group, and 27.9% overall versus 13.9%. So factor V Leiden turned out to be a protective factor. What’s going on?

**Title: Factor V Leiden in the prowess study - Baseline characteristics**

![Factor V Leiden in the PROWESS Study - Baseline Characteristics](image)

Was it that these patients weren’t just as sick, didn’t have a coagulopathy or what? So we looked at a variety of the characteristics. The APACHE II score is a clinical index that’s used to judge severity and risk of death in patients in this setting. It was virtually identical between these two groups. The rate of shock was about the same.

Interestingly, the use of vaspressors were significantly lower in the factor V Leiden group. Now remember one of the things I had mentioned is that in that endotoxin model, you could prevent hypotension by administration of APC. So, something going on here. Cardiovascular SOFA really is a reflection of vasopressor use. Overt DIC was identical. Not more likely not less likely.
And indeed, if you looked at the coagulation parameters between V Leiden and the non-V Leiden patients, really no difference.

One thing I would point out here though is look at the PTs in this trial. The PTs were up at mean of about 8.2 to 8.7. The average INR in this trial was about 1.6. Common cause of prolongation of the PT in your hospital setting is sepsis.

This was actually a novel finding, because I had, the PI for the study kept calling saying now why is the PT long? Is that real? Or because they weren’t, clinicians were not used to recognizing this. But patients who have severe sepsis almost universally have a coagulopathy reflected by prolongation of PT, aPTT.

D-dimer, as you might expect was high. Very high, 3.8, antithrombin F1.2 were also elevated, consistent with the increased thrombin generation, but nothing to distinguish the groups. So factor V Leiden, where you would think there might be more thrombin generation, you might expect to find higher levels of TAT and F1.2, didn’t find it.
Protein C, protein S, you can look in your handout. So, next question. Why would patients with factor V Leiden who theoretically ought to have increased thrombin generation have better survival? Well, here we have to go back to the mouse.

Title: Question: Why was the mortality rate lower in patients with factor V Leiden?
And this is a murine model of sepsis, where a dose of E. coli endotoxin is given by intraperitoneal injection and at this dose, 40 mg/kg causes about 50% mortality rate in the wild-type mice. And I think this might be a typo here. Now this model was evaluated with several, what we call, knock-in mice.

These are genetically modified mice. There was, there were mice that had a mutation in thrombomodulin called the thrombomodulin proline or TMPPro mutation. What this mutation does is to significantly reduce the functional activity of thrombomodulin.

If you knock out thrombomodulin in the mouse, it’s a lethal genetic trait. They don’t survive. If you decrease the function, the mice can survive, be born and so forth. So these have, the TMPPro have reduced thrombomodulin function. Then, heterozygous V Leiden mutation was knocked in and a homozygous V Leiden was knocked in, all into the separate population of mice.

Now, what happens when you give endotoxin to these mice? Thrombin-antithrombin is a measure of thrombin formation. So in the factor V Leiden, heterozygous, you give endotoxin compared to the wild-type, there is a significantly greater amount of thrombin formation.

Even higher in the homozygous and also in the thrombomodulin mutated mice, that the amount of thrombin formation goes up. When you look at the amount of activated protein C that’s generated, in the factor V Leiden heterozygous, it actually goes up but no it’s not statistically significant.
In the homozygous V Leiden, the amount of APC goes way up. Whereas, in the thrombomodulin mutated mice, the amount of APC generated goes down. Now, why does the APC concentration go up here? Any thoughts?

More thrombin. More thrombin can bind to the normal thrombomodulin in these mice and generate more APC. Whereas in the thrombomodulin mutated mice, they can’t generate APC and the concentration of APC that you get is greatly reduced.

Title: Effect of FV Leiden and TMPPro on cytokine release following endotoxin

If you look at cytokine release, here’s IL6, IL1 beta, both of these are common cytokines that go up early in sepsis. Compared to the wild-type, the heterozygous factor V Leiden slight decrease but not statistically significant.

Homozygous V Leiden, significant decrease. You look in the thrombomodulin mutated, there’s a significant increase in IL6. Similar pattern for IL1 beta. What’s going on here?

Higher levels of APC in the homozygous, remember in vitro studies would suggest that APC can decrease cytokine release, and this would be in vivo evidence that the increased generation of APC actually fed back to inhibit cytokine production. Whereas if you had increased thrombin production with low levels of APC, you actually enhanced cytokine production.

Title: Effect of FV Leiden on survival in murine endotoxemia

Well, here’s what happened mortality wise. The factor V Leiden heterozygous mice had a much lower mortality. The mortality rate was but half the wild-type. So they were protected, just like in the clinical trial. Interestingly, the homozygous were virtually indistinguishable from the wild-type.
Now what’s not showing on here is the mortality curve for the TMPro, but basically that was 100%. So the thrombomodulin mutation was associated with increased mortality.

We can try and summarize this as follows. In the presence of factor V Leiden, you had increased generation of thrombin and APC; you had decreased generation of cytokines, especially evident in homozygous; you had increased survival of the heterozygous factor V Leiden, but the homozygous factor V Leiden was similar to the wild-type.

In contrast, thrombomodulin mutation increased thrombin but decreased APC, increased generation of cytokines and decreased survival.

And that might be easier put together on this chart here. So, the heterozygous for Leiden increased thrombin but also increased APC, and better survival. Increased factor V Leiden, great increase in thrombin, great increase in APC, same as controls, and then here worse outcome.

