DIALOGUE AND DISCUSSION

2 Unification of the NCA and the ASCP Board of Registry
Susan Morris

CLINICAL PRACTICE

5 Heparin-Induced Thrombocytopenia (HIT): A Case Study
Catherine E Newkirk

RESEARCH AND REPORTS

12 CLS to Higher Education Administrator: The Right Navigational Skills
Suzanne Campbell, Barbara Y LaCost

21 Platelet Function Testing: Auditing Local Practice and Broader Implications
Emmanuel J Favaloro, Soma Mohammed

32 Effects of Glucosamine and Celadrin on Platelet Function
Pei-Chun Lin, Samuel O. Jones, David L. McGlashon

FOCUS: Obesity and Metabolic Syndrome

37 Obesity and Metabolic Syndrome Overview
Wayne Gade, Jean Gade, Melissa Collins, Jessica Schmit, Nicole Schupp

39 Failures of Feedback: Rush Hour Along the Highway to Obesity
Wayne Gade, Jean Gade, Melissa Collins, Jessica Schmit, Nicole Schupp

51 Beyond Obesity: The Diagnosis and Pathophysiology of Metabolic Syndrome
Wayne Gade, Jean Gade, Melissa Collins, Jessica Schmit, Nicole Schupp

CONTINUING EDUCATION QUESTIONS

62
Unification of the NCA and the ASCP Board of Registry

SUSAN MORRIS

ABBREVIATIONS: AGT – Association of Genetic Technologists, ASCP – American Society for Clinical Pathology, BOC – Board of Certification, BOR – Board of Registry, NCA – National Credentialing Agency.

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Introduction
Professional credentials are the cornerstone for defining and achieving recognition of an independent scope of practice. When multiple competing credentials exist, that identity is less clear to those outside the profession. The combination of the two largest laboratory certification organizations in 2009 was a landmark event for increasing recognition of laboratory professionals.

During the 1970’s, irreconcilable differences erupted between pathologists and technologists regarding the composition and operation of the ASCP Board of Registry, which led to the formation of the National Credentialing Agency. NCA was founded as a free-standing certification organization, based on the principles of peer certification, exam development based on practice analyses and psychometrics, and mandatory re-certification to demonstrate continued competency.

Over time, there were outcries from educators, students, employers, and professionals in the field. The complexities that resulted from having two competing certifications for NAACLS-accredited programs were making their lives and careers more difficult. They wanted the two organizations to find a way to come together.

After thirty years of fierce competition, both organizations began to recognize that the differences that created the separation, and loomed so large at that time, had all but disappeared. Although there were still pathologists participating on the Board of Registry (BOR), they were clearly minority stakeholders on the board. Both NCA and the BOR had chosen to participate in an accreditation program that ensured best practices, including the requirement of re-certification. Both organizations began to realize that the way to have the greatest impact on our profession was to focus the passion for excellence of our committed volunteers – together – at the same table.

Unification!
On July 21, 2009, a Combination Agreement was signed to merge all of the certification activities and operations of the NCA and the BOR into a single organization, to be known as the ASCP Board of Certification (BOC). The new organization is a modification of the previous BOR structure. Certification activities will operate with complete autonomy from the three sponsoring organizations of the BOC – ASCP, ASCLS, and the Association of Genetic Technologists (AGT). Daily operations will be maintained through ASCP.

The agreement became effective October 23, 2009. At the first meeting of the BOC on October 31-November 1, six former NCA representatives joined the Board of Governors. Four are representing ASCLS, and two representing AGT. ASCP has five laboratory profes-
sional representatives, and five pathologist representatives. There are eight additional governors representing participating organizations. These include AABB, AACC, American Association of Pathology Assistants, American College of Microbiology, American Society for Cytopathology, American Society for Hematology, CLMA, and National Society for Histotechnology. In addition, there is one public member, for a total of twenty five.

The BOC remains committed to peer credentialing, and to responsiveness to the needs of the profession, adding exams for new specialties and practices levels as healthcare, medicine, and the science warrant. Examination Committees of the BOC now include members from both the relevant NCA and BOR exam committees. It will be their responsibility to hybridize the exam content from both former exams.

**Implications**

As of October 23, 2009, several changes occurred in credential terminology as comparable active certification designations were reconciled. The ASCP suffix will attach to each certification designation, maintaining that strong brand recognition with employers. The baccalaureate-level certification designations Medical Technologist (MT) and Clinical Laboratory Scientist (CLS) were replaced by Medical Laboratory Scientist, MLS(ASCP)CM. This new terminology is consistent with that used in most of the rest of the world. Associate-level certification designations were converted to Medical Laboratory Technician, MLT(ASCP). The cytogenetics certification designation became Technologist in Cytogenetics, CG(ASCP)CM, and the molecular certification designations became Technologist in Molecular Biology, MB(ASCP)CM. The superscript CM is included after the (ASCP) as (ASCP)CM to designate the continuing certification maintenance by the laboratory professional. At the first BOC meeting, a decision was made to convert active NCA Clinical Laboratory Director and Clinical Laboratory Supervisor designations to Diplomate in Laboratory Management, DLM(ASCP)CM. Anyone holding an active NCA credential, or an ASCP CM credential was automatically converted to the new designations.

Any individuals who were formerly certified by NCA, but allowed their credential to lapse, may re-activate their credential (without taking another examination) by completing the certification maintenance/recertification requirements. This will be important for all still in practice to do, because records at the BOC will only allow credential verification for “active” credentials. Because re-certification was always mandatory for NCA credentials, any lapsed NCA certified individuals will be reported as having no valid credential on record if a request for verification is received by the BOC.

There will be communication to all certificants strongly encouraging all to participate in recertification by continuing education, the hallmark of all recognized professions within and outside of healthcare.

Individuals who were credentialed by the BOR prior to 2004, when certification maintenance became mandatory, are encouraged to convert their credential to the new designation by completing the certification maintenance requirements. For the MT(ASCP) individuals credentialed prior to 2004 who become “active” in their certification maintenance, their credential will be revised to MLS(ASCP)CM and will be shown as that for the three-year certification maintenance period at which time they will be required to update for the next three year cycle.

Because there were differences in the NCA and BOR re-certification CEU procedures, both procedures will be accepted through Spring 2010, allowing the Certification Maintenance Committee time to review and reconcile those procedures. This means that NCA certificants who were due to re-certify in February 2010 can submit the CEU’s they have been accumulating over the past three years under the NCA procedures.

NCA ceased operations and is dissolving as a corporation, in compliance with their by-laws. On October 31, 2009, ASCLS and AGT officially began sending representatives to the new BOC. All records and examination materials have been transferred to the BOC. Any remaining financial assets, after all expenses
have been paid, will revert to the two sponsoring organizations.

Our Work Is Not Finished
Not forgetting the lessons from the past, we are now moving forward, together, to adopt best practices and enhance pride and respect for our profession. Our constituents are the winners. When competition becomes the focus, no one wins. Standardizing credential designations and the new descriptive generalist credential will decrease confusion, enhancing our credibility and respect from other healthcare professionals and the public. It will also simplify employers' tasks when setting entry-level standards for job descriptions and recruitment. Students will no longer have to choose between competing exams, or incur the expense and stress of taking two exams. Program Directors can focus their efforts on getting all of their graduates to become certified. They will also only need one performance report, decreasing cost and complexity for their program. Within the profession, we will now speak from consensus on entry-level standards. There will be additional issues to be resolved, and that will be part of the on-going work of the BOC.

Taking a lesson from nature, birds flying in formation conserve energy, protect and support each other, and combine their strength to advance all in the group toward a common destination. We had been behaving more like a flock of sea gulls than a flock of geese. In the area of professional certification, we will now commence to create a unified formation, and move forward at a faster pace. We truly have reason to celebrate.
Heparin-Induced Thrombocytopenia (HIT): A Case Study

Catherine E Newkirk

ABSTRACT: Heparin-induced thrombocytopenia (HIT) ranges from an asymptomatic reaction to heparin with a transient mild thrombocytopenia (HIT I) to a life- and limb-threatening immunological reaction, heparin-induced thrombocytopenia with thrombosis (HITT or HIT II). HITT can occur in patients with any heparin exposure and must be recognized and treated quickly and appropriately to prevent symptomatic and/or fatal thrombosis. HIT will be discussed using a case study approach.

ABBREVIATIONS: APTT = activated partial thromboplastin time; AT = anti-thrombin; ¹⁴C = carbon-14; CT = computerized topography; DTI = direct thrombin inhibitor; Fc = fragment crystallizable; FDA = Food and Drug Administration; HIPA = heparin-induced platelet aggregation; HIT = heparin-induced thrombocytopenia; HITT = heparin-induced thrombocytopenia with thrombosis; IgG = immunoglobulin G; INR = international normalized ratio; IU/ml = international units per milliliter; IV = intravenous; LMWH = low-molecular-weight heparin; PF4 = platelet factor 4; RBC = red blood cell; SC = subcutaneous; SRA = serotonin release assay; UFH = unfractionated heparin.

INDEX TERMS: argatroban, heparin, lepirudin, thrombocytopenia, thrombosis.

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INTRODUCTION

The use of heparin to treat and prevent thromboembolic disorders is well established. Heparin, however, is also associated with heparin-induced thrombocytopenia (HIT), a potentially life- and limb-threatening immunological reaction. If recognized early and treated appropriately, the risks of HIT-associated thrombotic complications can be significantly reduced.

CASE HISTORY

A 70-year-old male in good health arrived in his local Emergency Room with a table saw injury to his right hand resulting in a near complete amputation of his right thumb and laceration of his middle finger. He was immediately transferred to the regional medical center where an attempt was made to reattach and revascularize the thumb. He was given a bolus injection of heparin intraoperatively and an intravenous infusion of heparin preoperatively and postoperatively. On day 8, he was sent home. On day 18, he had a follow-up visit with the surgeon. Finding the thumb showing signs of failure, he was scheduled for surgery. The patient complained of pain in his left leg with increasing
intensity over the past week. The surgeon ordered X-rays of the lateral spine which showed advanced degenerative arthritis. Prior to surgery, the whole blood platelet count was $135 \times 10^9$/liter, (reference range: $160-410 \times 10^9$/liter). This platelet count, performed on day 19, was the first recorded measurement of platelets for this patient. Partial amputation of the right thumb, due to poor vascular supply, was performed on day 20. On day 26, he complained that the pain in his left leg was getting progressively worse and his right heel was tender. His physician noted arthritis, spur and/or gout and ordered Celebrex and Hydrocodon. His platelets were $108 \times 10^9$/liter. Two days later, he was admitted to his local hospital with a pulseless, cyanotic left leg. An angiogram revealed an embolism in his left superficial femoral artery. The next day, an embolectomy was performed to attempt revascularization. The arteries, described as appearing “full of sand”, were irrigated with a heparin solution and 48 hour Fragmin therapy was initiated (Fragmin, given by subcutaneous injection, is a low-molecular-weight-heparin.). Platelets were measured at $70 \times 10^9$/liter. The patient was transfused with 6 units of platelets and 2 units of packed red blood cells. On day 31, amputation of his left leg below the knee was performed due to a failed revascularization attempt. Platelets were $121 \times 10^9$/liter. Heparin was continued during debridement, revising and closing the stump. On day 48, the patient was discharged to a rehabilitation hospital where an exam on admission revealed an extremely swollen left leg and thigh. An ultrasound revealed acute venous thrombosis and the patient was transferred to the vascular service where IV heparin was started. On day 51, platelets had decreased to $32 \times 10^9$/liter and the patient exhibited diaphoresis, chest pain and shortness of breath. On day 52, the patient had an abrupt onset of slurred speech with weakness in the left upper and lower extremity. A CT scan of his brain revealed a blocked right middle cerebral artery and an electrocardiogram showed acute anterior wall transmural infarction. A CT scan of his lungs demonstrated pulmonary edema. His platelets measured $41 \times 10^9$/liter. Neurology, hematology, pulmonary and cardiology consults were called in and the first suggestion of heparin-induced thrombocytopenia with thrombosis (HITT) was made. Heparin was discontinued and an emergency order for Hirudin from the regional medical center was placed. On day 53, a repeat CT scan of the brain showed marked edema, infarction of the right internal carotid artery and the right middle cerebral artery, brain stem herniation, herniation of the right frontal lobe, and dilation of the left lateral ventricle. The prognosis was listed as “abysmal” and the patient expired that afternoon. His presumed cause of death was listed as “White Clot Syndrome” secondary to Fragmin and heparin therapy with multiple thromboses.

