THE INTERNATIONAL NORMALIZED RATIO

A KNOWLEDGE BRIEF
VERSION 20190108

MANX Enterprises, Ltd.®
P.O. Box 7323
Huntington, WV 25776-7323
304 521-1980

mark@manxenterprises.com
www.manxenterprises.com

A BIOCHEMICAL, MATHEMATICAL, OPERATIONAL, AND TECHNICAL OVERVIEW

WITH RECOMMENDATIONS FOR ATTAINING OPTIMAL SERVICE TO OUR PATIENTS

HUMAN FOIBLE

IN ORDER TO CHECK THESE THINGS OUT…

YOU HAVE TO CHECK THESE THINGS OUT.

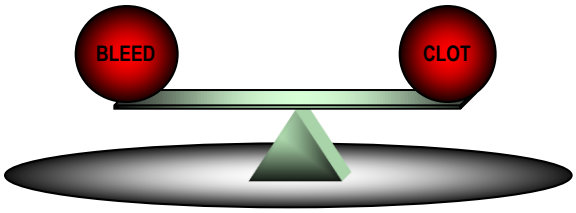
Written on the triage blackboard

The George Washington University Hospital Emergency Room

July 1979
by
Dr. Bruce Van Bockel, M.D. Resident

1 SITUATION

Anticoagulation to prevent patients at risk from thrombosis has been a key therapeutic modality for many decades. Clinicians and laboratorians have been searching for a reliable method of assuring that this effort is effective and safe. This is because the risk for spontaneous hemorrhage is greatly increased as control over the tendency to clot is abated by therapy with anticoagulants. And, the two risks of thrombosis and hemorrhage both threaten the patient's wellbeing and life if not properly balanced.



Over the last thirty years there has been a significant increase in the need to treat numerous thrombogenic diseases and prosthetic devices using long term outpatient anticoagulation. As a result, the number of patients that have experienced an adverse outcome has gone up and this has led to a demand for an accurate and precise method of monitoring this high-risk therapy. Complicating this is the increasing number of older patients who suffer from multiple co-morbid states that further affect their clotting system.

This includes but is not limited to:

- Coronary Artery Disease
- Congestive Heart Failure
- Liver Disease secondary to Hepatitis C, Alcohol, and various drug usage
- Chronic inflammatory processes secondary to Diabetes, Rheumatoid Arthritis, and other processes
- Malignancy with or without chemo-radiation therapy
- Multiple drug therapy

Recently the Center for Disease Control [**CDC**] noted that the two most common visits to an Emergency Department for a medication misadventure were Insulin and Coumadin. In addition, the Joint Commission on National Safety Goals for 2008 included the following:

> "3E Reduce the likelihood of patient harm associated with the use of anticoagulation therapy."

The Prothrombin Time [**PT**] is the test of choice for monitoring chronic non-heparin anticoagulation. To provide accurate and precise result to clinicians, most laboratories use highly sensitive thromboplastin reagents run on automated coagulometers using optical sensors and mathematical algorithms to recognize fibrin clot formation. Furthermore, most have adopted a World Health Organization [**WHO**] calibration standard and mathematical equation to normalize **PT** results as the International Normalized Ratio [**INR**].

Attempts to improve the **PT** test has led clinicians to establish Coumadin treatment protocols that, in many cases, rely on changes as small as 0.1 **PT/INR** value to adjust dosages and levels as high as 20 to determine if and what type of intervention should be pursued to reverse anticoagulation on an emergent basis.

However, when reviewed in depth, the literature of the past forty years combined with the experiences of individual laboratories reveals that we are still far from the level of accuracy and precision that would justify such confidence in this test. So, while we struggle to maintain good calibration and control over our local results, clinicians demand a level of service that isn't realistically attainable.

2 PROBLEM

So, the question we face is how to:

RISK	***Maximize patient safety*** with a coagulation test that provides accurate and precise results linked to the clinical propensity to bleed or clot
QUALITY	***Minimize pain and suffering*** due to wrong or delayed adjustment of their anticoagulation therapy
UTILITY	***Minimize expenditure*** of scarce resources on avoidable adverse coagulation events as well as follow on liability costs

More specifically:

How do we provide clinicians with the means to monitor coagulation therapy with available technology to minimize the tendency towards bleeding (over anticoagulation) and thrombosis (under anticoagulation)?

3 SOLUTION

Within the limitations placed on us by the present state of the art in coagulation testing we can optimize the service we provide through appropriate literature review, instrumentation evaluation, rigorous validation, reagent calibration, and ongoing improvement in operator proficiency. Finally,

As consultants to our clinicians we can educate them as to the significant limitations, appropriate use, and reasonable interpretation of **PT/INR** results in service to their anticoagulated patients.

4 PHILOSOPHY

My position on providing optimal laboratory service is to prioritize:

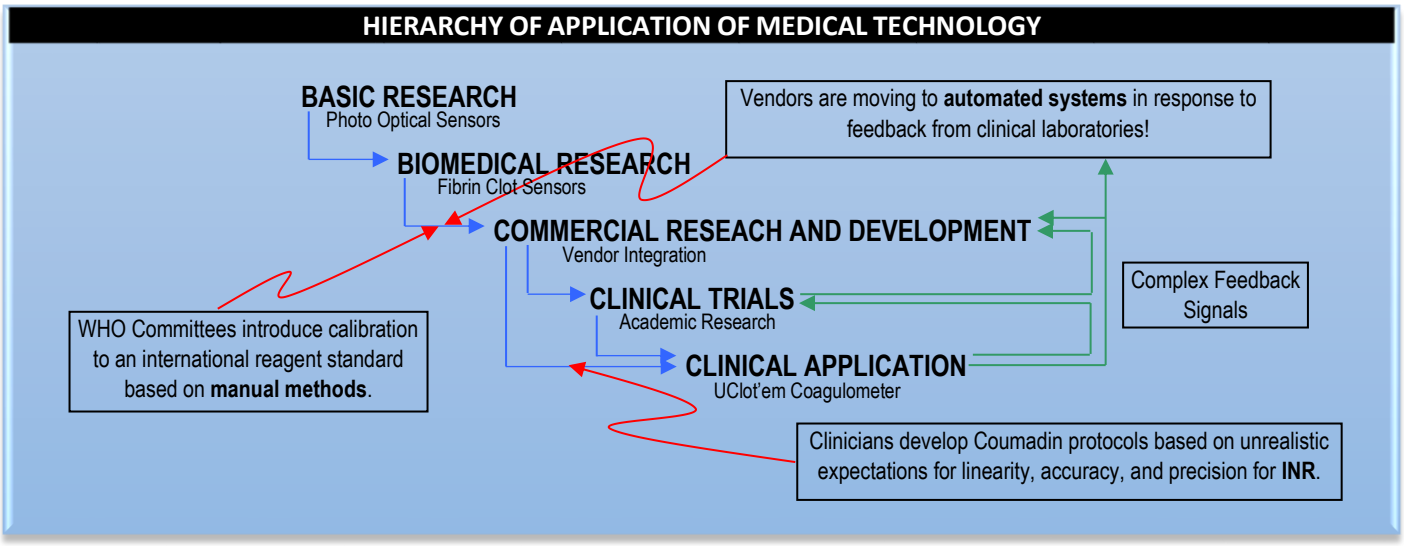
THE PATIENT'S SAFETY COMES FIRST.

The client's (clinician) and customer's (patient) quality of experience must necessarily come second and the cost of the testing for this high-risk therapy last. One optimizes patient safety through an objective scientific approach to the solution of the problem. This requires:

- **RESEARCH**: Careful and critical review of the literature
- **CONSULTATION**: Obtaining expert opinions
- **EXAMINATION**: Evaluate testing modalities, vendor technical data, and clinician expectations
- **EXECUTION**: Draw appropriate conclusions and implement change as necessary

This approach is not always easy because there is often a disconnect between the knowledge and terminology used by the various participants involved in the discovery, development and use of clinical laboratory technology. And this can lead to a significant misunderstanding as to the appropriate implementation of technology, its actual reliability, and its utility value in diagnosis or monitoring of therapy.

There are several steps that lead to the application of basic scientific principles to technological innovation in the laboratory. In the case of anticoagulation monitoring there have been numerous disconnects between some of these steps as shown schematically below.



5 BACKGROUND HISTORY

The following is a brief overview of the history of coagulation and anticoagulation to orient us to the present:

COAGULATION – THE EARLY YEARS

Rudolf Virchow [1821 – 1902]

- Coined term Fibrinogen.
- Pathogenesis of pulmonary arterial thrombosis.
- Virchow's Triad leading to clot formation:

  Vessel wall injury
  Blood flow disturbance
  Blood constituent alteration

Paul Morawitz [1879 – 1936]

- Factor I Fibrinogen
- Factor II Prothrombin
- Factor III Thromboplastin (thrombokinase)
- Factor IV Calcium

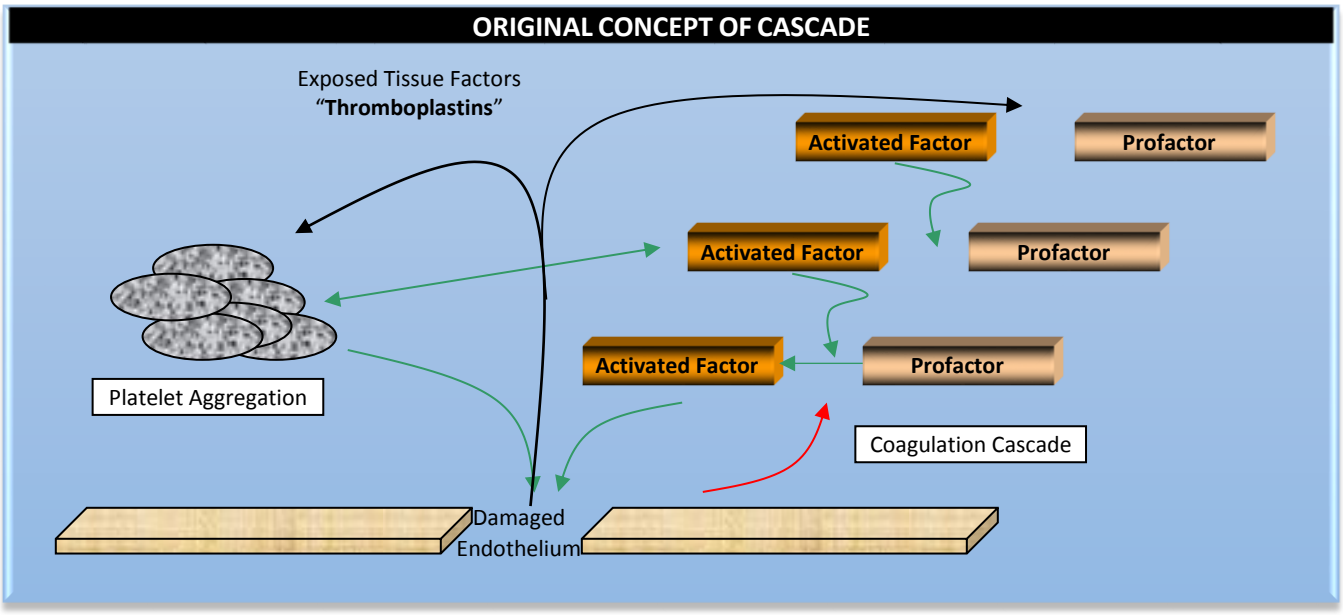
Robert Macfarlane [1907 – 1987]

- Postulates the Cascade theory

During the early to mid-1900's clinical and laboratory evaluation of numerous patients with coagulation defects lead to the elucidation of the causes of the hemophilia's, Factor V deficiency, Stuart Factor X, Hageman Factor XII and other procoagulant deficiencies or functional abnormalities.

COAGULATION "CASCADE" ESTABLISHED

By the early 1970's it was determined that there was an intrinsic and an extrinsic coagulation pathway each feeding into a final common pathway. It was described at that time as a primarily humeral multifactorial amplification cascade system where very small initial signals at the top of the biochemical pathway of as little as one molecule could, if uninhibited, lead to a final production of Fibrin from Fibrinogen several magnitudes larger in volume. The fibrin cross linked and, in synergy with a platelet thrombus forming system, provided a means of rapidly walling off disruptions in the integrity of the vascular system.



During this time parallel investigations pertaining to the tissue injury pathway known as the **Kalikrin System** and the **Complement System** added to our knowledge about the coagulation/clotting system. It was found that both could be activated by humeral and cellular mechanisms that were specific (Antibody-Antigen) and nonspecific (Lysozymes and Cellular Activators). Furthermore, it was found that both systems fed into the coagulation/clotting system causing both chronic low-level activation as well as acute thrombo-hemorrhagic events.

These two pathways are linked together with the clotting pathway to form a very complex biochemical super system that responds to disruptions of structural and functional equilibrium of the body.
And this makes effective in vitro monitoring of anticoagulation therapy difficult.

Since that time further research and clinical investigations have shown that, just as with the immune system, the coagulation/clotting system includes cellular based as well as humeral factors with numerous feedback loops that modulate its functional integrity and activity. This has led to the development of the modified Tissue Factor [**Extrinsic**] Pathway and the Contact Activation [**Intrinsic**] Pathway that integrates platelet and vascular endothelial surface factors into the cascade feed forward and feedback loops.

ANTICOAGULATION – THE EARLY YEARS

Francis Schofield [1889 – 1970]

- 1922 Hemorrhagic disease of cattle eating spoiled clover after a very cold winter.
- A chemical called Coumarin had been oxidized by a spoiling mold.
- Laboratory modification of this chemical by cross-linking into Dicoumarol using formaldehyde lead to increased potency.
- Warfarin was the result - **W**isconsin **A**lumni **R**esearch **F**und + "arin" and introduced therapeutically.