And what this really indicates is that the generation of APC was probably critical for improving survival in the factor V Leiden heterozygous mice. And that this was probably due to things other than thrombin generation because thrombin generation is higher in this group of animals.
So it can’t be that the APC levels for the V Leiden somehow results in decreased thrombin generation, so the APC has to be working elsewhere. So the non-anticoagulant properties of APC are what allow survival.

But notice that you can only use that to a certain degree, because when you get to the homozygous with again thrombin formation and very high levels of APC, you start to lose that benefit.

So at some point you cannot compensate for that increased thrombin generation with increased APC. And then in the TMPro mice, you have increased thrombin generation, similar to the Leiden heterozygous mice, but without that increase in APC, the mortality gets very bad.

### Title: FV Leiden survival advantage in sepsis

![Graph showing the relationship between FV Leiden and APC generation](image)

And this really would suggest that activated protein C has significant effects other than its anticoagulant effects. We can try and put this together this way.

That in the presence of factor V Leiden, you get some increased thrombin generation. That will feedback to increased activated protein C, and this increased activated protein C will then be able to modulate the inflammatory response in a beneficial way.

And that could explain the survival benefit that we saw in the factor V Leiden-positive patients in the trial. And indeed the survival advantage in sepsis may well explain why there is such a high rate of factor V Leiden that has been maintained in the population.

Now, let’s go back to the various agents that have been used, anticoagulant agents that have been used in the clinical trials. Antithrombin inhibits thrombin and thrombin formation by inhibiting Xa. What is the effect of inhibiting thrombin going to be on APC generation?
They would drop it in all likelihood. So, the potential is that you would inhibit APC generation and the non-anticoagulant activities of APC. So you may gain a benefit from inhibiting thrombin. But you pay a price because you’re knocking out APC generation, and you end up with no treatment effect.

TIFP inhibits thrombin generation and again potentially inhibits APC generation and its non-anticoagulant effects. Same thing. However, when you replace with APC or when you treat with APC, you inhibit thrombin generation at the same time that you increase or enhance the non-anticoagulant properties, because you’re bypassing the need for endogenous thrombin to generate the APC.

And also in the setting in of sepsis, you’re bypassing the need for the thrombomodulin, which has been decreased on the endothelial cell surfaces.

Now, how does APC accomplish these non-anticoagulant effects? It is thought to be mediated principally through cell signaling. Interaction with cells. And it’s been shown that APC can interact actually with a variety of cells to alter gene and protein expression.

The cell signaling is dependent on the protease activity of APC. In other words, if you modify the active site of APC, chemically modify it so it can’t, has no enzyme activity, it will bind to cells but it will not activate them. You have to have that active enzyme site to be able to activate the cells.
The other key is that this protein EPCR appears to be involved in cell signaling. And what is emerging is a model where APC in the presence of EPCR can interact with a protease-activated receptor 1 or PAR-1 to activate cells.

And the mechanism of activation here is that this enzyme will cleave a peptide in this terminal portion of the molecule, and once it cleaves that, the modified terminal end then will actually fold down and bind to the receptor itself and autoactivate it.

Title: Activated protein C mediated signaling

And that will then lead to cell signaling, and induction of pathways that inhibit apoptosis and inhibit neutrophil migration and so forth.

Title: Activated protein C mediated signaling - Cell signaling

The curious thing, is that this PAR-1 is also the same molecule that thrombin often uses to signal cells. And so when you signal with thrombin through this mechanism, you generate a pro-inflammatory signal, when you signal through the EPCR-APC complex, you generate an anti-inflammatory anti-apoptotic response.
And that’s where we are. How this, how these two pathways are differentiated within the cytoplasm of the cell is an area of active research, and an area to pay attention to. But we will learn more about the biology. It’s clear that these pathways that can be activated may be very important in settings other than sepsis.

And any time where you have signals that are generating apoptosis or cell injury, you may be able through this pathway of signaling through the EPCR-PAR1, to provide a protective signal for the tissues of the body.

And so, we’re opening up a whole new avenue of biology here, if you will, through this protein C system and that is under active exploration and it’s going to allow people to come back and give you talks in 5-10 years that will simply amaze you.

So activated protein C, it truly is more than an anticoagulant. And it’s going to be fun finding out all that it does and how it does it. Thank you. Yes. (Question from audience). A: Excuse me. (Question from audience). A: The question is, is that a product that’s available.

Yes, activated protein C was approved by the FDA, the commercial name for that is Xigris, X I G R I S. How much does it cost? How much is a life worth? Now, a course of therapy in the U.S., because it’s on a weight base, obviously will vary from individual to individual.
I’m an expensive candidate. It’s about $6,000 to $7,000 for a course of therapy. (Question from audience). A: The question is, what kind of laboratory tests do you do to monitor patients on Xigris. The answer is none. But be aware that APC can prolong the aPTT.

Be aware that how much it prolongs the aPTT will depend on how quickly you test that sample after it’s drawn from the patient. It is fairly quickly neutralized by serine protease inhibitors in plasma, so if you let it sit for an hour to two hours on the bench top, you will not see much of an effect.

It can interfere with factor assays if you do those promptly afterwards, because of its ability to prolong the aPTT. So it’s helpful to know if the patient, if you get one of those funny aPTTs and you’re scratching your head what’s going on here, find out if the patient is being treated.

Yes. (Question from audience). A: The answer to, the question has to do with is there a higher mortality rate in other ethnic populations where you find factor V Leiden not present. This study was done predominantly in North America and Europe, and the numbers of non-Caucasian subjects were too small really to do that kind of analysis.

Thank you very much.