HEPARIN OVERVIEW

Heparin, discovered over 80 years ago by McClean, is a heterogeneous mix of glycosaminoglycans with antithrombotic properties. It requires a plasma cofactor, anti-thrombin (AT). Heparin binds to AT causing a conformational change that results in its activation and this activated AT inactivates coagulation factors IIa, Xa, IXa, XIa, and XIIa (IIa and Xa are most sensitive).¹

Heparin use is well established in the U.S. to treat and prevent thromboembolic disorders. One trillion units are used annually. Heparin has proven efficacy, rapid onset of action, ease of monitoring with the APTT, rapid neutralization with protamine and low cost.¹,²,³

Two forms of heparin are commonly used (Table 1). Unfractionated heparin (UFH) is a purified preparation of glycosaminoglycans derived from beef lung or pork intestine. UFH is composed of a large number of related compounds with molecular weights from 1,000 to greater than 40,000.⁴⁵,⁶ UFH non-specifically binds to plasma proteins, macrophages, and endothelial cells, among other things, and thus must be monitored with the APTT to find out how much is acting as an anticoagulant. Low-molecular-weight heparin (LMWH) is derived from UFH by chemical or enzymatic depolymerization. LMWH has a molecular weight range from 2,000-9,000 and has a reduced ability to inactivate thrombin because inactivating thrombin is chain length dependent. LMWH does not need to be monitored because it lacks non-specific binding and, therefore, has excellent bioavailability and predictable distribution. Both UFH and LMWH are cleared by the kidney.¹,³
Table 1. Comparison of Two Forms of Heparin

<table>
<thead>
<tr>
<th>UFH</th>
<th>LMWH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inexpensive</td>
<td>Expensive</td>
</tr>
<tr>
<td>Non-specific binding</td>
<td>Lacks non-specific binding</td>
</tr>
<tr>
<td>Binds PF4</td>
<td>Less efficient binding of PF4</td>
</tr>
<tr>
<td>½ life - 60 min (IV, longer SC)</td>
<td>½ life 3-6 hours (SC)</td>
</tr>
<tr>
<td>Neutralized by protamine</td>
<td>Not fully normalized by protamine</td>
</tr>
<tr>
<td>Monitor with APTT</td>
<td>No need to monitor</td>
</tr>
<tr>
<td>HIT (porcine mucosa) 4.8%</td>
<td>HIT (enoxaparin) 0.6%</td>
</tr>
<tr>
<td>HIT with thrombosis 3.3-3.6%</td>
<td>HIT with thrombosis 0.3-0.6%</td>
</tr>
<tr>
<td>MW range 1,000-&gt;40,000</td>
<td>MW range 2,000-9,000</td>
</tr>
<tr>
<td>Anti-Xa/Anti-IIa ratio 1:1</td>
<td>Anti Xa/Anti-IIa ratio 2:1 to 4:1</td>
</tr>
</tbody>
</table>

HEPARIN INDUCED THROMBOCYTOPENIA (HIT) OVERVIEW
HIT I appears to be a benign reaction to heparin beginning 1-4 days into therapy with a transient mild thrombocytopenia (100 - 150 x 10^9 platelets per liter) that is not immune mediated. HIT I is not associated with thrombosis and the patient is asymptomatic. HIT I is manifested in 10-20% of patients treated with IV heparin.4

HIT II, on the other hand, is found in 0.5-5% of patients on IV heparin, 5-10 days into therapy6,7,8 (a distinguishing factor from HIT I); but can occur with any heparin exposure (heparin flushes, heparin coated catheters, etc.).2,9 Platelet counts are less than 100 x 10^9/liter or have a 30-50% decrease from baseline.2,4,10 Thirty to seventy-five per cent of patients with HIT II have thrombosis which can manifest as deep vein thrombosis, pulmonary embolism, skin necrosis, limb gangrene, myocardial infarction and /or stroke.2,9,10,11,12 There is a 20% mortality rate with an additional 10% amputations and/or major morbidity.6 HIT II is immune mediated and heparin must be stopped immediately with administration of alternative anticoagulation.2,4,6,13

HIT II was first described as “White Clot Syndrome” in 1958 by Weismann and Tobin. It was found in 10 patients on heparin; six of whom died. They described pale salmon-colored clots, rich in platelets. White Clot Syndrome is a misnomer because platelet-rich clots are uncommon in HIT II, in which the majority of clots are RBC-rich fibrin clots.5

HIT II has three classifications: isolated HIT with thrombocytopenia only, yet substantial risk of symptomatic and fatal thrombosis; HITT with thrombocytopenia and thrombosis; and rapid onset HIT with thrombocytopenia within 24-48 hours of heparin administration. Rapid onset HIT is associated with recent heparin exposure (within the past 100-120 days). It is not an anamnestic immune response but due to already circulating HIT antibodies.11,14,15

HIT MECHANISM OF ACTION
Alpha granules in activated platelets release PF4 which binds to other platelets activating them.5 Heparin binds to PF4 and causes a conformational change in the PF4 protein, “bundling” 4 or 8 molecules.5 An antibody (90% IgG) recognizes this PF4-heparin complex as an antigen and forms immune complexes with it.5,8,10,13 This antibody activates platelets through Fc receptors on the same or adjacent platelets.5,8,13,14,16,17 This activation causes the release of more PF4 and prothrombotic microparticles with resultant thrombin generation and fibrin clot formation.5,10,15 In addition, these antibodies can bind to Fc receptors on monocytes and endothelial cells, activating them to produce tissue factor, further stimulating thrombosis.5,8,14,17,18

HIT II is more commonly associated with UFH of bovine lung or porcine mucosa origin, showing up in 1-5% of recipients.5,6,7 HIT II can also develop with LMWH in 0.2-0.8% of recipients.4,6 Bovine lung UFH has a greater incidence of HIT II than porcine mucosal UFH and porcine mucosal UFH HIT II shows up more frequently than with LMWH.11,19 This appears to be because heparin chains larger than 12 saccharide units are required to form antigenic PF4-heparin complexes and greater polysaccharide chain lengths are thought to cause increased immunogenicity.6,17
Patients on heparin should have their platelets monitored, with a baseline count followed by counts every other day between days 4-14 or until the heparin is stopped, whichever occurs first. HIT should be suspected if platelets are decreased between days 4-14 of exposure to heparin (less than 100 x 10^9/liter or a 30-50% decrease below baseline) or if there are any clinical signs or symptoms of a thrombus. To assess a pretest probability of HIT, a clinical scoring system known as the “4 Ts” can be utilized (Table 2).

If HIT is suspected, all forms of heparin must be stopped immediately without waiting for further laboratory testing. Heparin cessation alone, however, does not reduce thrombosis or death because thrombin is still being generated. A direct thrombin inhibitor (DTI) must be administered for a minimum of 7 days. LMWH should not be used because it shows complete cross reactivity with HIT antibodies. The DTI should be continued until platelets return to normal and reach a stable plateau, at which time it is safe to administer warfarin (an oral Vitamin K antagonist). The DTI should not be stopped until there are at least five days of overlap with warfarin administration and until the International Normalized Ratio (INR) is in therapeutic range for at least 2 days to assure the warfarin is working to prevent thrombin generation. Overlapping treatments is 

<table>
<thead>
<tr>
<th>4 Ts</th>
<th>2 Points</th>
<th>1 Point</th>
<th>0 Points</th>
</tr>
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<tbody>
<tr>
<td><strong>Thrombocytopenia:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decrease</td>
<td>&gt; 50%</td>
<td>30 – 50%</td>
<td>&lt; 30%</td>
</tr>
<tr>
<td>and</td>
<td>and</td>
<td>or</td>
<td>or</td>
</tr>
<tr>
<td>Nadir</td>
<td>≥ 20 x 10^9/L</td>
<td>10 – 19 x 10^9/L</td>
<td>&lt; 10 x 10^9/L</td>
</tr>
<tr>
<td><strong>Timing of platelet count decrease:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No recent heparin (within 100 days)</td>
<td>Clear onset between days 5 – 10</td>
<td>Onset between days 5 – 10 but unclear history (e.g. missing platelet counts) or &gt; 10 days</td>
<td>≤ 4 days</td>
</tr>
<tr>
<td>Recent heparin</td>
<td>or ≤ 1 day (prior heparin exposure within 30 days)</td>
<td>or ≤ 1 day (prior heparin exposure 30 - 100 days ago)</td>
<td></td>
</tr>
<tr>
<td><strong>Thrombosis or other sequelae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>New thrombosis (confirmed); skin necrosis at heparin injection site; acute systemic reaction post-IV UFH bolus</td>
<td>Progressive (increase in size) or recurrent thrombosis; erythematous skin lesions at heparin injection site; suspected thrombosis (not proven)</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td><strong>Other causes for Thrombocytopenia</strong></td>
<td>None apparent</td>
<td>Possible</td>
<td>Definite</td>
</tr>
</tbody>
</table>

Note: Pretest probability score: 6-8, high; 4-5, intermediate; 0-3, low.
necessary because warfarin needs 4-5 days for effect and causes decreased Protein C and S (natural anticoagulants) before it decreases Vitamin K dependent coagulation factors (II, VII, IX and X), resulting in persistent thrombin generation linked to limb gangrene and blistering skin due to clots in the microvasculature. 2, 5, 8, 11, 14. The risk for thrombosis in HIT II persists for at least 6 weeks so warfarin treatment is recommended to be continued for a minimum of 2-3 months. 15 Infusion of platelets is contraindicated while the mechanism activating platelets persists due to possible resultant increased coagulation2 (though a recent retrospective study finds no evidence to withhold platelet transfusions, if clinically indicated11).

If there is a history of heparin use within the past 100-120 days and heparin is newly administered, the patient should be monitored for rapid onset HIT where the platelets decrease within hours.11, 15 It is recommended that a baseline platelet count be performed and repeated within 24 hours to catch this rapid decrease in platelets due to antibodies formed with the earlier heparin that are still in the circulation.10, 11, 14

DIRECT THROMBIN INHIBITORS (DTIs)

Direct thrombin inhibitors are key in the treatment of HIT because thrombin plays a central role in HIT. Activated platelets activate the coagulation sequence which generates thrombin. Thrombin enzyme activity depends on two domains: an active catalytic site and an anion binding exosite for fibrin recognition. Individual DTIs act differently on these sites. There are presently two DTIs approved by the FDA for the treatment of HIT: Lepirudin and Argatroban.2

Lepirudin is a recombinant form of Hirudin, an anticoagulant extracted from leeches.5, 15 Lepirudin was the first DTI approved by the FDA for HIT in 1998.2 Lepirudin is a bivalent inhibitor, binding both the catalytic site and the exosite of thrombin.5 In addition, Lepirudin inhibits both clot-bound and soluble thrombin, thus acting on existing and new thromboses and has no cross-reactivity with HIT antibodies;5, 17 although anti-hirudin antibodies are common (up to 84% of patients) and prevent repeated use.5, 11, 15

Argatroban is an arginine based synthetic thrombin inhibitor approved by the FDA in 2000 for the treatment of HIT.2, 5, 15 It is a monovalent inhibitor which only binds the active catalytic site on thrombin so it is not as effective as Lepirudin.5 It inhibits clot-bound and soluble thrombin; and has no cross reactivity with HIT antibodies.2 The high cost of both DTIs is minimal compared to the cost of major complications associated with HIT.2

LABORATORY TESTING

Laboratory testing includes temporal platelet counts, functional assays for serotonin release, heparin induced platelet aggregation, platelet aggregation, flow cytometry for activated platelet markers, and antibody detection with enzyme immunoassay.