Henrik Dam [1895 – 1976]

- Foods that prevented undue hemorrhage caused by Coumarin substances were discovered.
- Vitamin K is found to be the offending substance.
- The "K" in vitamin K from the German spelling of coagulation: Koagulation.
- Vitamin K is involved with the synthesis and activation of a family of coagulation factors in the liver.

Vitamin K dependent factors:

- II, VII, IX, X. Factor II – Prothrombin is found to be the key profactor through which Coumarin drugs act.
- More recently additional factors affected by Coumarin are Proteins C, S (and Z?).

Armand Quick [1894 – 1978]

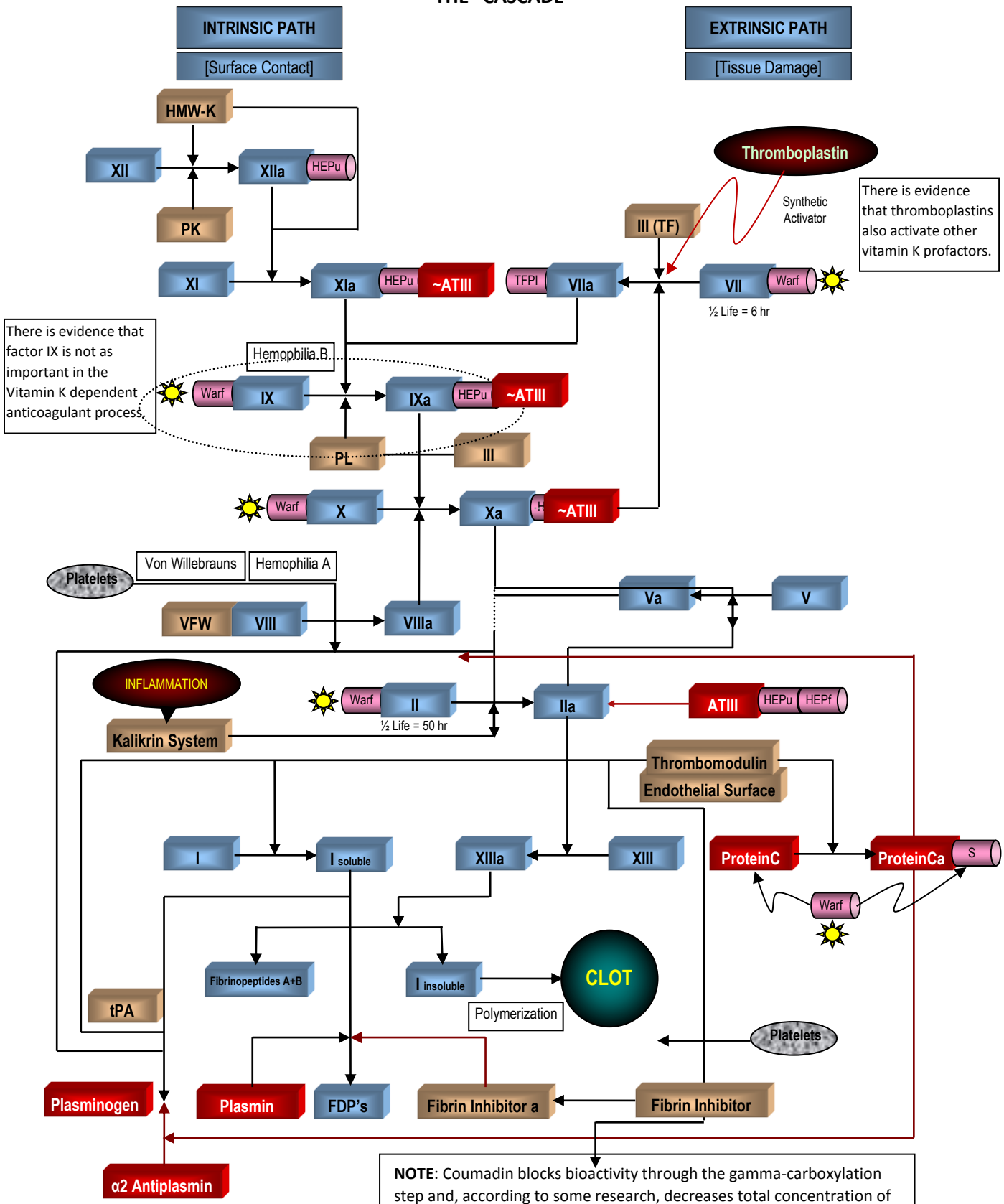
In 1935 Quick devised a laboratory testing methodology for abnormalities of the Tissue Factor and Common Pathway segments of the coagulation cascade. It utilized a thromboplastin or thromboplastin-like reagent to initiate the clotting cascade in vitro and relied on visual determination of clot formation to measure the time it took for Fibrinogen to be converted to Fibrin, the product that forms this clot. Since the amount of time it takes for activated Thrombin to cleave enough Fibrinogen to create a visually identifiable clot is so short, the test was, to all intents and purposes a measure of the amount of time it took the coagulation cascade to convert prothrombin to thrombin. Therefore, it was called the **Prothrombin Time [PT]**.

The **PT** test allowed for the evaluation of the Vitamin K dependent factor deficiencies as well as giving clinicians a means of monitoring and adjusting anticoagulation therapy both in and out of the hospital. In turn, this led to a dramatic increase in the number of patients treated with Coumarin based medications on a chronic basis, an increase in adverse outcomes, and the discovery that the **PT** was not as accurate and precise as first thought by the clinicians.

6 THE COAGULATION SYSTEM – A BRIEF OVERVIEW OF STRUCTURE AND FUNCTIONALITY

For the purpose of this paper an abbreviated yet rich coagulation schema has been provided. This schema includes most of the factors that are now generally accepted as being critical to the integrity of hemostasis. These are supplemented by a wide range of additional plasma and endothelial factors not included due to lack of full understanding of how they work.

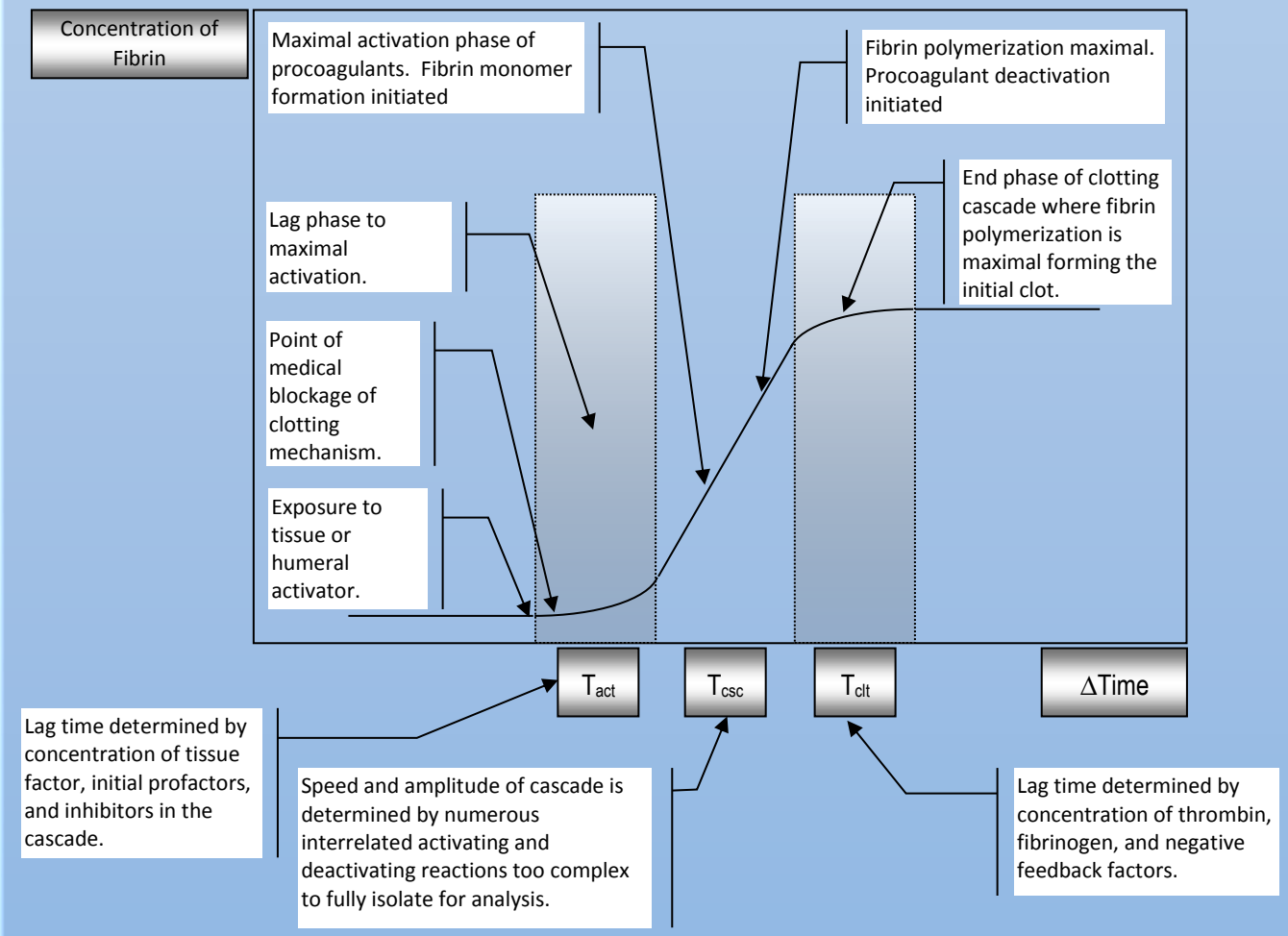
THE COAGULATION SYSTEM SCHEMA
THE "CASCADE"



BLOOD CLOTING FACTORS

Factor	Synonym	Category	Vitamin K
I	Fibrinogen	Structural Protein	No
II	Prothrombin	Proenzyme	Yes
III	Tissue Thromboplastin/Factor	Profactor	No
IV	Calcium	Mineral Electrolyte	No
V	Labile Factor/Proaccelerin	Profactor	No
VII	Proconvertin	Proenzyme	Yes
VIII	Antihemophilic Factor [AHF]	Profactor	No
IX	Plasma Thromboplastin Component [PTC] Christmas Factor	Proenzyme	Yes
X	Stuart Factor/Stuart Prower Factor	Proenzyme	Yes
XI	Plasma Thromboplastin Antecedent [PTA]	Proenzyme	No
XII	Hegeman Factor		No
XIII	Fibrin Stabilizing Factor [FSF]		No
HMW-K	High Molecular Weight Kininogen/Fitzgerald Factor		No
PL	Platelet or Phospholipid		No
PK	Prekallikrein/Fletcher Factor		No
Protein C		Regulator Protein	Yes
Protein S		Regulator Protein	Yes

CLOT CASCADE CURVE - IDEALIZE



7 THERAPEUTIC MODULATION OF THE COAGULATION SYSTEM

There are three general mechanisms that can be targeted in treating patients with a tendency to thrombosis or who will be undergoing therapy that could lead to excessive production of activated clotting factors. These are:

Tissue Factor	**EXTRINSIC**	Vitamin K dependent factor inhibitors - Warfarin/Coumadin/Coumarin **[Rat Poison!]**
Contact Activation	**INTRINSIC**	Heparin sensitive factors - Heparin
Platelet System	**PACKET**	Cyclo-oxygenase inhibiters - Aspirin/Clopidrogel

This talk focuses on monitoring the first pathway regarding physiologic, mathematical, and technical aspects of determining the effectiveness of the **Prothrombin Time** [**PT**] and **International Normalized Ratio** [**INR**].