Routine temporal platelet counts (a baseline and at least every other day between days 4-14) are more important than other studies.11 Platelets decrease before antibody titers rise and there is a high risk of thrombosis with a decreased platelet count (with or without detection of HIT antibodies).9, 11 Testing for antibodies without clinical indications of thrombosis is not useful.11

The serotonin release assay (SRA) is considered the gold standard for functional tests.8, 17, 21, 22 Platelets from normal donors are labeled with radioactive serotonin (14C serotonin) and added to different concentrations of heparin representing no heparin, therapeutic levels (0.1 to 0.5 IU/ml) and an overload (≥ 10 IU/ml).5, 8, 17, 22, 23 The overload inhibits binding and yields a negative test to assure that heparin is the culprit.23 Serum from a patient suspected of HIT is added and 14C serotonin released by activated platelets is measured by scintillation counter.22, 23 Positive results are confirmed by suppression with Anti-Fc receptor antibodies to assure that this is the site of activation.21,22 This assay is moderately-highly sensitive (a sufficiently high titer of antibodies is needed to activate platelets) and highly specific.2, 21
Heparin induced platelet aggregation (HIPA) involves combining washed platelets from healthy donors with sera from patients suspected of HIT and different concentrations of heparin (no heparin, therapeutic levels and an overload) in microtiter plates. If positive, platelets should fall out of solution as indicated by transparency on a microtiter plate.

Platelet aggregation, using platelet-rich plasma from healthy donors incubated with patient sera and known concentrations of heparin, is moderately-highly sensitive and highly specific. Aggregation is considered positive by a sharp slope (≥ 20% per minute) and maximum aggregation of ≥ 50% within 20 minutes.

An emerging method involving flow cytometry also uses platelets from healthy donors, different concentrations of heparin and sera from patients suspected of HIT. A monoclonal antibody labeled with a fluorescent tag to a platelet activation marker (such as the generation of microparticles) on the platelet surface is utilized. Binding of the antibody reflects activation of platelets and is analyzed by flow cytometry.

Enzyme-linked immunoassay methods utilize a microtiter plate coated with PF4 and heparin. Serum from a patient suspected of HIT is added, followed by anti-human IgG antibody with alkaline phosphatase and a chromogen tag. Change in absorbance corresponds to the presence of antibodies to the heparin-PF4 complex but does not demonstrate the ability of the antibody to activate platelets. Currently, the FDA requires reporting as positive or negative, but higher antibody titers are associated with the development of thrombotic complications. This method is extremely sensitive but only moderately specific.

When testing for HIT antibodies, an enzyme immunoassay should be performed first because it is extremely sensitive and, if negative, rules out HIT. If positive, it should be followed with the serotonin release assay which is highly specific for the HIT antibody.

HIT treatment, however, should be initiated upon clinical suspicion and not await laboratory testing confirmation because the potential is great for life- and limb-threatening complications.

CASE SUMMARY
This patient had a classic case of heparin-induced thrombocytopenia with thromboses, which escaped diagnosis at three different medical facilities and under the care of numerous doctors. He had his first exposure to heparin with emergency surgery to reattach his thumb. No baseline measurement of platelets was recorded at that time. He underwent a second surgery, with more heparin and his first platelet count, recorded on day 19, was 135 x 10^9/liter (reference range: 160-410 x 10^9/liter). Without a baseline for comparison, it is impossible to know if this represented a significant decrease in platelets. Over the next few weeks, platelets continued to decrease; more heparin was administered; an embolectomy was attempted; and Fragmin therapy followed. Fragmin is a LMWH that has high cross reactivity with HIT II antibodies. Platelets continued to fall and the patient was transfused with 6 units of platelets, possibly adding fuel to the fire. After amputation of his left leg below the knee with more heparin administered, platelets were recorded at 121 x 10^9/liter, probably increased due to the transfusions. Following discharge to a rehabilitation hospital, a heparin IV was started to treat acute venous thrombosis. Platelets continued to decrease and were recorded at 32 x 10^9/liter when the patient developed signs of stroke, myocardial infarction and pulmonary edema. A neurologist and hematologist, called in for consult, made the first suggestion of HITT. Heparin was discontinued and an emergency order of Hirudin was placed. Hirudin is an anti-coagulant extracted from leeches with anti-thrombin activity and low cross reactivity with HIT II antibodies. The diagnosis of HITT was considered much too late and the patient expired (53 days after the first heparin dose) before the Hirudin arrived.

Death of this patient could possibly have been avoided had a baseline platelet count been performed before the first heparin administration, with temporal platelet
counts performed every other day on days 4-14 following. A baseline platelet count is important in order to recognize a significant decrease in platelets and alert the physician to the possibility of HIT II. If HIT II was suspected early, all heparin could have been discontinued and a direct thrombin inhibitor administered with a transition to warfarin and continued anticoagulation therapy for 2-3 months. The patient’s outcome could have been decidedly better.

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REFERENCES

The peer-reviewed Research and Reports Section seeks to publish reports of original research related to the clinical laboratory or one or more subspecialties, as well as information on important clinical laboratory-related topics such as technological, clinical, and experimental advances and innovations. Literature reviews are also included. Direct all inquiries to David L McGlasson MS, MLS, 59th Clinical Research Division/SGRL, 2200Berquist Dr., Bldg. 4430, Lackland AFB TX 78236-9908, david.mcglasson@lackland.af.mil

The Right Navigational Skills

SUZANNE CAMPBELL, BARBARA Y LACOST

OBJECTIVES: To identify the experiences, training, and opportunities that directed and influenced the career paths of women clinical laboratory scientists that transitioned to higher education administrators.

METHODS: A multi-site case study design was selected for this qualitative research involving a purposive sample of eight research participants. Data collection was guided by ten open-ended questions in seven face-to-face and one telephone semi-formal interviews.

SETTINGS AND PARTICIPANTS: The purposive sample included women clinical laboratory scientists who held a current higher education administrative position at the dean’s level, including associate and assistant dean positions, in a university setting. The participants were located in eight higher education institutions in Nebraska, Illinois, Ohio, Tennessee, Missouri, and Texas.

MAIN OUTCOMES MEASURES: Leadership skills/characteristics, professional development opportunities, mentoring experiences, opportunities for advancement.

RESULTS: Possessing a doctoral degree, demonstrating competence and strong leadership skills, having a good role model and/or mentor, displaying the ability to see the big picture, and possessing exemplary communication skills were identified by this group of women as necessary requirements for obtaining and maintaining a position as a higher education administrator.

CONCLUSION: The participants in this study confirmed that by possessing a terminal degree and a defined skill set, they were able to obtain a higher education administrator position.

INDEX TERMS: career paths of women clinical laboratory scientists; women higher education administrators; women’s leadership skills.

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from being clinical laboratory scientists to becoming higher education administrators.

The purpose for conducting this qualitative study was to investigate and document the career paths of women clinical laboratory scientists who have transitioned from the clinical setting to the higher education arena and held administrative positions at the dean’s level, including assistant and associate dean positions. This research sought to identify the experiences, training, and opportunities that directed and influenced the career paths of these women.

Three major themes emerged from the data. They included: Getting to the Right Place at the Right Time, The Right Navigational Skills are Required, and The Right Place Comes with a Price. The Right Time – Right Place theme described the three different career stops experienced by each of the participants. These findings were published in the Summer 2009 edition of Clinical Laboratory Science. The Right Navigational Skills theme was developed from two categories: don’t wait for opportunity to knock and communication is the key. The findings that support this category are presented here. The Right Place Comes with a Price theme has been submitted for possible publication in a future edition of Clinical Laboratory Science.

**RESEARCH QUESTIONS**

To investigate the career paths of women clinical laboratory scientists who held higher education administrative positions, the following questions were considered.

1. What are the lived experiences of women higher education administrators with a background in clinical laboratory science during their career paths?
2. What skills, training, and/or professional development opportunities enabled these women to become successful higher education administrators when their initial academic area of study was clinical laboratory science?
3. What barriers and/or obstacles have these women experienced during their career paths as women clinical laboratory scientists who transitioned to higher education administration?
4. How has being a woman influenced their careers as higher education administrators?

This report focuses on the findings related to the research question that addressed the skills, training, and/or professional development opportunities that these women identified as contributing factors to obtaining a position as a higher education administrator.

**LITERATURE REVIEW**

Leadership has been studied since the time of the ancient Greeks. “Plato, Machiavelli, and Shakespeare offered images of leadership cast in the context of their times.” In 1979, James McGregor Burns stated, “Leadership is one of the most observed and least understood phenomena on earth.”

Modern day researchers such as Kouzes, Posner, Bass, Bennis, Stogdill, Bruce, and Avolio continue to research the topic of leadership. Much of the information resulting from the research has identified the delineation and overlap of management and leadership and the determination of a variety of leadership styles. However, numerous definitions of leadership and attempts to identify the qualities and skills one should possess to be an effective leader still remain.

The literature reviewed to support this study included studies of women’s career paths in their pursuit of administrative positions in higher education and research findings that focused on the skills required to be successful administrators. Several themes emerged that contributed to the success of women as educational leaders: earning a doctorate degree, developing a mentoring relationship, participating in leadership development programs, and demonstrating high levels of skill and competence. Women who earned doctoral degrees were seen as highly credentialed and often were rated as more competent than those that did not possess doctoral degrees. The doctoral degree is essential for women to succeed in higher education.”
Development of a mentoring relationship with either a male or female mentor was identified as a successful strategy for women wanting to obtain educational leadership positions. These relationships helped develop self-confidence and self-esteem and provided internship opportunities.

An additional strategy utilized by women in their quest to obtain leadership positions has been the participation in leadership development programs. The literature demonstrated the need for women in a variety of settings to experience leadership development opportunities. The American Council on Education Fellows program is a well-known, highly respected leadership development program for women in the higher education arena. Academic institutions also recognize the needs to develop women leaders from within their own ranks. The common solution is the formation of women’s leadership programs that aim to provide workshops focusing on leadership competencies and administrative internships to hone the newly developed skills.

The literature also indicated that women prefer the transformational style of leadership. The components of successful transformational leadership are more in line with the socialization processes that guide the development of female values. Transformational leaders act as role models/mentors, communicate goals, display optimism, encourage innovation, and build relationships to achieve a common goal.

Successful women leaders demonstrated high levels of skill in communication, problem solving, organizational savvy, team building, and instruction and curriculum. They utilized a team approach to formulate an atmosphere of openness, willingness to share power, and attentiveness to the needs of the institution.

Women need to be seen as leaders by presenting themselves as competent and task-oriented. They need to communicate intelligence and expertise. Women must establish the legitimacy of their authority without damaging their acceptability. They must demonstrate control of various situations and themselves.

**METHODOLOGY**

This case study of women higher education administrators with a background in clinical laboratory science sought to illustrate topics using a descriptive mode of the common themes developed from the data. The semi-formal interviews were audio recorded for transcription and subsequent data collection, and analysis. The primary researcher reviewed the transcripts to ensure accuracy and precision. Any remaining need for clarification was sought from the participants.

After developing the initial categories, the researcher performed axial coding in which the data were assembled and reassembled. The process of relating categories to their subcategories, termed ‘axial’ because coding occurs around the axis of a category, linking categories at the level of properties and dimensions. The axial coding portion of the data analysis included looking for descriptive wording for the topics, turning them into categories, and determining relationships between the topics. The last phase of coding involved the writing of the story line.

To provide credibility to the data, a variety of validation mechanisms was employed. For this study, member checks, rich-thick descriptions, and an external audit validated the data. A review of the participant curriculum vitas allowed for verification of the data indicated on the participant demographic information form. The member check allowed the participants to review the transcription documents for accuracy.

The use of rich-thick description of the data was used as a second validation method. Descriptive narratives play a large role in the reporting style of qualitative research. The rich descriptions were developed from the interview transcripts and the field notes. The intent of the narrative portion of the findings of the study was reported in such a manner as to allow the reader to be present in the setting and to “give the discussion an element of shared experiences”.

14 VOL 23, NO 1 WINTER 2010  CLINICAL LABORATORY SCIENCE
A third method of data validation was the use of an external audit performed by an experienced auditor not involved in the research project. The auditor employed a method of checking the recordings with the transcription documents and the conclusions determined by the researcher.

**Participant Demographics**
All participants had a previous history of experience in the clinical laboratory. One participant had a formal degree in chemistry while the other women were formally trained clinical laboratory scientists. Position titles of this group of women included dean, assistant dean, and associate dean.

The majority of the participants were between 50 and 59 years of age. Five of the eight clinical laboratory scientists were married at the time of the study; seven had children. All of the participants had earned advanced degrees: two possessed masters degrees, three earned doctorates of philosophy, one was all but dissertation (ABD), one held a doctorate of arts, and one held an educational doctorate. One woman was an assistant professor, two were associate professors, and five women were full professors. All had a minimum of ten years experience as a faculty member.

Each of the eight participants was assigned a pseudonym and a university name that corresponded with a Greek letter. The selected pseudonyms included: Ann at Alpha University, Brianna at Beta University, Debra at Delta University, Gwen at Gamma University, Kelly at Kappa University, Lynn at Lambda University, Olive at Omega University, and Teresa at Theta University.

**RESULTS**
The Right Navigational Skills are Required theme was supported by two sub-categories: Don’t Wait for Opportunity to Knock and Communication is the Key. These categories were supported by sixteen codes, and each was explored.

This select group of women higher education administrators provided a plethora of information when asked about their skills, training, and professional development opportunities. They indicated that communication, listening, interpersonal skills, and leadership skills were vital to their success. They further stated the need for strong leadership capabilities especially when they sought buy-in for the vision of the college and university. Surprisingly, one skill they mentioned was humor. Additional items included being committed to their position as well as the institution, and the opportunity to mentor others – especially other women.

**Don’t Wait for Opportunity to Knock**
The first category that supports the right navigational skills is seeking and accepting a variety of opportunities. The data codes include seek and accept opportunity, seek professional development opportunities, being visible/visibility, seeing the big picture, having a mentor, learning at all levels, earning an advanced degree, and leadership training.

Seek and accept opportunity. The data revealed that these women obtained their administrative positions by seeking and accepting opportunities that broadened their knowledge of and visibility throughout the entire organization. They did not sequester themselves to their faculty duties and teaching responsibilities. Instead, they looked for opportunities for personal and professional growth. They volunteered for committee work and professional development opportunities. Ann advised women to take advantage of every opportunity to grow. She commented,

> I know some people tell young faculty to focus on a particular research project or something, on a research area, and essentially to get narrower and narrower. Maybe that is where you should go if research is going to be your number one goal. But as a faculty person, I think getting lots of experiences is a little bit better. The opportunities to serve on different committees, to find out more about the organization, that’s what you are going to need to know if you get in an administrative role. If you focus too much, you won’t have any of those experiences.
Debra advised,

I think that any project, any job that someone asks you to do, do it, volunteer for it. If there is anything special going on the college or university campus, volunteer. Get as much experience as you can in various areas.

Teresa believed that accepting opportunities should fit your career goals. She commented,

I will step forward and take what I consider reasonable risks and take as many opportunities as I can. There may have been opportunities there that I have not recognized as opportunities and maybe didn’t pursue them where I could have. But generally speaking, I’ve been willing to make the next step into an opportunity. Unless…it was something that just did not fit with the career goals of where I was going.

Seek professional development opportunities

This group of women administrators was quick to point out that taking advantage of formal professional development opportunities was important. Examples of the various professional development experiences included: Toastmasters, an institutional cooperation committee, workshops conducted by state education coordinating boards, an American Council on Education workshop, and an ethnic leadership program and diversity workshops, along with formal education at the master’s and doctoral level. To achieve a position as a higher education administrator, these participants indicated that their prior involvement with professional organizations and campus committees allowed them to demonstrate skills that are desirable for academic leaders.

Being visible/visibility

The participants reported that by being visible and having a reputation for possessing the desirable skills and traits of leaders, they were able to obtain their administrative positions. Brianna indicated,

The big issue was getting me known on campus. I was very well known in the college but really [needed] campus exposure and getting the campus perspective. So I [was] sent to every committee and every meeting known to man.

Ann shared, “Take advantage of things like being the [non-profit organization] co-chair. I would personally go visit everyone in our pathology department. I started to learn who the people were and just more about what was happening.” They stayed visible on campus and sought the big picture.

Seeing the big picture

The opportunities that were sought or presented to these participants played a role in their ability to see the big picture. Several of the participants indicated that their roles in professional organizations and campus committees provided a mechanism for them to become known across campus. As a faculty member, department chair, and division chair, the view can be somewhat narrow and focused on specific areas. Brianna observed that in serving as an acting dean “…you really get a global view of campus and you don’t get that kind of insider information as a faculty member.” She noted a key item to her success as an administrator was the ability to “…see the big picture and to help other people see their role in it.” The opportunity to serve on various high level committees allowed Brianna to grasp “…the sense of the feel of the campus and how things work.” The transition to the administrative arena required these women to see the big picture, to see beyond their clinical laboratory science programs, and to fully comprehend the institutional mission and purpose.

Having a mentor

Mentoring was identified as a necessary component for success. This group of women had been mentored through various stages of their higher education careers. Four of the participants were mentored by men, while three were mentored by women. Most described their mentoring relationship as an informal arrangement. Their mentors encouraged them to seek learning opportunities at all levels of their careers. Debra, an adjunct faculty member, recalled that her mentor, the dean of instruction “took me under her wing. She
preceded me. She left the position, then I applied and got it.” Debra’s relationship with her mentor was informal and the advice was very beneficial. “If she saw that I was getting ready to do something she didn’t feel was good for my career, she would let me know.” Lynn’s mentoring relationship with her dean was grounded in his support for her advancement. “When the opportunity came to turn that program into a department and to take on a department chair position, he was there along the whole way saying ‘you need to do that’.” Lynn further commented, “This individual was important in influencing those types of decisions along the way and that made those decisions easier, more comfortable, more certain.”

Although the women considered these experiences as forms of mentoring and referred to the individuals as mentors, the term that may more accurately describe this process and the individuals is modeling. The participants identified ideal leadership strategies in the administrators with which they worked and modeled those characteristics and practices.

Learning at all levels
Five of the eight participants indicated that to achieve their position as a higher education administrator, they had demonstrated the ability to take on a variety of projects and experiences. They were not afraid to explore unfamiliar areas and willingly accepted the opportunity to expand their knowledge of the institution where they were employed. Olive shared, “Maybe I was fortunate when I was at a small university. You had more of an overview – the coordinating board funding - there are a lot of chairs who still don’t understand how higher education is funded. I think learning those things is key to understanding how the university operates. Really, just paying attention. I try to learn as much as I can about the other programs. When they’re talking, I don’t tune them out.

The main thrust of their responses indicated a need to volunteer, to be active on campus committees, to participate in professional development, and to “never say no” when asked to take a special assignment or project. These women indicated that they were often very busy with their existing responsibilities but were able to take on additional tasks and to do them well.

Earning an advanced degree
Early in their faculty careers, these women recognized that an advanced degree was a necessity. Lynn indicated, “I knew right away that if I would have wanted to stay in the university and ever get ingrained into the mainstream of academia, I needed that doctorate.” Debra stated, “I should have gotten my PhD much earlier in my career. I think I would have been more open to some of the opportunities that came my way.” Two participants completed course work toward earning their doctoral degrees but did not finish the program requirements. During the interviews, when asked about their career choices and if they would do anything different, both individuals indicated that they would obtain the terminal degree. They identified possessing a doctoral degree as “a must” in today’s higher education administrative arena. All of the participants strongly urged young women striving for an administrative position in higher education to earn a doctoral degree. Kelly said, “It is something that you really, really, really need. Some of it is for credibility with other faculty.” Additionally, Kelly stated, “It’s [the PhD] a must in today’s market.”

Leadership training
The majority of these women shared their experiences related to learning to be a leader. For some, it was on-the-job training in a very informal manner. The participants described their mentoring relationships as informal opportunities to develop their leadership skills. For others, it was attending structured conferences and workshops to develop their leadership skills.

Communication is the Key
The second category that supports the right navigational skills is communication. The data related to this category includes communication skills, listening, being of service, teamwork, interpersonal skills, humor, commitment, and mentoring others.
Communication skills
One participant described her necessary communication skills as formal public speaking and conducting meetings along with the informal communication with volunteers and faculty. The ability to communicate with faculty was one aspect that this participant thought was very important in her nomination for the associate dean position. Another participant emphasized that communication is key for all healthcare professionals to be effective. Gwen became very interested in communication skills. She commented, “I started doing a lot of lecturing about [the importance of communication]. I did a fair number of workshops talking about communication.” Gwen admitted, “Not to say that I’m a good communicator, but I sure learned a lot about it, and I sure learned how to help people become better communicators, to frame their message both verbally and in writing.” She was adamant that this skill assisted her as an educator and now as an administrator.

Listening
When the participants spoke of communication skills, they included the importance of listening. Four of the participants identified their ability to listen to their faculty and to hear their concerns as very important in their roles as administrators. Gwen said, “I think I listen well. My faculty have told me that I listen well.” Kelly viewed listening as one aspect of her position as an administrator. She indicated “… there are things that you cannot resolve and cannot work through, but I think to listen is important …” She further stated, “Maybe that’s communication skills, but I see that as an important part of what I do. I have no problem working with people to resolve their issues or listening to what they see as an issue and seeing if there are ways to work that through.

The participants realized their ability to listen to the faculty and department chairs was key to their success.

Being of service
Three of the participants spoke of their administrative position as one of service. Brianna believed that communicating with people and convincing them that you can provide them with a service was vital to being a higher education administrator. She sensed a mentality of “us versus them” between faculty and department heads and the dean’s office. Brianna “really tried to change the perception of the dean’s office to a culture of service. We are here to facilitate, to help guide you.” Kelly, in describing her position as one of service, stated, “I see administration in general as a service position. I don’t know that everybody agrees with that.” Teresa’s vision of providing service to her people was to gather information and to make a decision. She described herself as “one that can make a decision and be firm” when required to, while hoping that she is always fair.

Teamwork
Another component of communication was the ability to form viable, effective teams. Debra shared that “… really being able to work with people to get them to work together and being able to go from A to Z on a project” is important in her role as assistant dean. Olive recognized that every “organization is made up of a variety of people and that everyone has different contributions to make.” She noted that this “is a much better way to utilize people than to go against the grain.” She added, “… putting those people in those areas where they have strengths, I think is very important.”

Interpersonal skills
Trust, honesty, and respect were identified as necessary interpersonal skills. Being able to deal with people on an honest basis to build a level of trust was extremely important. Gwen described her interpersonal skills as “essentially how to work with people, how to listen to what they’re saying and incorporate their ideas, how to respect what they’re doing, and yet lead them to where they need to be.”

Humor
Surprisingly, when asked about their leadership skills and characteristics, three of the participants identified humor as a skill. The humor described was not slapstick or being funny. It was more related to seeing things in
perspective and not taking yourself or your position too seriously. An additional description of humor included having an openness and willingness to laugh and being willing to bring some lightness into somebody else’s life.

Commitment

The participants were acutely aware of the level of commitment that was required to be successful at the administrative level. Gwen demonstrated her level of commitment by describing her relationship with her staff.

Some people you’re just never going to change. You have to get them to focus on the things that are really important and let go of the other stuff. Sometimes it is hard, but I love what I do. If I moved up, I wouldn’t be able to do the kind of things that I really love doing.

Teresa discussed her commitment to the job and the necessity of others considering a move to administration to develop an awareness of the position requirements. They [future women administrators] need to consider whether they like that. It’s very challenging but can be very exciting. If you don’t like decision making, if you don’t like conflict — not that any of us like conflict, but you may feel uncomfortable of trying to work through conflict. [It is like] being in the middle of the sandwich because you have people below you, you have people above you, and you have to advocate for both groups. They just really need to understand what they’re getting into. And not go into it because ‘I’m the best thing they’ve got’. Those are things they really need to avoid and know that they are going to make a commitment.