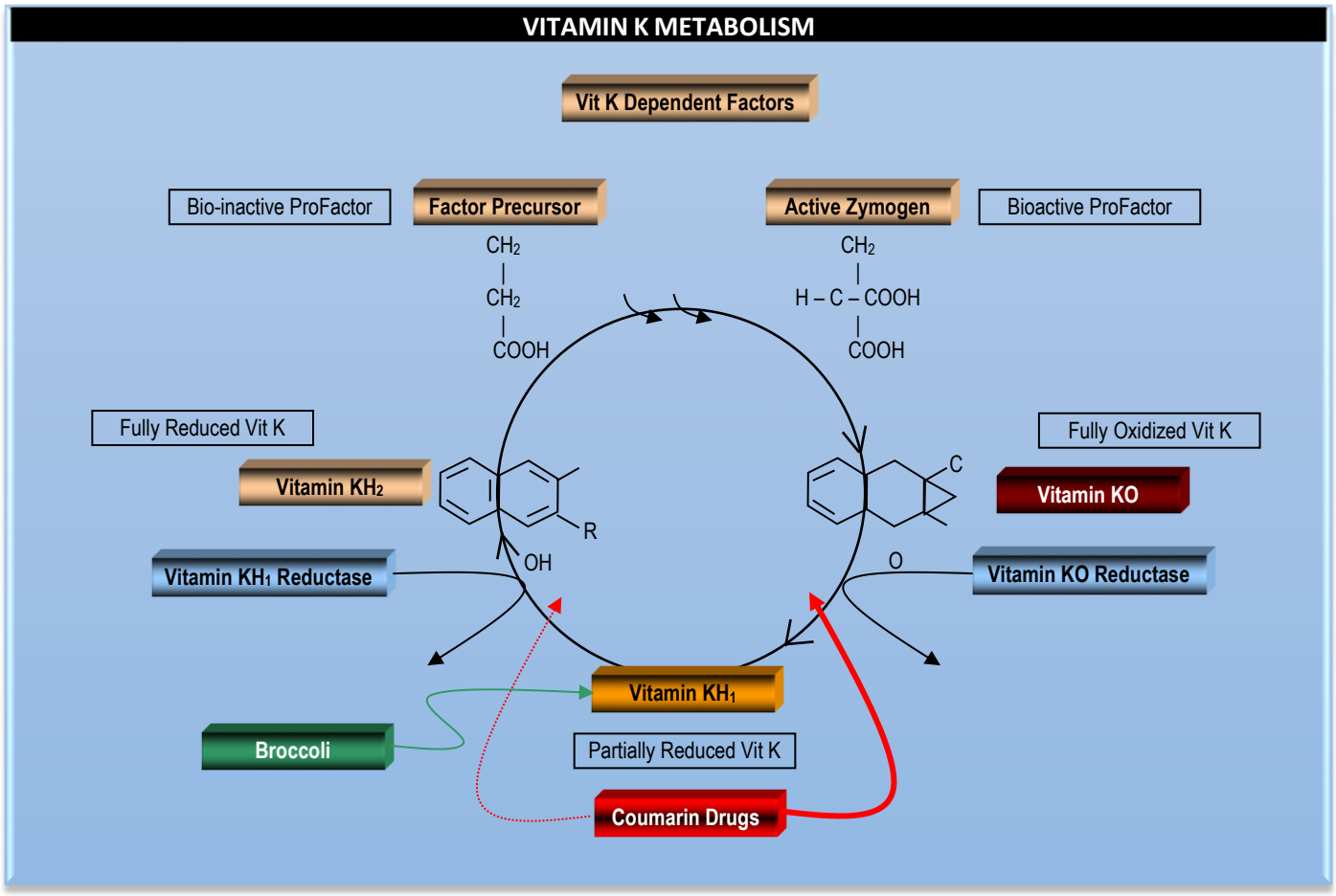
8 BIOCHEMICAL BASIS OF COUMARIN ACTION ON VITAMIN K DEPENDENT COAGULATION FACTORS

Vitamin K dependent coagulation factors are serine protease enzymes synthesized in the liver and modified through several post translational events that lead to fully active pro factors. One key step is carboxylation of glutamic acid moieties on the N-terminal region of each factor to Y-carboxyglutaminate allowing them, in the presence of adequate concentrations of plasma calcium ions, to bind with exposed phospholipid vascular endothelial surface factors which act as coagulation activators. Once activated each serine protease acts on one or more cofactors and profactors in the plasma either activating or inactivating them. Depending on their initial concentrations, bioactivity, and concentrations of activating substrate, the cascade will produce a final product through activation of Fibrin to Fibrinogen.

COUMARIN DRUGS AS VITAMIN K ANTAGONISTS: **Modality of Action**

The Coumarins interfere with the recovery of biologically active vitamin KH_2 from its 2.3 epoxide state formed during carboxylation of the coagulation profactors. The point of maximum interference is at the first reductase step that converts 2.3 epoxide K to KH_1 - the form found in the vitamin K we get from our diet. In contrast the second reductase step is relatively insensitive to the Coumarin medications. This means that the intake of Vitamin K containing foods or administration of significant amounts of vitamin K intravenously, even on a one-time basis, may rapidly increase the concentration of biologically active vitamin KH_2 reconstituting enough active procoagulants to rapidly normalize the **PT**. In addition, vitamin K is a lipid-based cofactor that is not rapidly cleared from the body and is, instead, stored in the liver. It may take approximately one week or more for the effects to wear off depending on amount given, Coumarin levels, and rate of synthesis in the liver.

Below is a simplified schematic of how this system works:



COUMARIN CHARACTERISTICS:

Warfarin as the most commonly used version of Coumarin in the United States and is characterized by:

- High bioavailability
- Peak serum concentration by approximately 90 minutes
- Unlike heparin, the anticoagulant effect is delayed.
- Half-life 36 - 42 hours [it takes approximately 4.5 – 5.0 days to adequately clear]
- It binds to albumin in the plasma
- It accumulates in the liver

GENETIC VARIATIONS IN PATIENT RESPONSE TO ANTICOAGULATION THERAPY:

There appears to be considerable genetic variation in patient response to Coumarin therapy. Most of this response may be secondary to the P450 enzyme (2C9) oxidative metabolism of Warfarin S-isomer which appears to be the source of genetic variability. This variability of the P450 system can be amplified by many disease processes that either activate or inhibit its function. Some patients undergoing Coumarin therapy show an unusual response requiring from approximately 5 up to 20 times the dosage to achieve adequate control by PT/INR measurements. In addition, there is a rare situation where factor IX pro-peptide mutations lead to bleeding without significant prolongation of **PT** due to a dramatic drop in factor IX activity to 3% whereas other factors may show only a 20 - 30% drop in activity. Even though the patient appears to be at risk for an adverse bleeding event they are paradoxically at risk for a thrombotic event since the other procoagulants are still at adequate physiologic concentrations.

Recently CMS began to develop and implement recommendations for determining the clinical validity and utility of pharmacogenetic testing for Warfarin metabolism to provide a means of predicting patient response to anticoagulation therapy. Initial studies show some promise. However, currently, there is no agreement on this matter in the literature.

MODE OF ACTION:

There appears to be several separate processes by which Coumarin therapy leads to modification of hemostasis. This includes inhibition of profactor activation by a blocking effect, possible inhibition of synthesis in the liver, plus one secondary effect that has not been fully researched but is often cited in the literature called PIKAS. However, the key effect is antithrombotic:

- **ANTICOAGULANT EFFECT**: Factors VII & IX half-lives 6 - 24 hours = 2-4 days
- **ANTITHROMBOTIC EFFECT**: Prothrombin half-life 50 - 96 hours = 4-8 days
- **ANTICASCADE EFFECT**: Protein C & S half-lives 8 – 30 hours = 2-4 days
- **INHIBITION OF SYNTHESIS**: Not well characterized - controversial
- **PIKAS/PIVKA-II**: Proteins induced by Vitamin K absence/antagonists – Factor II

NOTE: This is extensively published but not well understood by most clinicians:

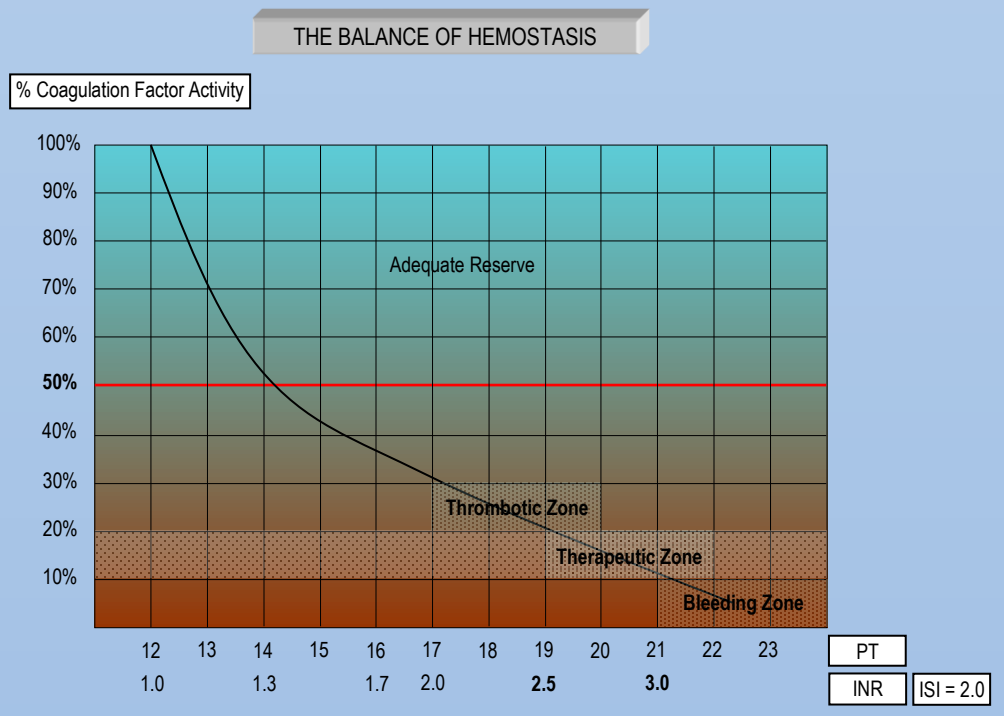
INITIATION OF THERAPY: loading dosages of Coumadin may reduce protein C and Factors VII and IX significantly while not having the same effect on factor II. This paradoxical increases the risk for thrombosis despite a more rapid rise in **PT/INR** and concomitant risk for hemorrhage. Therefore, the standard of practice requires approximately four days of heparin cross over therapy to prevent undue **risk of thrombosis**.

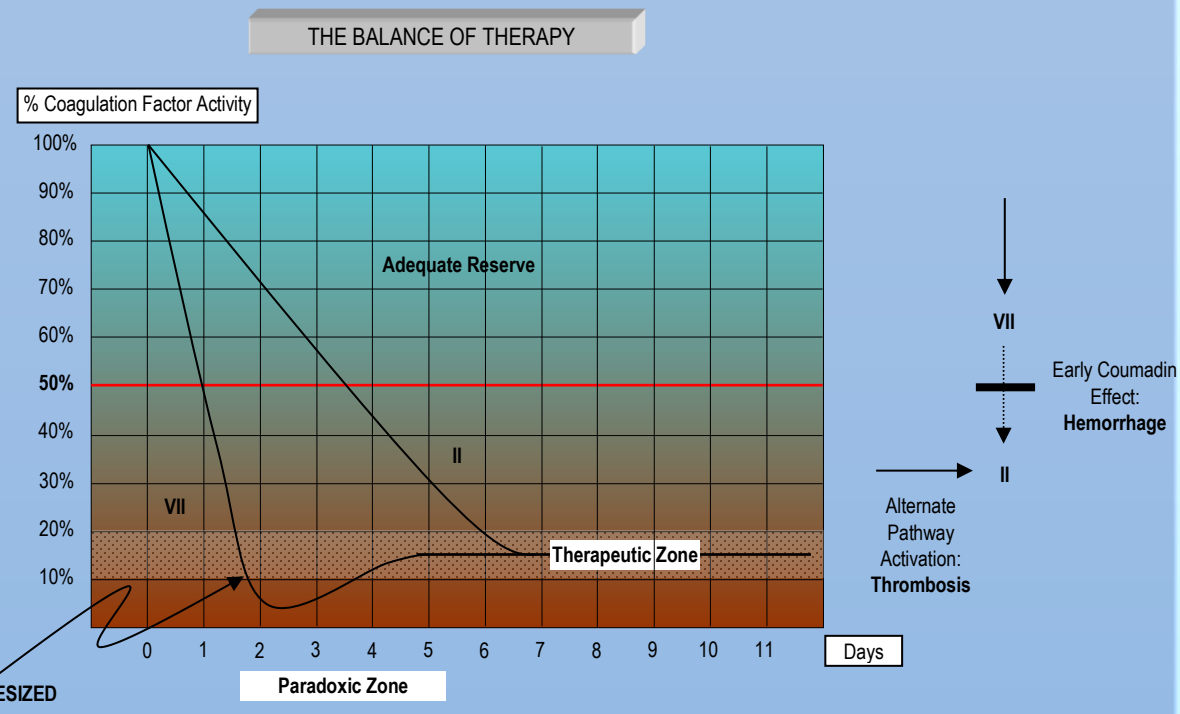
CESSATION OF THERAPY: discontinuation of Coumadin requires several days before levels fall enough to allow for the rebound in Vitamin K dependent factors unless the patient ingests adequate Vitamin K in the diet or is given intravenous Vitamin K to rapidly reverse the effects. Yet, even here, the time it takes for Factor II to rebound will open a window where the **PT/INR** appears to approach normal levels while there is still a **risk of hemorrhage**.

SIMPLIFIED OVERVIEW OF THE EFFECT OF COUMARIN THERAPY

Below is a schematic overview of what we are measuring and what is happening in vivo and illustrates some of the problems of therapeutic monitoring with PT/INR.

THE TWILIGHT ZONE!





Coumadin therapy blocks biologic active profactors leading to feed back up modulation of profactor production leading to rebound profactor

9 OVERVIEW OF THE PROTHROMBIN TIME

REAGENT TYPES AND THEIR EFFECT ON THE PROTHROMBIN TIME

After the original thromboplastin reagent was developed for commercial use, many vendors moved to a variety of different sources such as rabbit, bovine, porcine, and human materials. In addition, this variation was widened through the use of different organs such as lung, brain, and placenta. Vendors also employed different harvesting and purification methodologies to isolate the thromboplastins while each suspended their product in a proprietary buffer. Presently some of the most common reagents are:

- Rabbit Brain thromboplastin [Tissue Factor and Phospholipids] – rarely used now
- Recombinant Human Tissue Factor and Phospholipids [**RHTF**] - most common

These variations lead to a wide range of biologic activity of the thromboplastins. At first this was not recognized by laboratorians and certainly not by their clinicians. This meant that each laboratory testing system didn't always correlate well with the risk for a thrombotic or hemorrhagic event and often lead to over or under treatment of patients with anticoagulants. To further complicate this problem was the fact that there was even less comparability of **PT** results between laboratories. Diversity of biologic activity had led to a diversity of **PT** ranges leading to adverse patient events and even death. Today, this isn't a serious a problem but still poses a serious risk. So, we can see that there were problems with:

- **ACCURACY**: Closeness of result to the actual biologic condition of the patient [also known as bias].
- **PRECISION** Spread and variability of results around the actual biologic condition of the patient.