They talked about how hard they work, the stress related to the position, and the challenge of being a female in a male environment.

Mentoring others

During their career paths, the mentoring experiences of these women have varied. However, the majority of the participants have been involved in mentoring both students and staff. Brianna described experiences mentoring her graduate students. She commented,

One of my own students was all but dissertation, had a baby, and then disappeared. I tracked her down and got her to continue writing. She was almost there when she got pregnant again. I didn’t hear from her. I [contacted] her and said ‘You have to finish this for yourself.’ She was getting no support from her mother or her husband. I dragged her through by her teeth. I hounded her.

Brianna also mentored her faculty. She used the same tactics with them as she had with the student. She indicated,

I dragged those others through it. I beat them up. I said, ‘You have to finish.’ You have to finish for a variety of reasons and I dragged at least four people through. I kept telling them ‘You’re going to need it later whether it’s just self accomplishment or whether it’s professional development. It’s a life jacket. It’s a life vest.’

They believed mentoring others was an important aspect of their roles as higher education administrators.

CONCLUSION

Time and time again these talented and productive women described the opportunities they sought out and accepted. They indicated that the skills, knowledge, and experience they gained from these opportunities assisted them in moving to the administrative level. They emphasized the need to be visible on campus, to do everything well, to possess the ability to see the big picture, to be mentored, and to seek the opportunity to learn at all levels.

By displaying the desired leadership and communication skills and competencies, these women were groomed for positions in higher education administration. During their ascension to administrative offices, they actively sought and accepted numerous opportunities to expand their knowledge and experiences. They promoted themselves by participation in campus committees so they could be more visible
and to gain the “big picture” perspective of the institution. Learning the skills and gaining the education required for promotion were necessary tasks.

To be successful higher education administrators, these women emphasized the importance of communication skills, listening, interpersonal skills, and leadership skills. To gain buy-in for the vision of the college and university, having strong leadership capabilities was seen as a vital skill. The participants described their level of commitment to their positions and to their institutions. Although most of the discussion was of a serious nature, these women also stressed the importance of humor in the workplace.

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Platelet Function Testing: Auditing Local Practice and Broader Implications

EMMANUEL J FAVALORO, SOMA MOHAMMED

BACKGROUND: Platelet function testing is a common test procedure used for assessing patients with mucocutaneous bruising and/or bleeding and for monitoring anti-platelet therapy. However, standardization of practice is poorly applied, and experts differ on several aspects of its application.

OBJECTIVE: This study reports on a local audit of current practice in consideration of recent reports, expert opinion and current CLSI guidelines.

METHODS: We undertook an assessment of our laboratory test practice for light transmission aggregometry (LTA) as a diagnostic screening process for platelet function, as well as performance of PFA-100 closure times used for screening primary haemostasis. For LTA testing, we wished to assess the validity or otherwise of platelet count adjustments using autologous platelet poor plasma (PPP), as used by some experts and as also recommended by the current CLSI guidelines for performance of platelet aggregation. For PFA-100 testing, we assessed the effect of different blood collection tubes.

RESULTS: For most test cases undergoing LTA, platelet count adjustment using autologous PPP resulted in considerable diminution of detectable platelet function using several agonists, and in particular collagen, ADP and epinephrine. These effects could result in differing conclusions regarding the likelihood or severity of a platelet function disorder. For the PFA-100, different blood collection tubes resulted in slightly different closure times that could also potentially influence the conclusion of ‘normality’ or otherwise for investigated patients.

CONCLUSIONS: This audit of local practice indicates that the process of platelet count adjustment using autologous PPP provides adverse outcomes related to identification of platelet dysfunction. Accordingly, we recommend that all laboratories validate this practice if used at their facility. For PFA-100 testing, local validation of the normal reference range is required according to local conditions and collection practice. Otherwise, laboratories may inappropriately identify platelet function disorders when these may not exist.

ABBREVIATIONS USED: ADP - adenosine diphosphate; CLSI – Clinical and Laboratory Standards Institute; CTs - closure times; C/Epi - collagen/epinephrine; C/ADP - collagen/adenosine diphosphate; Epi – epinephrine; fc -final concentration; LTA - light transmission aggregometry; PPP - platelet poor plasma; PRP - platelet rich plasma; VWD - von Willebrand disease; WBA - whole blood aggregometry.

INDEX TERMS: platelet function tests, PFA-100, light transmission aggregometry.

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INTRODUCTION
Platelet function testing is a common test procedure used for assessing patients with mucocutaneous bleeding and/or bruising and in some laboratories for monitoring anti-platelet therapy. However, standardization of practice is poorly applied, and experts differ on several aspects of its application. The most common test methods applied for diagnostic screening of platelet function are light transmission aggregometry (LTA) and whole blood aggregometry (WBA). In addition, several primary haemostasis or platelet function screening procedures are available, of which the PFA-100 is probably the most popular. WBA has several advantages over LTA, including test performance under ‘more physiological’ conditions (i.e. using whole blood, albeit typically diluted 1:1 with buffer) and reduced processing of blood and platelets prior to testing. This reduces test time, and also the likelihood of test artefacts (i.e. of identifying false reductions in platelet activity due to over-handling of platelets, which are highly sensitive to adverse processing effects). Nevertheless, LTA remains the gold standard because it represents the original test methodology and thus has a longer history than WBA, inclusive of research and publication data.

Standardization of platelet function is poorly applied, and experts differ on various test practices. In addition, although internal quality control is feasible, this is difficult for most laboratories to apply in practice, given the time required for testing (up to 2 or more hours for each individual platelet function test) and the need to collect normal individuals (usually laboratory staff) on a regular, sometimes daily, basis. External quality assurance of platelet function testing is also largely lacking, despite some recent initiatives from external quality assurance providers.

One of the main differences in regards to expert opinion related to platelet function testing using LTA is the need to adjust the platelet count. Some experts undertake such adjustments as a standard practice, whereas others warn of the dangers related to loss of platelet activity because of over-handling and refractoriness. The latter is potentially related to dense granule release (or other events) following the high speed centrifugation required to obtain the platelet poor plasma (PPP) needed to dilute the platelet rich plasma (PRP) in order to obtain a standardised platelet count. The current CLSI guideline on platelet function testing by LTA recommends undertaking a platelet count adjustment in order to standardise the platelet count to between 200 and 250 x 10^9/L, while also noting that some experts disagree with this practice.

Our facility ceased performing platelet count adjustments several years ago, when it became apparent that we were observing ‘minor’ platelet abnormalities in a high proportion of patient samples, as well as in controls, and potentially related to a change in collection practice. We have therefore undertaken an audit of current practice in order to reinvestigate this issue in relation to LTA testing, while reflecting on current CLSI guidelines, and recent published reports and differing expert opinion. We also decided to briefly re-evaluate local performance of the PFA-100 in the context of platelet function testing. Although these audits have particular relevance to our own test practice, and most likely reflect changes to blood collection practice at our institution, we also propose that these local findings hold substantial implications to the globally applied practice of platelet function testing at many facilities.

MATERIALS AND METHODS
Patients and blood collection: The patients included in this study were those referred to us for investigation of mucocutaneous bleeding or bruising, other adverse bleeding event(s), or other clinically justified reason(s), and for which platelet function testing was performed by both LTA and PFA-100. Our laboratory does not undertake regular monitoring of anti-platelet therapy within the context of treatment, in agreement with the International Society on Thrombosis and Haemostasis.
(ISTH) Scientific Standardisation Committee (SSC), who advise that the clinical utility of such monitoring requires further evaluation. It is our standard practice for our platelet function investigations to query recent medication history and to generally decline testing if there is evidence of recent medication likely to affect platelet function. If possible, we also undertake a review of personal and familial history related to bleeding and bruising.

For platelet function testing, we now typically collect a number of ‘small-draw’ blood collection tubes (2.7ml ‘Vacutainer’ tubes containing 3.2% buffered sodium citrate; reference number 363095; Becton Dickinson, Sydney, Australia) as well as ‘large-draw’ blood collection tubes (9ml ‘Vacuette’ tubes containing 3.2% buffered sodium citrate; reference number 455322; Greiner, purchased from Interpath, Sydney, Australia). Blood is collected by venesection by experienced phlebotomists on site at our facility and generally under our guidance. The above reflects a change of collection practice over the years, due to occupational health and safety concerns related to handling of blood, since in the past, blood was collected by needle into syringes and then gently dispensed into anticoagulant containing tubes without vacuum. This earlier practice aimed to minimise adverse effects on platelet function potentially due to vacuum collection and resulting shear effects.

The ‘small-draw’ collection tubes are those routinely contracted to our organization. These are used for our general haemostasis and PFA-100 testing, and reflect the test system for which we have previously undertaken normal reference range estimations. The ‘large-draw’ blood collection tubes represent those tubes specifically purchased to perform platelet function testing by LTA, and for which validation of usage was previously established. The current report relates to the use of this material differentially within LTA studies or in PFA-100 testing.

In addition, a small blood sample is always routinely collected (3ml ‘Vacutainer’ tubes containing 5.4 mg potassium EDTA (K,EDTA); reference number 367838; Becton Dickinson, Sydney, Australia) for routine blood parameters as part of a full (or ‘complete’) blood count workup. In particular, the platelet count and morphology, as well as the hematocrit, is particularly relevant for LTA and PFA-100 testing.

Platelet function testing by light transmission aggregometry:
This was performed similarly to that previously reported, using a Chronolog platelet agregometer (Model 560-VS, Chronolog Corporation, Havertown, PA, USA), with the stirrer speed set to 1000rpm. In brief, citrate anticoagulated whole blood (4x9ml Greiner Vacuette tubes) was allowed to rest for 15 minutes post-collection and then centrifuged at low speed (120g, 15 minutes, without brake) to isolate PRP. Care was taken not to disturb the plasma cell interface and the top two mls of PRP from each tube was gently transferred (pooled) to a fresh plastic tube, which was then capped to prevent changes in pH. The PRP tube was then gently mixed to homogeneity by inversion and a platelet count performed. The remaining whole blood sample was centrifuged at high speed (1200g, 15 minutes, without brake) to isolate PPP. Care was again taken not to disturb the plasma cell interface and the top two mls of PPP remaining in each tube was gently transferred to a second fresh plastic tube, which was then also capped to prevent changes in pH.

For LTA, 450ul volumes of PRP were transferred to 10 aggregometry tubes containing a siliconised stirrer, and 500ul of PPP was transferred to a separate aggregometry tube without a stirrer and used as the assay blank tube. All samples were incubated in the resting position of the aggregometer for a minimum of 5 minutes to equilibrate to 37°C. A test aggregometry tube containing 450ul of PRP was then transferred to the test position to assess any spontaneous aggregation. Subsequent testing involved adding in sequence a variety of platelet agonists to each separate aggregometry tube, typically at 50ul volumes, to assess specific platelet aggregation responses. Each aggregometry test is monitored for a minimum of 5 minutes, but longer as required to enable the maximum response to be determined. 100% (‘theoretical maximum’) aggregation is taken as 100% light transmittance and is assessed with a PPP blank as the ‘test’ sample in the test position versus PPP in the blank position. 0% aggregation is taken as the PRP in the test
RESEARCH AND REPORTS

position (without added agonist) versus PPP in the blank position. All tracings are visualised using a chart recorder, and any aggregation above 80% is considered a maximal response. A minimum response requires at least a 20% shift in the aggregation response.

The response to the following agonists are now routinely assessed by LTA in our laboratory: (a) ristocetin (Catalogue number 5199; 15mg/ml; Helena Laboratories, Melbourne Australia), used at two or more concentrations to assess minimum and maximum thresholds (typically over the range 0.2-2.0mg/ml final concentration [fc]; (b) arachidonic acid (Catalogue number 5364; Helena Laboratories, Melbourne Australia), used at a single concentration (1.6mM final); (c) adenosine diphosphate (ADP; Catalogue number 5366; Helena Laboratories, Melbourne Australia), used at two or more concentrations to assess minimum and maximum thresholds (ie primary and secondary waves of aggregation (typically assessed over the range 0.6-20.0uM fc)); (d) epinephrine (Catalogue number 5367; Helena Laboratories, Melbourne Australia), used at a single or multiple concentrations to assess primary and secondary waves of aggregation (typical range 10-300uM fc); (e) collagen (HORM Collagen, purchased from Optigen Scientific, Adelaide, Australia), used at two or more concentrations to assess normal thresholds (typically assessed over the range 1.0-10.0ug/ml fc).

To assess the effect of platelet count adjustments using autologous PPP, a volume of PRP (typically 2-4 ml) was gently mixed with a volume of PPP (typically 2-4 ml) to obtain a platelet count of between 200 and 250 x 10^9/L, as recommended by the current CLSI guidelines.13 LTA was performed as per the unadjusted PRP testing described above using the same agonists at either the same and/or alternate (e.g. higher) concentrations, as required to elicit similar or maximal and minimal responses. This audit was performed over a period of around eight months, and assessment of PRP adjustment was only performed on a subset of LTA investigations during this period.

All platelet function testing was performed within 4 hours of collection, as recommended by the CLSI guidelines.13

For the purpose of this study, a ‘normal’ LTA was that which provided test results within our normal limits,16 as identified in Table 1 (viz: normal (>80%) aggregation to arachidonic acid (@ 1.6mM fc), normal aggregation with a secondary wave to the ‘weak’ agonists ADP and epinephrine (respectively @ ≤ 5uM and 10uM fc), normal aggregation with the ‘strong’ agonist collagen (@ minimum of 1ug/ml fc) and normal aggregation to ristocetin (@ between 1.0 and 1.5 mg/ml fc).

Table 1: Definitions for current LTA study

<table>
<thead>
<tr>
<th>Normal LTA</th>
<th>possible ‘mild’ platelet dysfunction</th>
<th>‘moderate’ platelet dysfunction</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal (&gt;80%) aggregation to arachidonic acid (@ 1.6mM fc), normal aggregation with a secondary wave to the ‘weak’ agonists ADP and epinephrine (respectively @ ≤ 5uM and 10uM fc), normal aggregation with the ‘strong’ agonist collagen (@ minimum of 1ug/ml fc) and normal aggregation to ristocetin (@ between 1.0 and 1.5 mg/ml fc).</td>
<td>test results within our normal limits for all the tested agonists excepting for one of the weak agonists and/or collagen</td>
<td>abnormal aggregation results obtained for the weak agonists plus collagen</td>
</tr>
</tbody>
</table>

Ristocetin aggregation is used to primarily assess for the possibility of Bernard Soulier Syndrome or variants of von Willebrand disease (VWD). Aggregation using arachidonic acid helps access potential defects in the arachidonic acid pathway or recent aspirin intake.

Aggregation using ADP, epinephrine and collagen is primarily helpful to provisionally identify storage pool deficiency or primary secretion defects for further study if required. For the purpose of this study, a possible ‘mild’ platelet dysfunction potentially related to these possibilities was defined where test results were within
our normal limits for all the tested agonists excepting one of the weak agonists and/or collagen, and a ‘moderate’ platelet dysfunction was defined where abnormal aggregation results were obtained for the weak agonists plus collagen (Table 1). A significant change in platelet function between unadjusted and adjusted platelet count was defined as a change from ‘normal’ to ‘mild’ or to ‘moderate’ platelet dysfunction, or a change from ‘mild’ platelet dysfunction to ‘moderate’ platelet dysfunction (Table 1).

**PFA-100 testing:** This was performed essentially as recommended by the manufacturer except that we routinely use 900ul whole blood (instead of 800ul as ‘recommended’), since this results in fewer error codes related to ‘insufficient sample’. This is consistent with the maximal tolerance (1ml whole blood) of this instrument as advised by the manufacturer, and has been previously validated. For this study, we employed both test cartridge types, namely collagen/epinephrine (C/Epi) and collagen/adenosine diphosphate (C/ADP), and were interested in any differences in closure times (CTs) obtained with our standard ‘small-draw’ Vacutainer tubes versus those of our LTA-usage ‘large-draw’ Vacuette tubes. The data for this part of the report therefore includes all cases investigated by both LTA and PFA-100 over the past 12 months.

**Other haemostasis tests:** Other associated haemostasis tests, including those for VWD, coagulation factor levels, and routine coagulation tests including the prothrombin time and activated partial thromboplastin time were also performed as required or requested by the clinician referring the patients for investigation, and as per previous reports from our laboratory. As the results of these tests are not specifically reported for the cases under investigation, neither is their test methodology. It is our standard practice to undertake tests related to VWD investigations on all cases referred to us for investigation of mucocutaneous bleeding or bruising, including cases where LTA and/or PFA-100 are performed.

**RESULTS**

A total of 11 cases were assessed for LTA over the period of this study with and without adjustment of PRP platelet count using autologous PPP (Table 2). Although this represents a small sampling, test results post-adjustment were substantially altered in 9/11 (82%) cases, and so further comparative assessments for this audit were felt to be superfluous. Nine of 11 cases (ie Cases 1-9) represented individuals with personal and/or family history of mucocutaneous bleeding and/or bruising, of varying severity. Using our standard test processes for VWD investigation and LTA without adjustment of PRP platelet count, 2/9 (22%) of these cases were identified to have possible mild platelet dysfunction, 1/9 (11%) possible mild type 1 VWD, and 1/9 (11%) possible mild type 1 VWD plus moderate platelet dysfunction. Only two of these cases (Cases 1 & 2) provided clearly abnormal PFA-100 CTs (with both C/ADP and C/Epi), with both cases also yielding test findings suggestive of possible mild type 1 VWD.

One case (Case 10) was a female at 34 weeks gestation with a previous history of Essential Thrombocytosis and thrombosis, referred for investigation because the haematologist had concerns regarding delivery, and this case was found to have normal platelet function plus normal (albeit high levels of) von Willebrand factor. One case (Case 11) had a single event of unexplained but serious bleeding (neural haematoma) plus a previous abnormal platelet function whilst on medication, referred for investigation largely to exclude what may have been a falsely identified (medication related) platelet dysfunction. Interestingly, a possible mild type 1 VWD plus mild platelet dysfunction was identified by testing on this repeat (but medication free) occasion.

Normal aggregation traces from a laboratory control donor using unadjusted PRP is shown in Figure 1. The cases identified in Table 2 as normal for LTA using unadjusted PRP yielded patterns similar to these. Some examples of changes observed in these cases post-platelet count adjustment for arachidonic acid, collagen, ADP and epinephrine, are shown in Figures 2-4. Aggregation in response to ristocetin was normal for all 11 cases, for both unadjusted and platelet adjusted LTA (data not shown). Accordingly, platelet adjustment did not appear to influence aggregation induced by ristocetin. All 11 cases also responded normally to arachidonic acid using both adjusted and unadjusted PRP, confirming
### Table 2: Summary of Case investigations for current LTA study

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Clinical information</th>
<th>PFA-100 Closure Times</th>
<th>VWD investigation</th>
<th>LTA (unadjusted)</th>
<th>LTA (adjusted)</th>
<th>Significant LTA difference?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Personal and family history mucocutaneous bleeding (post surgical, menorrhagia, nose and gum bleeds); abnormal PFA-100 tests on two previous occasions</td>
<td>abnormal</td>
<td>possible mild type 1 VWD</td>
<td>moderate platelet dysfunction</td>
<td>moderate platelet dysfunction</td>
<td>no</td>
</tr>
<tr>
<td>2</td>
<td>Personal and family history mucocutaneous bleeding (post surgical, menorrhagia, bruising); previous abnormal PFA-100 tests and VWD investigation suggesting mild Type 1 VWD</td>
<td>abnormal</td>
<td>possible mild type 1 VWD</td>
<td>normal</td>
<td>moderate platelet dysfunction</td>
<td>yes</td>
</tr>
<tr>
<td>3</td>
<td>Personal history mucocutaneous bleeding (menorrhagia, bowel and recent spontaneous bruising); previous VWD studies equivocal; negative family history</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
<td>moderate platelet dysfunction</td>
<td>yes</td>
</tr>
<tr>
<td>4</td>
<td>Personal and family history mucocutaneous bleeding (post surgical, menorrhagia, nose, gum) and spontaneous bruising</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
<td>moderate platelet dysfunction</td>
<td>yes</td>
</tr>
<tr>
<td>5</td>
<td>Personal and family history mucocutaneous bleeding (post three pregnancies) and bruising; probable Type 1 VWD</td>
<td>normal</td>
<td>normal</td>
<td>mild platelet dysfunction</td>
<td>moderate platelet dysfunction</td>
<td>yes</td>
</tr>
<tr>
<td>6</td>
<td>Personal mild history mucocutaneous bleeding (nose bleeds in youth); previous equivocal VWD test results</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
<td>moderate platelet dysfunction</td>
<td>yes</td>
</tr>
<tr>
<td>7</td>
<td>Personal history mucocutaneous bleeding (post cuts, post delivery, menorrhagia, nose) and bruising; previous abnormal PFA-100 tests</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
<td>moderate platelet dysfunction</td>
<td>yes</td>
</tr>
<tr>
<td>8</td>
<td>Recent personal history bruising; possible mild menorrhagia; possible family history (bruising); ?VWD</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
<td>moderate platelet dysfunction</td>
<td>yes</td>
</tr>
<tr>
<td>9</td>
<td>Personal and family history mucocutaneous bleeding (menorrhagia) and bruising; ?VWD</td>
<td>normal</td>
<td>normal</td>
<td>mild platelet dysfunction</td>
<td>moderate platelet dysfunction</td>
<td>yes</td>
</tr>
<tr>
<td>10</td>
<td>Previous Essential Thrombocytosis and previous portal vein thrombosis post splenectomy; now 34 wks gestation; current normal platelet count</td>
<td>normal (high)</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
<td>no</td>
</tr>
<tr>
<td>11</td>
<td>Unexplained serious bleeding (neural haematoma); previous abnormal platelet function while on medication</td>
<td>equivocal</td>
<td>possible mild type 1 VWD</td>
<td>mild platelet dysfunction</td>
<td>moderate platelet dysfunction</td>
<td>yes</td>
</tr>
</tbody>
</table>
All cases except Case 1 responded normally to collagen (ie maximal response observed @1mg/ml) when using platelet unadjusted LTA. Interestingly, Case 1 responded normally to a higher concentration of collagen (@2mg/ml) both when using PRP unadjusted and adjusted LTA. Of the remaining 10 cases who had responded to collagen @1mg/ml using unadjusted LTA, eight failed to aggregate to collagen at the same concentration using platelet adjusted LTA (two examples are shown in Figure 3), with three of these achieving maximal aggregation only above 2mg/ml.

Finally, of those cases (n=7) yielding a normal secondary wave of aggregation to ADP and/or epinephrine using unadjusted LTA, all either responded with a secondary wave only at a higher concentration of agonist or else failed to generate a secondary wave in response to the agonist even when used at a higher agonist concentration (two examples for each agonist are shown in Figure 4).

Data related to the PFA-100 audit is shown in Figure 5. Test results using the ‘large’ draw blood collection tubes gave generally higher closure times compared to our standard ‘small’ draw collection tubes, although in most test cases there was no change in interpretation of test findings (eg originally normal PFA-100 closure times using small draw tubes were also generally normal with the large draw tubes).
RESEARCH AND REPORTS

Figure 4a. Overlapping aggregation tracings for ADP (using 5uM fc) comparing unadjusted (‘NA’) and platelet adjusted (‘A’) LTA for Cases 4 and 7 as examples, showing normal aggregation with a secondary wave in the former, but disaggregation in the latter.

Figure 4b. Overlapping aggregation tracings for epinephrine (using 10uM fc) comparing unadjusted (‘NA’) and platelet adjusted (‘A’) LTA for Cases 7 and 11 as examples, showing normal aggregation with a secondary wave in the former, but primary wave only in the latter. Horizontal marker line for each case shows one minute.