Once recognized, the bias in reactivity was termed the '***sensitivity***' of the thromboplastin reagent. That is, the capacity for the reagent to detect a drop in the concentration of one or more Vitamin K profactors.

TESTING MODALITIES:

The problem with accuracy and precision were addressed with limited success. However, Prothrombin testing has gone through a series of improvements since the early 1950's when electromechanical sensor systems were introduced leading to intralaboratory uniformity:

VERSION	PRINCIPLES
ZERO	Manual addition of reagent, tilt tube agitation with visual observation of clot formation.
FIRST	Manual addition of reagent, tilt tube agitation with electromechanical sensor systems.
SECOND	Manual or automated addition of reagent, automated agitation and warming with photo-optical sensor systems to identify optical density rising to a predefined arbitrary reference level – **highly sensitive to fibrinogen levels**.
THIRD	Automated addition of reagent, blanking of cuvettes, dye-based volume check, real time optical density sensing with clot determination based on mathematical algorithms that analyze the clot waveform – **less sensitive to fibrinogen levels**.

REFERENCE RANGE – COMPOSITE FROM MANY SOURCES:

- 7.0 - 15.0 Seconds with ~12 second average. The average **PT** has been somewhat stable between most but not all instrument/reagent pairs. The range was never comparable between instruments or and even between laboratories using the same instrument making it dangerous to send a patient to more than one laboratory to have their **PT** measured when monitoring Coumadin therapy.

WHAT PROTHROMBIN TIME ACTUALLY MEASURES LEADS TO LIMITATIONS ON ACCURACY AND PRECISION: What is being tested for is a proxy for what we want to test for:

- **DIRECT**: Time to clot formation – nothing else!
- **INDIRECT**: Putatively the reductions in factors II, VII, IX and X. In fact, we are testing much more.

KEY ASPECTS OF THE PROTHROMBIN TIME THAT LIMIT ITS CLINICAL APPLICATION

The Prothrombin Time does not use a single monoclonal antibody matched to a single, well defined and homogeneous analyte. There is no attached signal molecule that provides a highly accurate and precise measurement between reagent lots, or between different vendor instrument/reagent systems. Nor does the Prothrombin Time use a single substrate to the analyte or a single enzyme that modifies the analyte leading to production of a signal molecule or change in optical characteristics that would provide a similar level of accuracy or precision.

Instead we are measuring the time it takes to:

- Initiate a complex multifactorial, nonlinear, enzymatic cascade system with more than one entry point
- The collective bioactivity of the four Vitamin K dependent clotting factors to be activated
- The polymerization of a fibrin monomer product – a stochiometric process
- An incomplete in vitro system where in vivo factors are not present [protein C or cofactor S]
- A reagent that is made up of a complex of incompletely understood initiators that are not standardized
- Using optical or other sensor system to signal the presence of a change in the reagent/specimen system
- Using a vendor's proprietary algorithm to decide when a sensor signal means a clot has been formed.
- Identifying clot formation where amount of thrombin – fibrinogen enzyme pairing is not known.

Since the final polymerization of fibrin monomers is a stochiometric process, even instruments that analyze the optical waveform will be affected in those cases where there is a very low level of fibrin or a dysfibrinogenemia. In the earlier methodologies the endpoint was determined by electromechanical or optical density measurements. Here, even moderate changes in fibrinogen, an acute phase reactant, caused significant variation in **PT** independent of the concentration of bioactive Vitamin K procoagulants. In addition, systems based on Nephelometry appear not to be comparable to photo-optical systems.

10 INTERNATIONAL NORMALIZED RATIO – HOW AND WHY IT WAS ADOPTED

During the early 1970's many clinicians, laboratorians, and researchers carried out studies that showed promise in mitigating the problems associated with the unmodified Prothrombin Time by "normalizing" results through comparison to a locally determined sample mean. Based on this research, laboratories across the world began to collect "normal" plasma from moderate sized samples of 20 - 40 patients not on Coumarin therapy to calculate an average Prothrombin Time. Now the clinician had an intra-laboratory "standard" against which to compare the individual patient result.

$$PT_{ratio} = PT_{patient}/PT_{average}$$

However, even this was not enough for, although it helped mitigate much of the intra-laboratory reliability of **PT** results using a single instrument/reagent pair, it did nothing to mitigate differences between different instrument/reagent pairs regardless of whether it was within the same laboratory or between laboratories.

Further clinical investigations during the late 1970's lead to the discovery of the two key problems underlying variation in **PT** results on the same patient plasma:

- **Vendors used reagents with different capacity to initiate and drive the coagulation cascade in vitro**
- **Different clot sensing and testing methods lead to different determinations of clot formation time**

Additional research was done and the World Health Organization [**WHO**] entered the fray as arbiter of national and international standardization. The following two committees developed "**recommendations**":

- International Committee for Standardization in Haematology [**ICSH**]
- International Committee on Thrombosis and Haemostasis [**ICTH**]

THE THEORETICAL: The goals of these recommendations adopted by the committees were twofold:

- **MAXIMIZE ACCURACY**: Assure each lab is measuring the same aspects of the coagulation cascade
- **MAXIMIZE PRECISION**: Reduce inter and intra laboratory variability in serial testing

THE OPERATIONAL: Before we can go on we need to define these goals in terms of laboratory and clinical operations:

- **MAXIMIZE ACCURACY**: Determine the patient's actual risk for thrombosis versus hemorrhage
- **MAXIMIZE PRECISION**: Assure reliable monitoring of Coumadin therapy over time and between labs

PROPOSED SOLUTION: Two means of maximizing the accuracy and precision of **PT** testing were proposed:

- **SINGLE INTERNATIONAL REAGENT:** Used by all vendors to standardize reagent sensitivity
- **SINGLE INTERNATIONAL STANDARD:** Used by all vendors to match or calibrate their reagents against

Neither solution is optimal. However, the first solution was not feasible both for technical and commercial reasons. It was not feasible to be able to produce a single standard reagent by commercial methods in more than one facility. A single standard reagent would force competing vendors to go to the same instrument technology and most likely, the same instrument. Therefore, the second solution was adopted. This required the creation of the following:

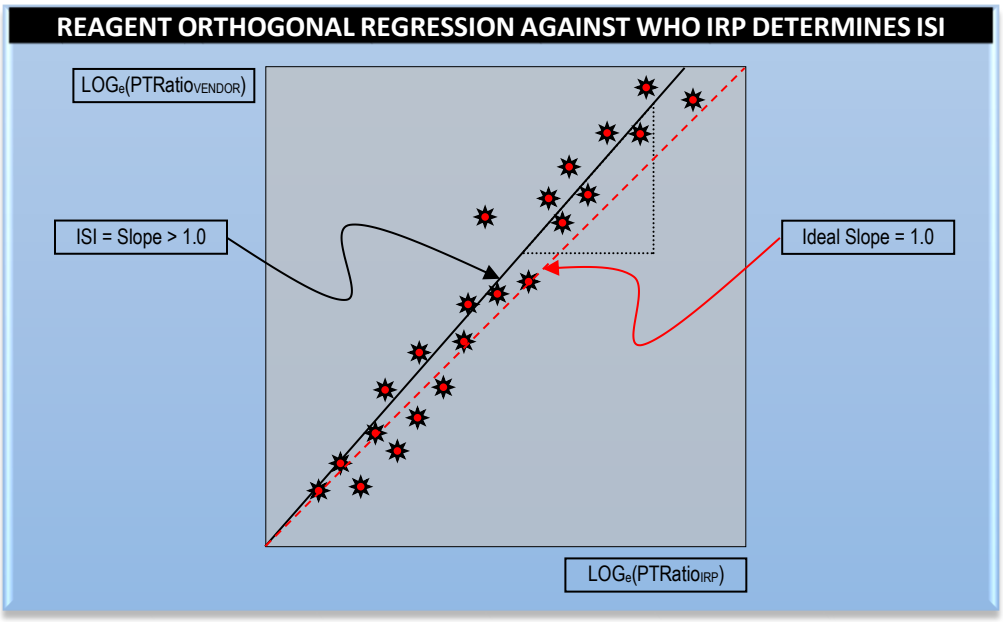
- **THE STANDARD**: International Reference Preparation [**IRP**] coded 67/40 to calibrate reagents
- **THE EQUATION**: A mathematical calculation to normalize vendor results to match the **IRP**

In theory, the solution appears to be adequate to solve the problems with the **PT**. However, as we shall see below, the actual results have consistently fallen short of what has been both expected by laboratorians and communicated to our clinicians.

Below we will investigate the ins and outs of what became known as the International Normalized Ratio [**INR**] to determine its strengths and weaknesses and so, determine what laboratorians need to do to maximize the effectiveness of the test while minimizing the risks of an adverse outcome inherent in its intended use.

11 MATHEMATICAL BASIS OF THE INTERNATIONAL NORMALIZED RATIO

Research by British investigators during the late 1970's showed how the different thromboplastin reagent sources and preparation methodologies led to wide variance in results. It was also noted that, in general terms, each instrument/reagent pair could be compared to another pair by carrying out orthogonal linearity studies and that a mathematical adjustment could be made to assure the results of any two different modalities comparable. The resulting mathematical equation is based on the original **PT** ratio elevated to a power as determined by comparison of vendor reagent to the WHO standard. The statistical evaluation to determine this power is an orthogonal plot of the natural log of the Prothrombin times for a sample population of normal and Coumadinized plasmas or chemically depleted plasmas:



CALCULATION OF THE INR: The final equation is a unitless power function calculated in one of two ways:

ARITHMETIC: $INR = (PT_{patient}/PT_{geometric\ mean})^{ISI}$

LOGARITHMIC: $INR = ISI * Log_e((PT_{patient}) - Log_e(PT_{geometric\ mean}))$

WHERE: **ISI stands for International Sensitivity Index = Slope of orthogonal regression line**

REMEMBER: The geometric mean normal plasma **PT** is NOT the same as the vendor's PT control.

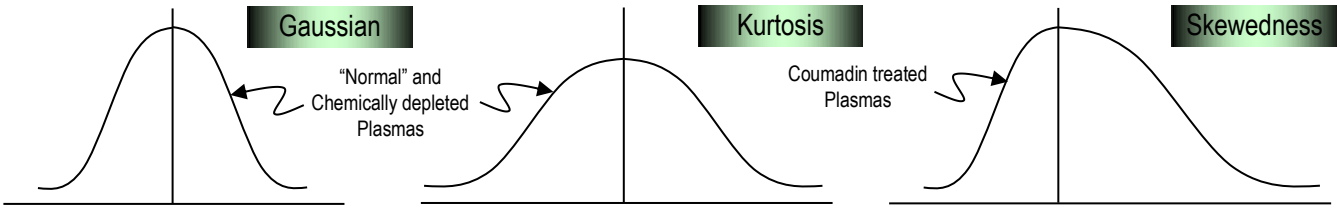
CALCULATION OF THE GEOMETRIC MEAN:

The geometric mean can be calculated in one of two ways:

ARITHMETIC: $GEOMETRIC\ MEAN\ NORMAL\ PT = (PT_1 * PT_2 * PT_3 * \ldots * PT_n)^{1/n}$

LOGARITHMIC: $GEOMETRIC\ MEAN\ NORMAL\ PT = (Log_e(PT_1) + Log_e(PT_2) + Log_e(PT_3) + \ldots + Log_e(PT_n))/n$

The geometric mean is meant to be used where the distribution of the data is not Gaussian. That is, the distribution of the data points is either not symmetrical about the arithmetic mean – **SKEWEDNESS** – or is very widely or narrowly distributed about the arithmetic mean - **KURTOSIS**. In addition, it is really meant to smooth out running average calculations over time:



Therefore, I carried out both arithmetic and geometric mean calculations in a laboratory on twenty specimens determined they are very close. Calculation of **INR**'s based on the two different means did not show any statistical or clinical difference. A review of the literature does not turn up any research that shows that normal patient **PT** studies are not Gaussian. In my opinion, the Coumadin treatment plasmas will be non-Gaussian and somewhere along the way someone made the same determination and mistakenly applied this conclusion to the distribution of normal patient plasma **PT**'s without checking.

The **WHO** recommendation for the laboratory geometric mean **PT** is for the laboratory to measure:

- At least 20 **PT**'s of health individuals
- Who are not on medications that are known to affect **PT**
- Of both genders (unless the patient population is mostly one or the other)
- Using a range of ages that matches that of the patient population to be tested.

They recommend laboratories determine a new mean for any significant system change such as:

- Each new lot of thromboplastin reagent where the **ISI** changes.
- When there is any major maintenance, repair, or upgrade of the instrument.

SOME KEY ASSUMPTIONS MADE IN FORMULATING THE INR EQUATION:

There are several key assumptions made in the **WHO INR** model and these include but are not limited to:

- There is a single straight-line relationship between the natural logarithms of **PT** measurements for both anticoagulated and normal patients – this has been proven in the literature not to be valid.

- Vendor reagents calibrated to the **WHO** standard manually can be recalibrated on their automated instruments to generate an **ISI** that will normalize client laboratory **PT**'s to the **WHO** standard – also proven invalid.

- The original **WHO** standard will last long enough to allow all vendors to do calibrations over a period of decades – false again.

- The vendor will be able to maintain their **ISI** calibration to the **WHO** standard between lots of reagent – variable. Again, not monitored by the vendors.

- Changes in vendor instruments secondary to maintenance, repair and upgrades will not require another complete recalibration – proven not to be valid in the literature.