DISCUSSION

LTA is utilized as a diagnostic or diagnostic-screening process for a wide variety of platelet disorders as well as for some cases of VWD. Nevertheless, standardization of platelet function is poorly applied, internal quality control is feasible but difficult to apply, external quality assurance is largely lacking, and experts differ on various test practices including the issue of platelet count adjustment. In this respect, the current audit has clearly shown that the practice of platelet count adjustment using autologous PPP for our local practice results in adverse outcomes for the vast majority of cases under investigation. Thus, using platelet adjusted LTA would have resulted in a conclusion of possible ‘mild’ or ‘moderate’ platelet dysfunction in 10/11 cases evaluated in this audit, whereas use of non-adjusted LTA would have identified only 4/11 of these cases as possible ‘mild’ or ‘moderate’ platelet dysfunction. Most detected abnormalities when using non-adjusted LTA related to lack of a secondary aggregation wave to weak agonists, which is otherwise evident in some 10-20% of normal individuals, but which is more common in individuals with mucocutaneous bleeding. There is no doubt in our mind that the non-adjusted LTA findings represent the more diagnostically correct findings, and that the post-adjusted LTA findings represent several false positive events related to handling issues.

Although the CLSI guidelines recommend adjusting platelet count to help standardize the assay, several workers in the field advise against doing so because this may lead to diminution of platelet responsiveness, potentially due to dense granule release. The likelihood of such events would depend on the blood collection process and the extent of platelet manipulation. In the past, our practice was to collect blood for LTA by a large bore (e.g. 19 gauge) needle directly into syringes and then to gently dispense this blood into tubes containing anticoagulant. Due to occupational health and safety concerns related to handling of blood in this manner, our facility was required to change practice and to utilise commercial blood collection tubes with blood collected under vacuum with guards. Prior to undertaking this change, we validated the use of such collections, but noted ongoing high rates of ‘mild’ defects, namely lack of secondary aggregation waves to ADP and/or epinephrine, when samples were platelet count adjusted. Accordingly, since platelet unadjusted LTA using the new collection process better correlated to our previous experience we made a conscious decision to stop undertaking such adjustments.

Therefore, it is very likely that the high rate of detection of these ‘false’ mild platelet function defects using platelet adjusted LTA within our study reflects local
Figure 5: PFA-100 closure times (CTs; y-axis in seconds) using collagen/epinephrine (C/Epi) and collagen/adenosine diphosphate (C/ADP) test cartridges and comparing 'small' draw (2.7ml) blood collection tubes versus 'large' draw (9.0ml) blood collection tubes. Figures A and B show data as a paired dot plot and separating data according to test samples providing originally normal CTs using small draw tubes (Figure A) versus those providing originally abnormal CTs using small draw tubes (Figure B). There was a modest rise in CTs for most test cases using the large-draw tubes, which was especially evident for normal CTs (Figure A). Figures C and D respectively show the same data as A and B, but graphed as bars showing mean and SEM of data set. There was a significant change between the means for normal CTs (Figure C).

issues related to use of blood collected into large collection tubes under vacuum. It is therefore possible that platelet count adjustment will not universally result in such high rates of false positive defects when collected at other facilities using different collection processes. Nevertheless, our findings do have potential implications for at least a proportion of facilities undertaking LTA, and also highlight the danger in undertaking such adjustments without confirming that this is without adverse consequence. Thus, despite the current CLSI guidelines recommending platelet count adjustments, we recommend that laboratories undertaking platelet adjusted LTA audit this practice at their institution to validate this process is without adverse consequence. If platelet count adjustment is considered to still be required, a potential proposed alternative to the use of autologous PPP is a suitable isotonic buffer.13

In a previous audit of our geographic locality, 30/36 (83%) laboratories undertaking LTA perform platelet count adjustments.21 However, for our own facility, we...
find platelet count unadjusted LTA to provide the more acceptable diagnostic screening procedure, providing that the platelet count is within the range 150-600 x10^9/L. This is also the experience of others. Platelet counts above this for PRP for unadjusted LTA are rarely encountered for investigation of mucocutaneous bleeding, but may still provide valid results, although a parallel assessment using a platelet count adjusted LTA would be considered on a case by case basis.

For the PFA-100 audit, we observed a slight prolongation of CTs using the ‘large-draw’ tubes compared to the ‘small-draw’ tubes, particularly for normal CTs (Figure 5). The reason for this is unknown, given these tubes reflect identical citrate concentrations. However, PFA-100 CTs are recognised to be influenced by a variety of analytical variables, including platelet count, hematocrit, platelet activity and von Willebrand factor level and activity. Accordingly, subtle changes to any of these parameters between the collection tubes could feasibly affect CTs (e.g. tube fill characteristics could feasibly result in slight differences in relative citrate:blood volume fill; alternatively, large fill vacuum effects may cause a slight diminution of platelet activity). In any case, laboratories are advised to validate normal reference ranges whenever a change in collection tube is undertaken, even if the collection tubes contain the same additive, and especially if there is a change in the size of the blood draw per tube.

CONCLUSION AND RECOMMENDATIONS

In conclusion, platelet count adjustment using autologous PPP can lead to a high rate of false identification of platelet abnormalities. Although our findings may largely reflect local issues, and not be representative of LTA testing using platelet count adjustment at all facilities, we recommend that laboratories undertaking platelet adjusted LTA, audit this practice at their institution to validate that this process is without adverse consequence. We also believe that the CLSI guidelines on platelet function testing should be reviewed in light of such findings, particularly if further validation of these findings comes to light.

Finally, it is important to recognize that, despite an extensive laboratory investigation of individuals suffering from mucocutaneous bleeding/bruising, laboratories will fail to identify a laboratory defect in upwards of 50% of these individuals. Accordingly, the failure of a laboratory to define a defect in any given individual does not necessarily reflect a failing of that particular laboratory’s practice, but rather the limitation of currently available laboratory testing.

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REFERENCES

Effects of Glucosamine and Celadrin on Platelet Function

Pei-Chun Lin, Samuel O Jones, David L McGlasson

OBJECTIVE: The purpose of this study was to determine the effects of glucosamine and celadrin on platelet function.

DESIGN: Baseline values were determined on the Chronolog 570VS platelet aggregometer with whole blood aggregation impedance readings using 2 different concentrations of ADP (5µM, 10µM), collagen (1µg/mL), arachidonic acid (0.5mM/L) and an Accumetrics whole blood platelet aggregation cartridge assay for P2Y12 receptors were obtained from 24 healthy volunteers. These subjects then took the suggested doses of Glucosamine with Celadrin (Stockbridge Naturals) as advertised (estimated 1500mg daily) for 2 weeks. Platelet aggregation analyses, as described above, were obtained after treatment. Statistics performed via a McNemar test.

MAIN OUTCOME: Five of twenty-four subjects had at least a 20% difference in whole blood aggregation using the 5µM concentration of ADP. A total of 6 and 7 subjects also showed a significant difference in platelet aggregation with administration of collagen and arachidonic acid, respectively. No significant differences were found with Accumetrics assay for P2Y12 in any of the subjects.

CONCLUSION: Glucosamine and celadrin may inhibit platelet aggregation in some individuals via aspirin-like effects as well as inhibition of ADP receptor P2Y1 but not P2Y12.

ABBREVIATIONS: ADP = adenosine diphosphate, NSAID = non-steroidal anti-inflammatory drug, EDTA = ethylenediaminetetraacetic acid, WBA = whole blood aggregation, CBC = complete blood count, PGE = prostaglandin-E

INDEX TERMS: Blood platelets, Platelet aggregation, Glucosamine, Celadrin

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INTRODUCTION

The use of dietary supplements in the United States is on the rise. A national survey conducted by the Center for Disease Control in 2007 found that 17.7% of American adults had used “natural products” in the previous 12 months as compared to 2.1% in 1990. The most popular products used were fish oil/omega 3/DHA (37.4%), glucosamine (19.9%), and echinacea (19.8%). Herbal and dietary
supplements are not regulated by the Food and Drug Administration, thus raising concerns over potential side effects and drug interactions with conventional medicines. This use of supplements by adults suggested to our cohort that glucosamine and celadrin may affect platelet function.

One of our subject controls, who has donated blood as a normal platelet aggregometry control for years, suddenly had abnormal values suggesting platelet inhibition. Whole blood aggregation using 5µM and 10µM concentrations of ADP showed 5Ω and 0Ω, respectively (normal aggregation is ≥ 8Ω). Upon further questioning, patient stated that she had started taking Glucosamine with Celadrin supplement for osteoarthritis. After cessation of the supplement, her platelet function returned to normal.

Glucosamine is an amino-sugar that is produced naturally in humans and is a common supplement used for treatment of osteoarthritis. Celadrin, a proprietary blend of esterified fatty acids, is often used in combination with glucosamine. The same population developing osteoarthritis is at also risk for cardiovascular diseases. Therefore, the likelihood for use of anti-coagulation or anti-platelet agents exists. Little is known about the effects of glucosamine on platelet aggregation in humans, and there is no literature of the effects of celadrin on platelet function reported. Given the potential drug interactions, the implication that glucosamine and celadrin may have effects on platelet function is an important one, especially in those who take concomitant anti-coagulation and/or anti-platelet agents, such as aspirin, clopidogrel, and newer agents like prasugrel and ticagrelor. This study is designed to evaluate only the effects of Glucosamine with Celadrin on platelet function.

MATERIALS AND METHODS
The presented protocol was approved through the local institutional review board in accord with the tenets of the Helsinki protocol for human subject experimentation. Informed consent obtained from all subjects. This study was monitored and approved by the United States Air Force Surgeon General’s Office. All authored materials constitute the personal statements of Pei-Chun Lin and are not intended to constitute an endorsement by the U.S. Air Force or any other Federal Government entity.

Participants
Inclusion criteria for the volunteers included age over 18 years, no contraindication to taking Glucosamine with Celadrin, not currently pregnant or breast feeding, not currently taking (or requiring from a medical standpoint) any anti-platelet regimen or anticoagulant, not taking any NSAID or aspirin for 7 days prior to the test, hematocrit > 33%, and platelets > 100,000. Twenty-seven normal healthy volunteers were asked to donate a blood sample consisting of approximately 15 mL drawn into 2 blue top tubes containing 3.2% sodium citrate, one 2.0 mL partial fill citrated tube and 1 lavender top tube containing (describe %, sodium or potassium, etc.) EDTA. Laboratory tests performed included whole blood platelet aggregation with 2 different concentrations of ADP (5µM, 10µM), collagen (1µg/mL), arachidonic acid (0.5mM/L) and Accumetrics whole blood platelet aggregation assay specific for P2Y12 receptors. After the baseline blood draw collection, the subjects were given Glucosamine with Celadrin (Stockbridge Naturals) supplement of a total daily dose of 1500mg for 14 days total. At the end of the 14 days, the blood tests were repeated. Concentration of glucosamine in the supplement was also analyzed in our CRD (Clinical Research Division) laboratory to ensure consistent amount of glucosamine taken by each patient. Pill counts were done before and after 14 days to measure level of compliance. Subjects were excluded from the study if more than 10% of the pills were not taken.