- Two different vendor instrument/reagent pairs with significantly different **ISI**'s will generate **INR**'s that are comparable over the most important part of the range of values – those critical to decisions on dosage of Coumarin – this most important assumption is also proven to be invalid throughout the literature. Unless, the ISI is very close to or equals 1.0.

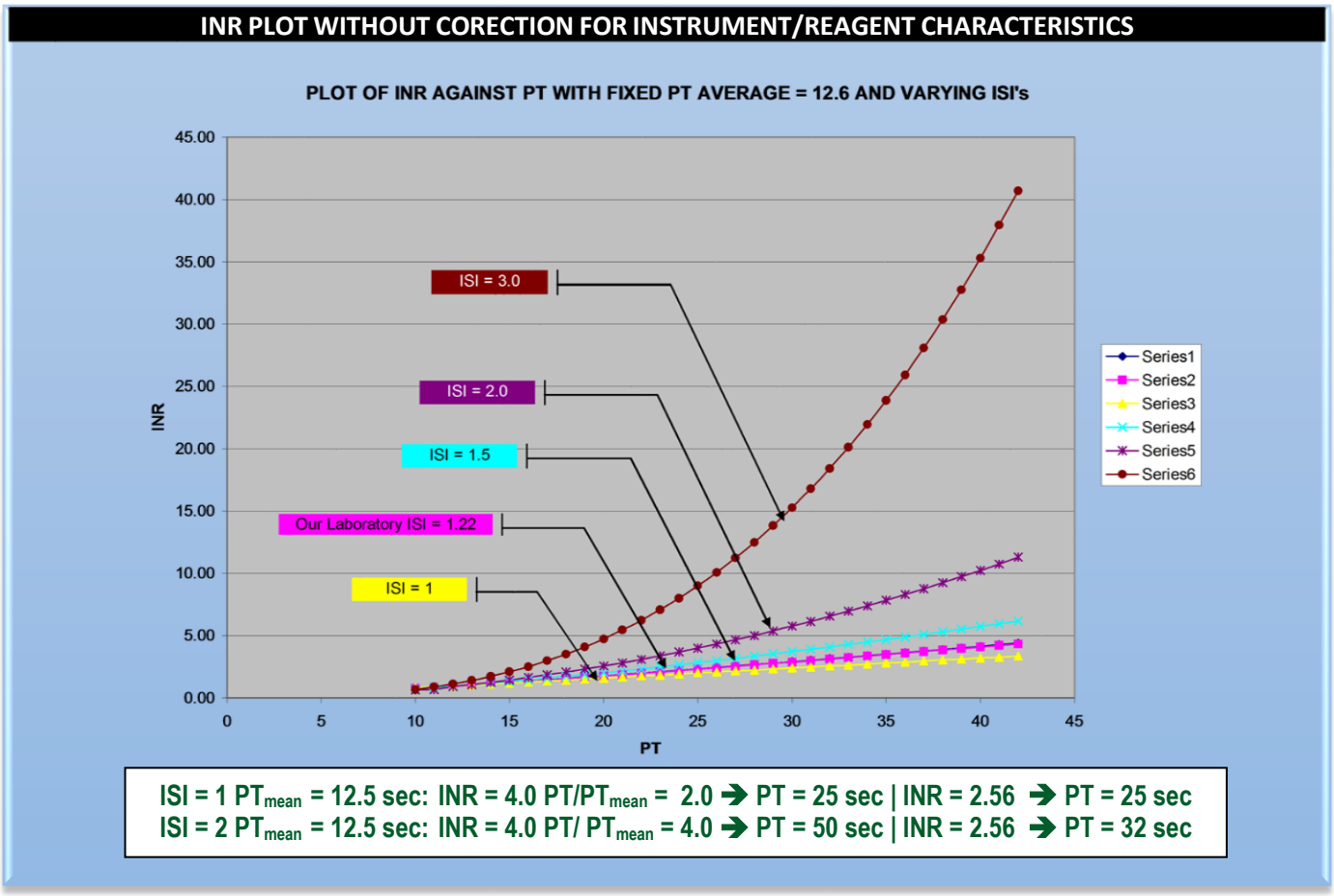
The first assumption is not based not on scientific principles but instead upon empirical data. This data shows good linear correlation only when measured over a short range of **PT** results generating **INR** values between 1.0 and 2.5. Those vendor **ISI**'s that are significantly different from each other and significantly greater than 1.0 show high imprecision especially above this range due to many causes but also due to the very mathematical basis of the **INR**. As the **ISI** moves further away from 1.0 the larger the calculated statistical variance regardless of the overall precision of the instrument/reagent pair. Measurements of **PT**'s above 50 seconds on any one instrument/reagent pair are often not reproducible over time due to this.

Furthermore, the range of **PT**'s that generate **INR** values above that for normal patient plasmas cannot be fully evaluated statistically except through chemically induced plasma depletion studies and these plasmas have also been shown not to be the same as Coumadin treated patient plasmas. At the very least their distribution curves are not comparable.

When two vendors calibrate their reagents against separate secondary or tertiary **IRP**'s and these are derived from different sources it has been found by researchers that, even if both reagents attain **ISI**'s very close to 1.0, their respective **INR**'s at or above the cutoff for treatment may not compare well.

Finally, when vendor **ISI**'s are at or close to 1.0 the literature shows that this does not always improve comparability in the lab. And this is due, in part to variation in calibration against 2° and 3° **IRP**'s and a second calibration against their instrument.

INR PLOT WITHOUT CORECTION FOR INSTRUMENT/REAGENT CHARACTERISTICS

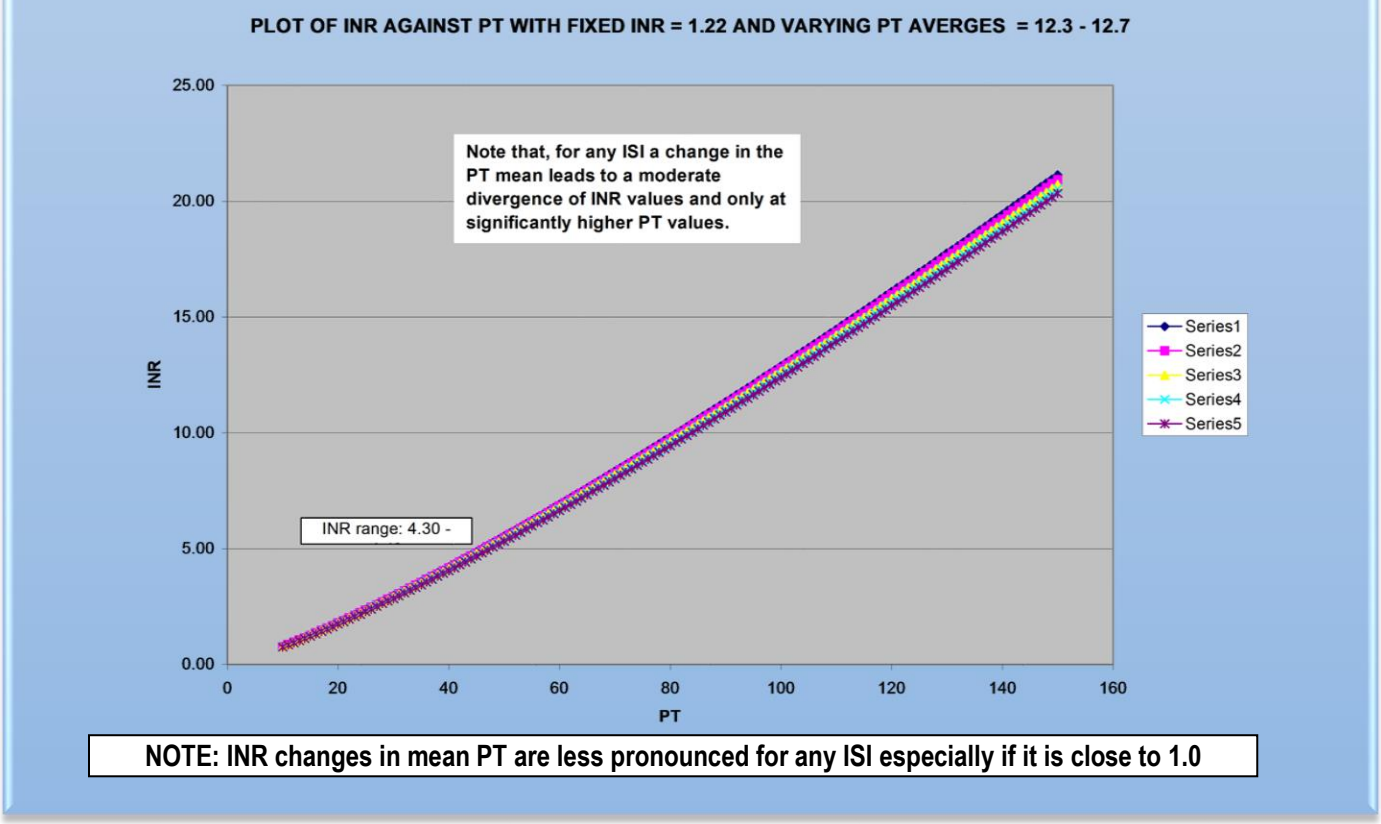


ISI = 1 PT_{mean} = 12.5 sec: INR = 4.0 PT/PT_{mean} = 2.0 ➔ PT = 25 sec | INR = 2.56 ➔ PT = 25 sec
ISI = 2 PT_{mean} = 12.5 sec: INR = 4.0 PT/ PT_{mean} = 4.0 ➔ PT = 50 sec | INR = 2.56 ➔ PT = 32 sec

NOTE: A mathematical correction for a seven second difference at an **INR** of 2.56 and twenty-five second difference at an **INR** of 4.0 is **nonlinear**. Therefore, it cannot be corrected for more than one point over the entire range by a change in the $\mathbf{PT}_{mean}$. Therefore, the rest must be secondary to other factors such as instrument principle and reagent variations.

Normalizing PT ratios with ISI leads to magnification of variations in INR's right where we don't want this: The therapeutic range!

INR PLOT WITHOUT CORECTION FOR INSTRUMENT/REAGENT CHARACTERISTICS



NOTE: INR changes in mean PT are less pronounced for any ISI especially if it is close to 1.0

Why is this? Answer: The **INR** is calculated as a first order power function based on only two variables whereas the coagulation cascade is a multiple order system that cannot be fully characterized experimentally where the rate of activation and inactivation of each enzyme in the cascade is determined by a large number of factors that cannot be directly measured or adequately controlled including but not limited to factor concentrations, bioavailability, and inhibitors.

Furthermore, **WHO** accepted the concept that the orthogonal regression model used defines a single linear relationship between the natural log of the Prothrombin Time of abnormal and normal plasmas. As noted, there have been many observations of a significant deviation of the slope and intercept of these two data sets. Many mathematical models have been proposed to correct for this. For example, Tomenson proposes both a multiplicative as well as a power factor:

MODIFIED EQUATION: $INR = \alpha\,(PT_{patient}/PT_{geometric\ mean})^{\beta}$

CONCLUSION: THE INR DOES NOT NECESSARILY IMPROVE COMPARABILITY BETWEEN LABORATORIES/INSTRUMENTS.

WHAT IMPROVES COMPARABILITY? Answer: An extensive review of the literature and our correlation studies reveals that there are a set of general characteristics of instrument/reagent pairs that, when closely matched yield better precision:

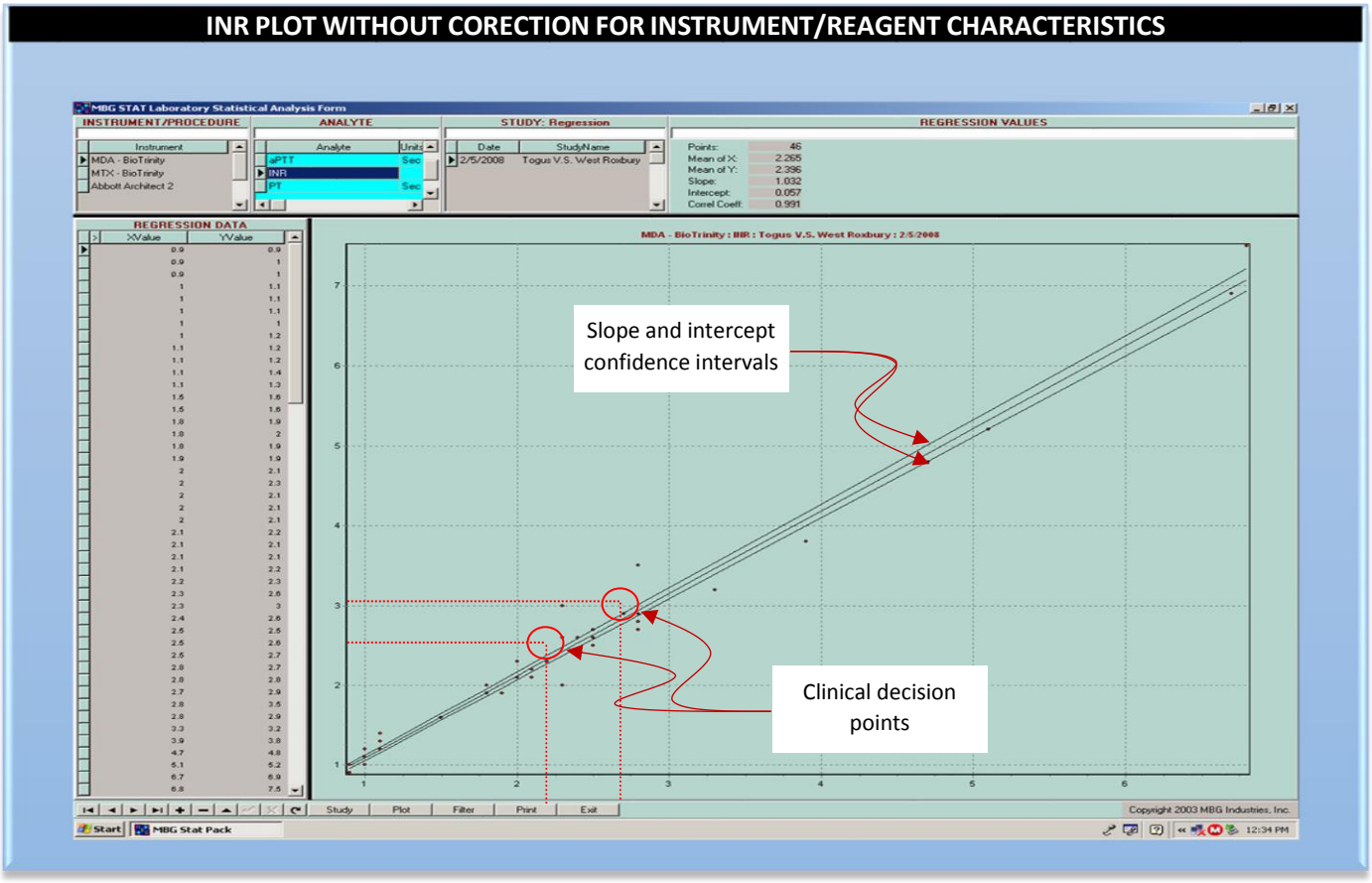
IF BOTH INSTRUMENT/REAGENT PAIR ARE:

INSTRUMENT	VERSION	REAGENT	LOT	PROCEDURE	PRECISION
SAME	SAME/SIMILAR	SAME	SAME/SIMILAR	SAME	**HIGH**
SIMILAR	N/A	SIMILAR	N/A	SIMILAR	**MODERATE**
DIFFERENT PRINCIPLE	N/A	DIFFERENT	N/A	DIFFERENT	**LOW**

What do we mean by precision? In this case there is no agreement in the literature. It includes but is not limited to:

- **REGRESSION CORRELATION COEFFICIENT**: Acceptable r^2/slope/intercept values not well defined
- **COEFFICIENT OF VARIANCE**: CV ranges from 3% – 6%
- **PERCENT DIFFERENCE BETWEEN INR'S**: Percent ranges from 5% – 10%
- **INDEX OR INTERRATER RELIABILITY**: Degree of agreement between instrument/reagent pair
- **PERCENT CLINICAL DECISIONS DIFFERENT**: Percent ranges from 5% to 10%
- **USE OF THE CORRELATION COEFFICIENT**: Below is an example of this approach

INR PLOT WITHOUT CORECTION FOR INSTRUMENT/REAGENT CHARACTERISTICS



This methodology is an excellent means of comparing and validating laboratory results where the analyte critical cut off is well above the reference range. Otherwise, even when there is excellent correlation for r^2, slope, and intercept, there may not be adequate correlation at the clinical decision point. **INR** is an example of this. As an example, the plot above is a correlation study between two laboratories using the exact same instrument model, reagent type, reagent lot, and procedure.

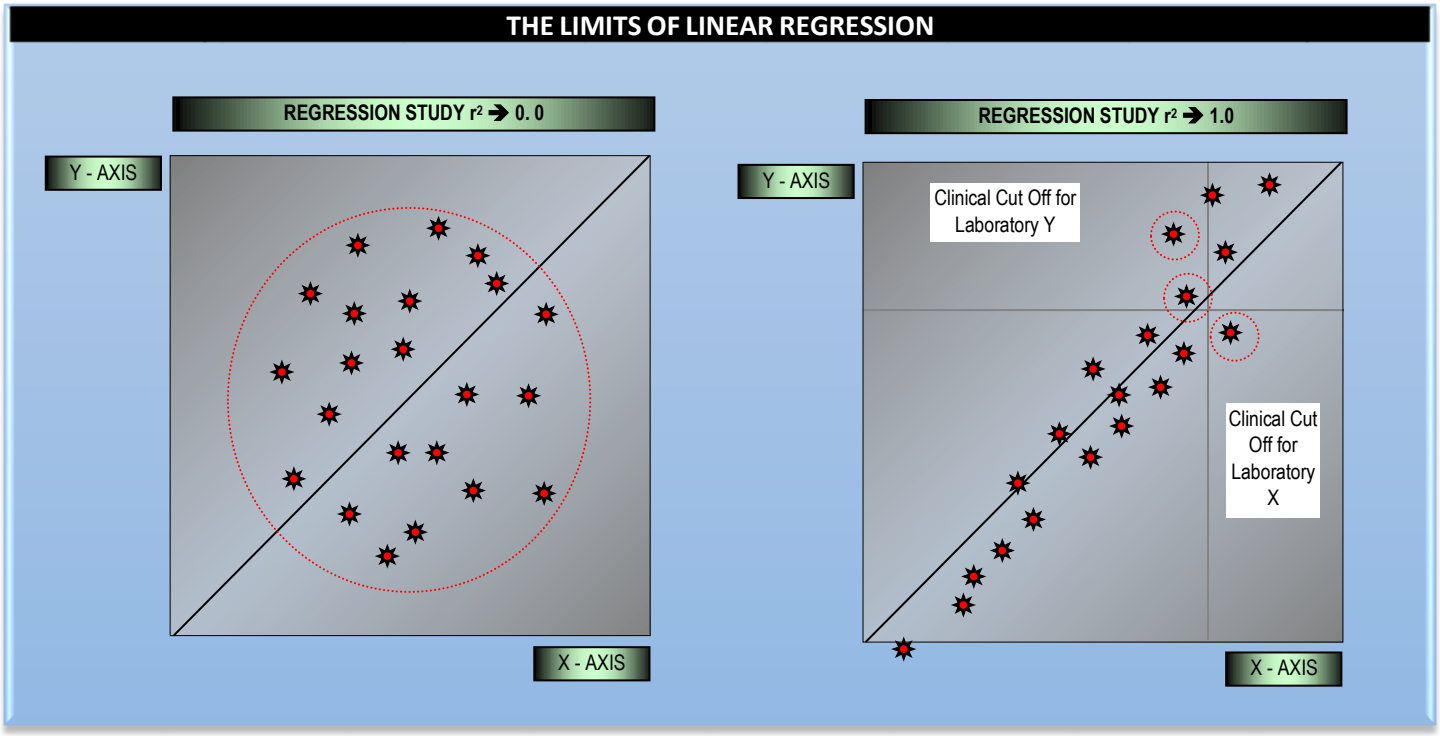
Note that, even though r^2, slope, and intercept values are as optimal as we could ever hope for, there are still a number of data points that lie outside the 95% combined slope and intercept confidence interval. Two

of these might be clinically significant depending on the exact content of the Coumadin therapeutic protocol being used by the ordering clinician.

HOW CAN THEIR BE SIGNIFICANT IMPRECISION AT THE POINT OF THERAPY WITH GOOD CORRELATION?

Answer:

- The linear regression method of correlation evaluates a data set and calculates an optimal fit for a theoretical line without determining if the data define a linear function.
- Therefore, this method attempts to put each data point as close to the theoretical line as possible and so tends to cause data points to be distributed evenly above and below it.
- Therefore, two different sets of data, one with much variation distributed evenly about the theoretical line and one with little variation having a few asymmetrical data points at either end can have very similar regression results.
- This is because of the principle underlying this method weighs values at the center of the range more than at the two ends of that range.
- An $r^2 = 0$ means the data is distributed relatively uniformly as a circular pattern within the data set range.
- An $r^2 = 1.0$ means that the data exactly correlates positively defining a perfect line.
- An $r^2 = -1.0$ means that the data exactly correlates negatively defining a perfect line.
- The more evenly the data points lie on either side of the theoretical line the closer r^2 is to 1.0.
- A subtle sigmoid curve can result in good correlation if deviation at each end is approximately the same.
- A linear pattern with a small set of outliers in the clinically critical zone can lead to an r^2 close to 1.0.



In the illustrations shown above the reader may think concordance of only 50% of clinically significant values on the right is an exaggeration. It is not. In the literature this is stated as ranging from 16% to 64% when the difference in **INR** values at the upper cut off is more than 10% different between two instrument/reagent pairs.

USE OF THE COEFFICIENT OF VARIANCE:

EQUATION: CV = σ/μ = (Standard Deviation/Mean) x 100 (Arithmetic mean NOT the geometric mean!)

This statistical tool is used to measure error where there is within-subject variation that is proportional to the magnitude of the measurement. As I have found in every lab and as is acknowledged in the literature, this occurs with the **INR** calculation when the **ISI** is significantly greater than 1.0. At first glance this appears to be an appropriate measure of the comparability of **INR** results. However, we cannot construct confidence intervals for the mean around which we are calculating the **CV,** so the standard deviation may well be a better measure. More importantly:

The **CV** does not give us or the clinician a good idea of what the risks are for an adverse outcome since it does not determine how often a clinical decision will be different with different sources of **INR** results.

CV DOES NOT PROVIDE A MEASURE OF THE RISK FOR OVER OR UNDER TREATING THE PATIENT

And that is exactly what we want!

USE OF THE PERCENT DIFFERENCE BETWEEN INR AT THE MEDICAL DECISION POINT FOR THERAPY:

This means of testing for comparability of **INR** results between two or more instrument/reagent pairs begins to address the problem we face. Here, several methods can be applied. One instrument/reagent pair can be used as the standard against which the second pair is compared and the number of **INR** results within a specified INR range (such as 2.5 to 3.0) vary from the standard by a certain percent are counted. This number is compared against the total number of results in that range to produce a percentage. The percent difference between the standard and comparison instrument/reagent pair is usually set in the literature as 5% or 10%. I cannot find how these standards were set in the literature. However, even at the more generous 10% mark many instrument/reagent pairs show up to a 50% divergence. For example, to calculate a value for a 10% difference

EQUATION:

$$\Delta_{PercentINRDifference} = (INR_{Standard} - INR_{Second}/INR_{Standard}) \times 100$$

$$\Delta_{Percent10\%Difference} = (\Sigma_{PercentINRDifference} > 10\%/N_{CriticalINR}) \times 100$$

The problem with this method is that it does not directly address whether and which results would lead to a different clinical decision between the two instrument/reagent pairs.

USE OF INTERRATER RELIABILITY:

This statistical approach is often called [Cohen's] Kappa [κ] and attempts to account for the potential for random agreement and disagreement between two observers – in this case two instrument/reagent pair – to calculate the degree of agreement, and therefore disagreement beyond chance. The concept is based on categorizing all outcomes into four categories:

- X INSTRUMENT/REAGENT PAIR **ABOVE** THE CUTOFF — Y INSTRUMENT/REAGENT PAIR **ABOVE** THE CUTOFF
- X INSTRUMENT/REAGENT PAIR **BELOW** THE CUTOFF — Y INSTRUMENT/REAGENT PAIR **BELOW** THE CUTOFF
- X INSTRUMENT/REAGENT PAIR **ABOVE** THE CUTOFF — Y INSTRUMENT/REAGENT PAIR **BELOW** THE CUTOFF
- X INSTRUMENT/REAGENT PAIR **BELOW** THE CUTOFF — Y INSTRUMENT/REAGENT PAIR **ABOVE** THE CUTOFF

These values are then compared to the total number of tests carried out to determine the Kappa value for the data set:

EQUATION: $\kappa = (O - C)/(1 - C)$ **Where O = Observed Agreement and C = Chance Agreement**

Below the regression plot for a hypothetical X and Y instrument/reagent pair displayed on page 18 above are used to illustrate how this approach is applied:

INR DATA GRID

		Instrument/Reagent Pair X		
		Above Cutoff	Below Cutoff	Instrument/Reagent Pair Y Totals
Instrument/Reagent Pair Y	Above Cutoff	3	2	5
	Below Cutoff	1	14	15
Instrument/Reagent Pair X Totals		4	16	20

OBSERVED POSITIVE: $\Sigma_{XYPositive}/N_{Total} = 3/20 = .15$

OBSERVED NEGATIVE: $\Sigma_{XYNegative}/N_{Total} = 14/20 = .70$

TOTAL OBSERVED: $(\Sigma_{XYPositive} + \Sigma_{XYNegative})/ N_{Total} = (3 + 14)/20 = .85$

CHANCE POSITIVE: $C_{Positive} = (\Sigma_{YPositive}/N_{Total}) \times (\Sigma_{XPositive}/N_{Total}) \times N_{Total} = 5/20 \times 4/20 \times 20 = 1.0$

CHANCE NEGATIVE: $C_{Negative} = (\Sigma_{YNegative}/N_{Total}) \times (\Sigma_{XNegative}/N_{Total}) \times N_{Total} = 15/20 \times 16/20 \times 20 = 12.0$

TOTAL CHANCE: $C = (C_{Positive} + C_{Negativ})/ N_{Total} = (1.0 + 12.0)/20 = .65$

EQUATION: $\kappa = (O - C)/(1 - C) = (.85 - .65)/(1 - .65) = .57$

CONCLUSION: **The two instrument/reagent pair agree more often than expected by chance**

Note that, if there were no disagreement between the two, then Kappa would be 1.0 and if all values were in disagreement Kappa would be – 1.0. So, the degree of agreement, when accounting for chance is moderate in this hypothetical example.

USE OF THE PERCENT CLINICAL DECISIONS CHANGED:

Understanding that we unfortunately don't know which instrument/reagent pair is the more accurate, this methodology comes closest to measuring clinically significant imprecision. Divide the number of data points that would lead to a different clinical decision by the total data points within the combined clinical range established by the institution's Coumadin protocol and multiply by 100.

EQUATION: $Clinical\Delta_{INR} = ((\Sigma INR_{ClinicallyDifferent}/N_{INRClinicallySignificant}) \times 100$

However, before implementing this method of determining imprecision between two instrument/reagent pairs we need to ask two very critical questions:

- **WHAT PECENT DIFFERENCE WILL YOUR CLINICIANS BE COMFORTABLE WITH? WHAT IS *ACCEPTABLE RISK*?**
- **DOES THE COUMADIN PROTOCOL ACCOUNT FOR IMPRECISION OF YOUR INR RESULTS?**

After all, it is the ordering clinician who needs to set the practice standard with regards to acceptable variation and then, to establish a safe Coumadin protocol based on general standards of practice adjusted to the capabilities of your instrument. Furthermore, given what will be presented below in reference to calibration of vendor reagents to **IRP**'s it will become even more apparent that not only do we have to provide our clinicians with data that will allow them to make a decision about the use of one or more instrument/reagent pairs to monitor and modulate their patient's Coumadin therapy, it will be necessary to monitor outcomes for any particular instrument/reagent pair and the cutoff(s) chosen for intervention.

12 OPERATIONAL BASIS OF THE INR – CALIBRATION TO INTERNATIONAL STANDARDS

As we noted above, the goal of moving to the International Normalized Ratio was to maximize accuracy and precision which we can now see means:

- Mathematical adjustment for reagent sensitivity and
- Establishment of comparability between two different instrument/reagent pairs – sometimes referred to as reliability

At this point the reader can now see:

INR CORRECTION FOR REAGENT SENSITIVITY DOES NOT NECESSARILY ASSURE RELIABILITY

And this is the key misunderstanding both in the literature and by many laboratorians and clinicians!

Despite these drawbacks use of the **INR** rose from approximately 20% of laboratories in 1992 to 97% of laboratories by 1997. Today it is a very rare event to see a laboratory report **PT** without **INR** values attached. And despite the limitation of the Coefficient of Variance as a measure of clinically significant variance between instrument/reagent pairs, this was chosen as the yardstick to measure test reliability. And, the measurable objective has been set as a **CV** below 3% in many research and review articles. Furthermore, the concept of reliability is not uniform in the literature. A review of the literature and vendor documentation shows it being defined as:

- Within run.
- Between run.
- Between laboratories

And this can also be for:

- Within reagent lot over a specified number of separate days
- Between reagent lots within one or over a specified number of days
- Between reagent types
- Between instrument types
- Between reagent/instrument pairs

It is immediately evident that the number of combinations of conditions that would need to be validated in order for there to be assurance of comparability of results within and between laboratories is beyond even the reach of the most lavishly equipped and funded institutions. Therefore, no matter how a laboratory establishes its reliability it needs to work with their clinicians to determine what is most important and what is feasible in terms of assuring ***Acceptable Risk*** first and, in more general terms:

- **RISK REDUCTION**: Patient safety
- **QUALITY EXPERIENCED**: Patient convenience in testing and therapy
- **UTILITY VALUE**: Total cost to the institution including liability for adverse patient outcomes

Even doing basic calibration studies has had some significant impact on reducing intra and inter-laboratory imprecision:

CALIBRATION AS A SOLUTION TO INTRA AND INTERLABORATORY RELIABILITY

Over time researchers and vendors have gravitated towards global and local calibration standards.

GLOBAL: Calibration that assures the greatest accuracy and precision of the vendors' instrument/reagent pairs so that, when implemented across several laboratories, the results will show moderately good correlation.

LOCAL: Calibration that assures the greatest accuracy and precision within and between laboratories through the calculation of a local **ISI**.

CAVEAT TO WHO CALIBRATION REQUIREMENTS - GLOBAL:

In 1977 WHO designated an International Reference Preparation of thromboplastin [**IRP**] coded 67/40. Many secondary and tertiary IRP's were established against 67/40 due to but not limited to the following problems:

- **INCONVENIENCE**: The inability of all vendors around the world to go to a single calibration laboratory
- **SCARCITY**: The limited amount of the original **IRP**
- **BIOVARIABILITY**: Variation in source of commercial thromboplastin

This was implemented using a variety of animal sources including:

- Human thromboplastin Plain
- Rabbit thromboplastin Plain
- Rabbit – Bovine thromboplastin Combined.

For example: **BCT/253**, **OBT/79**, and **RBT/90**. Since the establishment of the **INR** the original **IRP 67/40** has been exhausted and is no longer available for correlation studies. Therefore, the true value of **INR** results can only be approximated using secondary and tertiary reference thromboplastins. As each intermediate thromboplastin standard has been exhausted another component of error has been introduced and this effect on the accuracy of calibrations – either positive or negative – cannot be measured but is multiplicative.

Therefore, we don't know how much the **ISI**'s our vendors provide have drifted from the original **IRP** over the years simple due to this one factor. And therefore, we do not know how much the **INR**'s we report have drifted from their original values. So:

ALTHOUGH WE CAN TRY TO ADDRESS THE PRECISION OF INR, ACCURACY IS NO LONGER ASSURED.

As for precision, vendors and national institutions have begun to establish recombinant IRP's calibrated against such earlier secondary standards as **BCT/253**, **OBT/79**, and **RBT/90**. This has gone far to mitigate problems of **ISI** drift and has helped vendors maintain precision over longer periods of time because these recombinant **IRP**'s can be manufactured in large volumes under controlled conditions. However, recombinant technology itself is not without its own problems. The loss of a clone can require additional recombinant cultures to be developed leading to additional drift.

CALIBRATION REQUIREMENTS ESTABLISHED BY WHO:

With this background we can move on to the standards established for calibration. The **WHO** requires that the calibration between the thromboplastin reagent and the **IRP** be carried out:

TWO SAMPLE POPULATIONS	Using plasmas from both "normal" and "stabilized treated" patients.
CLOSEST MATCHING IRP	Against the **IRP** that most closely matches a laboratory's or vendor's thromboplastin.
MANUAL METHODOLOGY	Use the manual methodology not the vendors' actual automated instrument.

These three requirements are due to the following:

- Original research showed calibration results from normal plasmas could not be extrapolated to Coumadinized plasmas.
- The three basic International Reagent Preparation types show significant differences to each other:
- The original **INR** data upon which the **WHO** standard was determined was generated using manual methods only and it would take an enormous international effort to carry out these studies on the over 300 instrument/reagent pairs now available commercially.

In addition, there is a significant operational limitation on this calibration effort:

- **DIFFERENCES IN PLASMA SAMPLE POPULATION USED BY EACH VENDOR**.
- **DIFFERENCES IN COUMADIN PLASMA SAMPLE POPULATION USED BY EACH VENDOR**.

CALIBRATION METHODOLOGIES:

The **WHO** has recommended laboratories, or their vendors utilize one of the three following calibration methodologies:

- **LABORATORY ISI CALIBRATION**: with manual methods against one of the **WHO IRP**'s still available and then secondary calibration of the automated methodology in the laboratory with residual plasmas.

- **VENDOR ISI CALIBRATION**: with manual method using plasma pools that can then be preserved and used as standards against which their automated systems can be calibrated creating a set of vendor calibration and control standards that can be sent to the client laboratory for secondary calibration against their own instrument.

- **VENDOR INR CALIBRATION**: with manual method using plasma pools with the assignment of standard **INR**'s these and sending vendor calibration and control standards for that can be sent to the client laboratory to create a nomogram where the client laboratory plots their **PT** and reads off the **INR** data directly.

COMPARATIVE UTILITY AND ACCEPTABILITY OF THE WHO RECOMMENDED CALIBRATION METHODOLOGIES:

- Although optimal, the first option is not open to any but the most lavishly funded private laboratories and so is rarely used.
- The second option, used most often, requires an **ISI** value for each reagent and the calculation of a local mean **PT**.
- The third eliminates the need for an **ISI** and the local mean **PT** but is not widely used.

As a result, what we now do in the field of anticoagulation testing is to carry out three separate calibration steps:

CALIBRATION GUIDELINES:

The **WHO** procedure for calibrating reagents and calculating an **ISI** requires:

- 60 fresh plasma samples from stabilized orally anticoagulated patients or
- 60 chemically depleted plasmas
- 20 fresh plasma samples from "normal" subjects
- Manual tilt-top methodology to be carried out
- Orthogonal linear regression done comparing the natural log of the **IRP** and the vendors' reagent to obtain an **ISI**.

SECONDARY GUIDELINES REQUIRED FOR COMMERCIALIZATION AND INTRODUCTION OF AUTOMATION:

- Calibration of each vendor instrument/reagent pair against the results of the first calibration
- Production of calibration and control standards that can be stabilized and mailed to the client laboratory

ADDITIONAL WHO RECOMMENDATIONS TO ATTAIN THE MAXIMUM PRECISION INCLUDE:

PRECISION	RECOMMENDATION
HIGH	At least 10 laboratories against the three species of **WHO IRP** - **ISI** = average (**ISI** of three species).
MODERATE	Secondary standards against one or more species by at least two laboratories.
LOW	Calibration of individual reagents and batch-to-batch testing by the manufacturer using pooled plasmas.

PRECISION	WEAKNESS
HIGH	Cost and problems using present statistical methods to arrive at the ISI.
MODERATE	This is optimal in terms of cost versus precision but requires individual plasmas be used.
LOW	Pooled Coumadinized or depleted plasmas are not the same as individual Coumadinized plasmas

LIMITATIONS OF THE WHO CALIBRATION GUIDELINES:

A we can see, calibration guidelines recommended by **WHO** entail an expensive and complicated process that introduces many points where inaccuracies and imprecision can become additive leading to in appropriate **ISI** values. This has lead researchers and laboratorians to question the guidelines including but are not limited to:

- What does it mean to have stabilized orally anticoagulated patients?
- How are the normal subject plasmas chosen by each vendor?
- How can regression analyses using different patient plasmas assure comparability between different vendor reagents?
- How can calibration against secondary and tertiary **WHO** standards assure accuracy against the exhausted **IRP**?
- Knowing the limitations of regression techniques, how can the clinical reliability of the INR be assured?
- Are freezing and lyophilization methodologies of stabilizing calibration and plasma standards comparable?
- Are Coumadin treated and chemically depleted plasmas comparable?

GENERAL DISCUSSION OF LITERATURE FINDINGS:

The literature attempts to address each of these questions often separately and using disparate approaches. The results are not fully satisfactory. In each case there is at least one study that confirms that the above questions are significant depending on exactly how the research and clinical trials are carried out. And as noted above, **WHO** acknowledges normal and treated patient plasmas are not comparable to each other requiring a full sixty specimens from treated patients and an addition twenty from “normal” persons. In response to these requirements many vendors have gone to pooled plasmas. However, the average and distribution of sixty plasmas is not statistically comparable to a smaller number of pooled plasmas since mixing plasmas does not arithmetically average the Prothrombin times given that the relationship between **PT** and individual factor levels is itself not linear.

MANUAL VERSUS AUTOMATED METHODOLOGY:

Original research upon which the **INR** was certified was manual tilt-tube method. However, soon after this was established most vendors went to automated systems that employ a variety of principles for introducing the reagents and sensing the formation of a clot. The two most often used today are photo-optical that measure transmittance not absorption and nephelometry that measure light scatter. There has been a problem attaining comparability between these two methodologies when measuring single, well defined analytes so attaining comparability for **INR** is problematic. Even where the principle is the same, different technical implementation may introduce significant variance. For example, in one study there was as much as a 10% difference between two different instruments that employed a photo-optical principle.

VENDOR VERSUS LABORATORY DERIVED ISI’s:

Furthermore, studies have shown that the mean deviation of 95 client laboratories test systems using vendor **ISI**’s from the “true” **WHO** standard was 14.4% whereas locally calibrated **ISI**’s reduced this to 1.04%. The use of direct **INR** interpolated plots reduced the inter-laboratory **CV** from 12% to 6%. This is not entirely adequate since a **CV** over 3% usually considered a problem indicating that there will be significant variation at the clinically significant **INR** range where decisions to adjust Coumarin therapy are usually made. More recently, vendors have been able to get down to a **CV** of around 3% for some instruments.

WITHIN LABORATORY ERROR:

Vendors and laboratories need to consider imprecision in doing a single within "exercise" study to determine the **ISI**. It has been found that numerous studies done at different times leads to a significant variation in **ISI** as much as 5% and this can cause and additional component of variation and error leading to under or over treatment of patients at the clinical cut off values defined for that instrument/reagent pair.

BETWEEN LABORATORY ERROR:

Since vendors do an internal calibration and then send their reagents out to clients for use in their laboratories inter laboratory imprecision is the most important factor affecting clinical value of the **PT/INR**. Here, **CV**'s may vary from 1.7% up to 6.8% with values up to 3% considered acceptable.

LYOPHILIZED VERSUS FROZEN PLASMAS

In addition, use of lyophilized plasma provided by vendors may not match the original methodological outcomes from the foundational studies where fresh human plasma form health persons were used.

VENDOR ISI's THAT ARE THE SAME OR SIMILAR DO NOT ASSURE COMPARABILITY:

In almost all cases vendor reagents have had an **ISI** > 1 and in the beginning, many were even above 2.0. In 1992 the range in USA was 1.4 - 2.8. Now most **ISI**'s are below 2.0 and many are within 10% of 1.0 which shows an attempt to eliminate the **ISI**'s influence in the calculation of the **INR** in that an **ISI** = 1 defaults to the simple ratio of the patient's **PT** to the laboratory's mean **PT**. In combination with thromboplastins made from recombinant tissue factor, there is improved uniformity of the surface activator structures. Both advances have allowed for very good chronologic and inter/intra-laboratory comparability where the instrument/reagent pair are the same. However, despite these advances, different vendor instrument/reagent pairs with **ISI**'s close to 1.0 are not always comparable as noted above and the literature has begun to suggest that each laboratory define its own **ISI** through a final calibration step.

IMPACT OF BUSINESS CONSOLIDATION:

There have been numerous consolidations in the medical laboratory field over the last fifteen years, so an instrument may now be serviced by a tertiary or even quaternary vendor. This can lead to some confusion as to the actual operational characteristics of both the instruments and the reagents provided for their use. The laboratorian needs to be careful to distinguish between vendor publish theoretical maximum achievable values for **PT** and the maximum reportable **PT** based on control materials that provide actual validation. These often are far below the theoretical making **INR**'s above the 4.0 – 5.0 range questionable.

13 TECHNICAL APPLICATION OF THE INTERNATIONAL NORMALIZED RATIO – CALIBRATION OF ISI

As the field of anticoagulation monitoring progresses and more and more demands are being made on the laboratory to provide a high level of accuracy and precision, researchers have moved to establishing laboratory-based determination of **ISI** for the specific instance of instrument and lot of reagent. When applied under optimal conditions it appears that individual laboratories can attain a very high level of precision between within day runs and between daily runs allowing a more reliable evaluation of anticoagulant trending and pre-emptive modulation of Coumadin therapy before an adverse bleeding or thrombotic event occurs. **WHO** and research recommendations are:

- Exactly twenty patient samples are to be used in determining the average **PT**.
- A geometric mean is calculated instead of the arithmetic average.
- A logarithmic comparison of vendor reagent to certified plasmas calibrated against a **WHO IRP**.
- The results of the laboratory and certified plasmas are plotted using logarithmic scale.
- If the linear regression result is acceptable then a slope is calculated as the internal International Sensitivity Index [**ISI**].
-

CERTIFIED PLASMAS

There are two major types of certified plasmas. The actual preparation of certified plasmas will influence the outcome of client laboratory calibrations. There are two major types:

- **AVK**: **A**nti-**V**itamin **K** Treated Patients.
- **ART**: **ART**ificially Depleted of Prothrombin Complex Factors.

Their reliability depends on methodology used to collect and prepare them for storage and shipment. **ART** plasma is usually collected from healthy individuals and prepared by adsorption of vitamin K – dependent clotting factors with barium sulfate to create a series of plasmas with **INR** values that span the entire therapeutic range. In contrast, **AVK** plasma is collected from patients with stabilized Coumarin therapy at various therapeutic levels. This limits obtaining adequate plasma from abnormally high **INR**'s in contrast to **ART** where any level of depletion can be achieved. However, the **AVK** is more like the specimens that will be tested by the client laboratory and so, hypothetically, are more reliable.

COMPARISON OF THE TWO PLASMA TYPES:

ADVANTAGES OF AVK OVER ART:

- Closer agreement with fresh plasmas
- Better inter-laboratory agreement

DISADVANTAGES OF AVK OVER ART:

- Must be pooled.
- Inherent limitation range of therapeutic ranges of **INR** plasmas since they are taken from real patients.
- Inherent limitation on total volume of any one **INR** plasma since they are taken from a single real patient.

And these plasmas can be preserved and shipped in one of two ways:

- Frozen
- Freeze-dried – lyophilized

In comparison to lyophilized plasmas, frozen plasmas maintain good correlation with fresh plasmas when thawed and tested within a reasonable amount of time. The disadvantage is logistical in that lyophilized specimens have a long shelf life with no significant cost of shipping, storing, and risk of degradation due to accidental thawing. However, many studies have shown that lyophilized plasmas are very sensitive to the actual standard thromboplastin used to calibrate against, the reagent/instrument pair used by the client laboratory, and method of freeze drying.

NUMBER OF PLASMA SPECIMENS NEEDED FOR CALIBRATION:

SPECIMEN RANGE: The specimens used for calibration efforts should cover the therapeutic range and critical clinical decision-making point so this ranges from an **INR** of 1.5 to 4.5. Depending on specific methodology some researchers recommend determination of fibrinogen and factor V levels and assure that they are within the range of 60 – 140% to avoid significant variation in end point clot formation.

ISI CALIBRATION: **WHO** requirement: sixty lyophilized abnormal **AVK** samples or twenty lyophilized abnormal samples plus at least seven lyophilized normal samples. Pooling samples allows for fewer samples if several test runs are carried out.

INR CALIBRATION: At least four samples are needed; one normal and three abnormal pools of ten or more plasmas yielding **CV**'s of as low as 0.1 up to 4.6%.

NUMBER OF RUNS RECOMMENDED FOR CALIBRATION:

ISI CALIBRATION: **WHO** recommends **PT**'s be measured in quadruplicate in the same working session with the client reagent/instrument pair for the full set of normal and **AVT** plasmas. In addition, it is recommended to carry this out on three separate sessions or days to identify day-to-day variations and adjust for this.

INR CALIBRATION: **WHO** recommends **PT**'s be measured in duplicate in the same working session with the client reagent/instrument pair for the full set of normal and **AVT** plasmas. In addition, it is recommended to carry this out on three separate sessions or days to identify day-to-day variations and adjust for this.

SPECIMEN QUALITY AND PRESERVATION RECOMMENDATIONS FOR DOING COMPARISON STUDIES:

The most important aspects of specimen quality that will affect the **PT** and therefore **INR** comparison studies are:

- Platelet Poor Plasma — Incorrect tube will invalidate test results
- Calcium Poor Plasma through Citrate concentration – 0.105 M (3.2%)1:9 ratio by volume is optimal.
- Manipulation of draw site — Minimize and place tourniquet after cleaning site not before
- Size of needle — Maximize 18 gauge if possible
- Preanalytical Preparation — Centrifugation – varies by vendor
- Length of time from draw to testing — Minimize if possible
- Storage – Short Term — 18 – 24° C for up to 48 hours centrifuged or uncentrifuged
- Storage – Long Term — -20 ° C for up to 2 weeks centrifuged thawed to 37 ° C rapidly
- Type of specimen tube — Controversial as to glass versus plastic by vendor
- Interfering substances — Lipemia, hemolysis, bilirubin – may be compensated by vendor

VALIDATING INR'S BETWEEN INSTRUMENT/REAGENT PAIRS:

In order to determine if the INR results for between any two instrument/reagent pairs are comparable I recommend the following:

- Draw a set of twenty normal patients and split the sample testing in both systems simultaneously.
- As Coumadin treated patients with elevated INR's are identified, retain residual specimen to test on the second instrument/reagent pair.
- Collect at least twenty elevated INR's and test in both systems simultaneously.
- Do a linear regression and examine the r^2, slope and intercept. If there appears to be good linearity calculate the percentage of results that would lead to a change in therapy between the two instrument/reagent pairs.

14 RECOMMENDATIONS

RESEARCH AND CONSULTATION:

I recommend that laboratorians do due diligence on the contents of this presentation as a starting point for evaluation of their coagulometer testing methodologies. Appropriate consultation with the institution's Hematologist as well as experts throughout the field is also of value. This allows for the establishment of a foundational knowledge base that can be used to solve the problems facing them in anticoagulation monitoring in terms of clearly delineating what can and cannot be done for our clinicians using the **PT/INR**.

VENDOR DISCUSSIONS:

I recommend that laboratorians do due diligence on the technical and operational aspects of their instruments with focus on:

- Characteristics of Thromboplastin reagent.
- Reliability of **ISI** in terms of internal **CV**'s and **ISI**'s as close to 1.0 as possible.
- Principle of sensing clot formation.
- Methodology of maintaining instrument maintenance and calibration over time.

CALIBRATING ISI's:

This approach to increasing internal reliability of **INR**'s may be of real value. However, great care must be taken in developing the methodology and properly carrying out the testing necessary to obtain the local **ISI**. The downside is the frequency with which it will be necessary to do this given vendor changes of reagent sources, lots, and instrument maintenance and repair history.

VALIDATING COMPARABILITY BETWEEN INSTRUMENT/REAGENT PAIRS:

IN ONE LABORATORY:

There are two alternatives: Use of vendor supplied plasmas or collection of internally obtained patient plasmas. For the latter:

Due to operational limitations on the amount of plasma that can be collected from patients the following recommendation is made:

- Accumulate at least twenty plasmas from persons without history of anticoagulation and in good health.
- Accumulate at least twenty plasmas from patients on stable anticoagulation therapy.
- During accumulation store under appropriate conditions depending on the amount of time to testing.
- Split samples and run on both instruments simultaneously by the same technologist using standard operating procedure.
- If the instrument allows additional testing on residual plasma volumes, then repeat during a different day and shift.
- Do the linear regression correlations between the two instrument results for both data sets separately and together.
- Determine if the two populations have similar or different slopes and/or intercepts that could indicate a problem.
- If the correlation coefficient, slope, and intercept are acceptable calculate the percent clinical decisions changed.
- Present the results of the study to the clinicians involved with anticoagulation for review and decision making.
-

If there are significant differences between the two instruments that cannot be resolved to the clinicians' satisfaction or to meet locally acceptable practice, then consider:

- Using only one instrument (what do you do when that instrument is down?), or
- Assigning each patient to one instrument (many problems with this), or
- Obtaining two instruments that are comparable, or
- See below.

IN TWO LABORATORIES:

- Find a nearby laboratory that uses an instrument/reagent pair as close as possible to yours.
- Approach this institution and propose a joint comparability study for the purpose of providing reliable **PT/INR**'s.
- Develop a comparability study using elements from the protocol above.
- Coordinate a means of communicating changes that may occur in either laboratory to the other to assure maintenance of reliability through additional or routine ongoing studies on split specimens.

DEVELOPMENT OF ANTICOAGULATION PROTOCOLS WITH CLINICIANS:

- Establish the most highly precise **PT/INR** results you are capable of.
- Work with your clinicians to establish initial **INR** cutoffs for anticoagulation therapy and modulation based on vendor and literature recommendations.
- Develop a system to monitor clinical outcomes of hemorrhagic and thrombotic events in anticoagulation patients.
- Modify **INR** cutoffs in response to results of monitoring these outcomes.

AND MY FINAL RECOMMENDATION IS:

BEGIN TO ASK FOR OUR VENDORS TO MOVE TO AN ANALYTE TESTING MODALITY THAT DETERMINES THE LEVELOF BIOACTIVE PROTHROMBIN PRESENT IN THE PATIENT'S PLASMA.

15 APPENDIX: KEY ASPECTS DETERMINING ACCURACY AND PRECISION USING PT/INR

Numerous investigations published in the literature over the last 30 years have shown continuing challenges attaining the goals set by the WHO committees and our clinicians in serving their patients. These are but not limited to:

PARTIAL LISTING OF KNOWN PROBLEMS WITH THE PT/INR TEST

- Automation versus the original manual methods used to determine vendor calibration to the WHO IRP.
- Exhaustion of the original IRP requiring calibration to secondary and now tertiary IRP's.
- Proliferation of Multiple IRP's each with different sensitivities.
- Variation in thromboplastin reagent sensitivity to specific coagulation factors.
- Different final buffering and preservation of vendor thromboplastin reagents.
- Variations in vendor reagent sensitivity both within and between lot runs.
- Reagents with absorbed factor V and I leading to altered INR's depending on their sensitivity to these two factors.
- Variations between frozen and lyophilized calibration and control plasmas for instruments.
- Documented administrative inaccuracy of published vendor ISI's.
- Different methods of introducing the thromboplastin reagent into the plasma sample.
- Different principles used by vendor instruments to sense clot formation.
- High ISI values well above 1.0 leads to poor inter laboratory precision in general.
- Low ISI's close to or equal to 1.0 don't deliver the expected increased precision at the point of clinical decision making.
- Nonlinearity of INR's leading to difficulty in interrelating results by clinicians
- The INR Is a function that produces a unitless nonlinear result not interpretable as the PT seconds.
- Where the PT_{AVG} is identical, small changes in the ISI lead to very high imprecision where INR is above 4.0 – 5.0.
- INR increases variation between two different instruments in comparison to the PT and these differences widen at higher PT's because if we raise a number to a power greater than 1 as it rises the calculation rises faster leading to an asymptotic event.
- Depending on the established PT_{AVG}, ISI value, and actual test results two different instruments can have an INR range where instrument one is predictably lower than instrument two and a range where instrument one is predictably higher than instrument two.
- The difference between INR's for two different methologies can be shown to be nonlinear and therefore not a first order process that could easily be corrected by the addition of another mathematical factor.
- Variations in operator procedures.
- Methodology of phlebotomy that may cause undue hemolysis or low-level activation of coagulation factors.
- Phlebotomy competency leading to low level activation of coagulation factors.
- Citrate concentrations not uniform between vendor specimen tubes.
- Citrate concentrations not uniform due to incomplete draws
- Delay to testing.
- Specimen temperature.
- Genetic Variations in Factor activity by ABO type.
- Genetic and environmental variation between patients' vitamin K dependent factor activity.
- Genetic differences cause Coumarin to affects each patient's vitamin K dependent factors differently.
- Co-morbid states causing low level activation of coagulation factors by alternate humeral and cellular pathways.
- Liver disease with dysfibrinogenemia.
- Presence of lupus anticoagulant.

Want to know more? Contact me at mark@manxenterprises.com
See our website: www.manxenterprises.com

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