Whole-blood assays
Whole-blood impedance platelet aggregometry was performed using a WBA whole-blood impedance platelet aggregometer (Chrono-Log Corporation, Havertown, Pennsylvania, USA). Whole-blood citrated samples were diluted 1:1 with normal saline for
total volume of 1000µl in each aggregometry cuvette with added stir bar. Next, 10µl of an agonist was added to the cuvette and impedance was recorded for 6 min.\textsuperscript{7} Agonists used were ADP in 2 different concentrations (5µM and 10µM), collagen (1µg/mL), and arachidonic acid (0.5mM/L). For each agonist, normal aggregation was defined as ≥ 8Ω (ohms) impedance.\textsuperscript{8}

Accumetrics whole blood platelet aggregation assay for P2Y12 was performed using the VerifyNow Assay. This is designed to measure the level of P2Y12 receptor blockade. The VerifyNow instrument is a turbidmetric based optical detection system that measures platelet-induced aggregation by an increase in light transmittance as platelet aggregate.\textsuperscript{9} The self-contained sealed assay device employs microbead agglutination technology and contains a lyophilized preparation of human fibrinogen-coated beads, platelet activators (adenosine-5-diphosphate ADP/PGE1) and buffer. The patient specimen is aliquoted into Greiner Bio-One Vacuette partial fill blood collection tubes (2mL fill volume) containing 3.2% sodium citrate that is automatically dispensed and incorporated with the platelet activator reagent. The light transmittance increases as activated platelets aggregate and bind to fibrinogen-coated beads. The instrument then measures the baseline platelet function for each sample and reports the percent inhibition result.\textsuperscript{10}

Statistics and data analysis
SPSS Sample Power (version 2.0) was employed to estimate the samples sizes for detecting a treatment effect with glucosamine using two tests of platelet function. The criterion for significance (alpha) was set at ≤ 0.050 and power > 0.80. A McNemar test was applied to assess binary results of whole blood aggregation and Accumetrics platelet function assay. Platelet aggregation results used an impedance value of 8Ω (ohms) as criterion for normal limits. The Accumetrics P2Y12 cartridge normal limits were characterized as less than 20% of platelets affected (How/where was this determined?). A sample size of 25 paired observations was needed to yield a significant difference assuming that the treatment difference of platelet function is 20% in the sample population.

RESULTS
A total of 27 subjects were recruited over a period of one month, and all had baseline blood samples performed. Ages ranged from 22 to 56, median age of 35. Thirty-two percent were men and 68% were women. All had normal baseline CBC values. Of the included subjects, two had baseline whole-blood impedance platelet aggregation of less than 8Ω utilizing the 5µM concentration of ADP; one of these two also had < 8Ω with collagen and arachidonic acid agonists. All 27 subjects had normal baseline Accumetrics P2Y12 values with less than 20% platelet inhibition.

All lab values were retested in those who completed the two-week course of the Glucosamine with Celadrin. Three of the female subjects withdrew from the study prior to taking Glucosamine with Celadrin (two due to other medical reasons, one unknown). Of the 24 subjects who completed the study, a total of five (20.8%) and six (25%) subjects showed a significant difference with the 5µM and 10µM concentrations of ADP, respectively. Two out of the five subjects who showed a significant difference with the 5µM concentrations of ADP also showed a difference in platelet aggregation with administration of collagen and arachidonic acid. A total number of 6 (25%) and 7 (29%) subjects had decreased platelet aggregation post-treatment in response to collagen and arachidonic acid agonists, respectively. No significant differences were found with pre- and post-treatment CBC or Accumetrics assay for P2Y12 values in all the subjects (Table 1). Compliance was met in all 24 subjects.

DISCUSSION
Effects on platelets by various agents have been an interest in the recent past due to the potential for significant clinical implications, such as prevention of ischemic events or alteration of bleeding complications. Platelets are circulating anucleate disc-
Table 1. Response to agonists after taking Glucosamine with Celadrin

<table>
<thead>
<tr>
<th></th>
<th>5µM ADP</th>
<th>10µM ADP</th>
<th>Chronolog whole-blood aggregometry</th>
<th>Accumetric VerifyNow</th>
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<tr>
<td>≥ 20% decline from</td>
<td>20.8</td>
<td>25</td>
<td>1µg/mL Collagen</td>
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<td>baseline (% of patients)</td>
<td></td>
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<td>0.5mM/L Arachidonic acid</td>
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<td>Accumetric VerifyNow Assay for P2Y12</td>
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shaped cells that are responsible for the initiation of primary hemostasis, which repair injury to the vascular endothelium. When there is disruption to the integrity of the endovascular lining, platelets are exposed to the underlying collagen fibrils. These collagen fibrils not only provide a surface for platelet adhesion, but also serve as a strong stimulus for platelet activation, which includes secretion of thromboxane A2 and ADP into the circulation. The released thromboxane A2 and ADP stimulate neighboring platelets, causing them to become activated and secrete additional thromboxane A2 and ADP. ADP interacts with two G-protein-coupled platelet purinergic receptors, P2Y1 and P2Y12. When ADP stimulates the platelet G alpha-subclass q-coupled P2Y1 receptor, it causes protein phosphorylation, phosphoinositide hydrolysis, thromboxane A2 formation, and an increase in cytosolic Ca++. Likewise, ADP stimulates the G alpha-subclass i-coupled P2Y12 receptor that inhibits cAMP formation. Simultaneous activation of both P2Y1 and P2Y12 is required for the full ADP-response in platelets as seen in studies using platelets derived from knockout mice.

In this study, we proposed that glucosamine and celadrin, commonly used supplements for osteoarthritis, have inhibitory effects on platelets and can exacerbate effects of anti-platelet agents. We found that 6 out of the 24 subjects demonstrated at least a 20% decline in whole blood aggregation using the 10µM concentration of ADP and 5 out of the 24 subjects also showed the same response with the 5µM concentration of ADP post-treatment with Glucosamine with Celadrin. Several subjects also showed decreased platelet aggregation with the agonists, collagen and arachidonic acid (a total of 6 and 7 subjects, respectively). However, no response was found with the Accumetrics whole blood platelet aggregation VerifyNow Assay for P2Y12. This demonstrated that in some individuals Glucosamine with Celadrin will decrease platelet response to collagen and arachidonic acid (mimicking aspirin-like effects) as well as affecting ADP P2Y1 receptors, but not P2Y12 receptors.

Previous studies support our findings. Lu-Suguro et al utilized guinea pigs and found glucosamine decreased platelet aggregation in response to ADP by 51% and this study also prevented ADP-induced extracellular release of ATP and thromboxane A2 production by 91% and 96%, respectively. Another study done in rabbits by Kinlough-Rathbone et al also found decreased platelet aggregation induced by ADP with glucosamine administration. McNamara et al investigated the hemostatic effects of oral chondro-protective agents in dogs for 30 days. There were significant reductions of platelet aggregation in response to ADP and collagen as well as overall decline in total ATP release and platelet count. Hua et al took human platelet-rich plasma and stimulated it with ADP in the presence of glucosamine. They found that glucosamine suppressed platelet aggregation in response to ADP by inhibiting binding of its receptors. It also inhibited the extracellular release of granule contents (ATP and platelet factor 4) and production of thromboxane A2 from ADP-stimulated platelets. Moreover, glucosamine halted the intracellular calcium mobilization by blocking phosphorylation of Syk (tyrosine kinase in platelets) upon ADP-stimulation. Literature search via Medline from 1950 to current found no studies on celadrin and its effects on platelet function.
Our study is the first study, to our knowledge, showing Glucosamine with Celadrin may exhibit inhibitory effects on platelet aggregation in humans via possible aspirin-like effects and inhibition of ADP receptor P2Y1 but not P2Y12. This is an important finding in that many of those who take glucosamine and/or celadrin may also be those who take anti-platelet and/or anti-coagulation agents. The clinical implication of this cohort may be larger than we think, especially in the era of newer and more potent anti-platelet agents, such as prasugrel and ticagrelor. It is uncertain why some individuals in our study had platelet inhibition and others did not. This may be due to gender, age, race differences of the individuals as well as possible genetic predisposition not yet elucidated. This may also have been due to the addition of celadrin to glucosamine, which has not been previously studied in this manner. The fact that our sample size is small also precludes us from drawing definitive conclusions of susceptible individuals. Further studies are needed, with larger sample size, randomized-control design, and inclusion for clinical outcomes will better delineate the importance of this subject.

Clin Lab Sci encourages readers to respond with thoughts, questions, or comments regarding this article. Email responses to westminsterpublishers@comcast.net. In the subject line, please type “CLIN LAB SCI 23(1) RE LIN”. Selected responses will appear in the Dialogue and Discussion section in a future issue. Responses may be edited for length and clarity. We look forward to hearing from you.

REFERENCES
FOCUS: OBESITY AND METABOLIC SYNDROME

Obesity and Metabolic Syndrome Overview

WAYNE GADE, JEAN GADE

INDEX TERMS: Obesity, metabolic syndrome, cardiovascular disease, diabetes.

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Wayne Gade, PhD, MT(ASCP) is the Focus: Obesity and Metabolic Syndrome guest editor.

Modern societies suffer from an obesity epidemic, despite considerable societal pressure to be thin. The epidemic persists in spite of the widely accepted notion that obesity is unhealthy, unattractive, and shortens life expectancy. Conservation of energy (the first law of thermodynamics) tells us that obesity must result when caloric intake chronically exceeds caloric expenditures. Historically, physiologic control mechanisms suppressed appetite and promoted physical activity, enabling most people to avoid a positive energy balance. But what forces compel modern humans, the most intelligent and rational of beings, to chronically exceed their caloric requirements?

In the first article, we compare the development of obesity to a “highway” whose destination is obesity. This “Highway to Obesity” has many “entrance ramps” that represent the many biological, genetic, and psychological factors that underlie overindulgence. We explore the genetic deficiencies and biological tendencies that predispose one toward obesity. In recent decades, these inherent tendencies toward obesity are more likely to be expressed, due to easy access to high calorie foods and decreased physical activity. Complete genetic deficiencies rarely occur and generally result in early-onset, morbid obesity. More commonly, obesity involves a gradually acquired dysregulation of hormonal systems that initially limit obesity. However, when these fragile feedback mechanisms become overwhelmed and fail, hormone resistance actually favors weight gain.

Article one also examines the multitude of co-morbidities associated with obesity. These include type II diabetes, atherosclerosis, hypertension, congestive heart failure, pulmonary disease, several forms of cancer; renal disease, liver and gall bladder diseases, polycystic ovarian syndrome, coagulation disorders, sleep apnea, stroke, osteoarthritis, gynecological problems, and ocular diseases. But just how does obesity affect physiological changes that promote such a wide array of diseases?
The second article explores metabolic syndrome (MSX), an array of symptoms that are known to increase the risk of cardiovascular disease. These include insulin resistance, hyperglycemia, elevated lipid levels, and increased abdominal fat deposition, all factors known to compromise metabolic and cardiovascular health. The article describes numerous biochemical changes that damage cellular function, collectively termed lipotoxicity, which often translates into organ failure when obesity becomes extreme.

The next article in this series, which appears in a subsequent issue, will illustrate the clinical lab results (lipid panels, hormone levels, etc.) expected from several obese patients whose weight gain has resulted from different physiological or psychological conditions. A fourth and final article describes how a previously healthy individual's lifestyle choices have contributed to the development of obesity and metabolic syndrome. In this case, we follow the progression from an athletic teenager with normal metabolic and endocrine function to an obese middle-aged patient who has developed leptin and insulin resistances, glucose intolerance, abdominal obesity, hyperlipidemia, hypertension, and atherosclerosis.
Failures of Feedback:
Rush Hour Along the Highway to Obesity

WAYNE GADE, JEAN GADE, MELISSA COLLINS, JESSICA SCHMIT, NICOLE SCHUPP

Learning Objectives
After reading the following article, the reader should be able to:

1. Define obesity, in terms of body mass index or BMI.
2. Describe the hormones and functions of the HPA system and non-HPA hormones.
3. List and describe the three primary ways that the hypothalamus helps regulate body weight.
4. Describe the molecular types and tissue of origin for leptin, insulin and cortisol.
5. List and describe four “entrance ramps” to the “highway to obesity.”
6. Describe how leptin and insulin resistance are related to the development of obesity.
7. Describe “lipid buffering” and how it relates to ectopic fat deposition.
8. Describe how the dopamine “pleasure/reward system” is involved with such diverse behaviors as drug and alcohol abuse and overeating.
9. Discuss the impact of inheritance on an individual’s tendency to become obese.
10. Identify analytes that are typically elevated by the metabolism of obesity versus analytes that are decreased or unchanged.

Abstract
From hot dogs to Hashimoto’s and inheritance to inactivity, many “entrance ramps” converge onto the “Highway to Obesity”, each contributing caloric intake that exceeds expenditure. Initially, the hypothalamus regulates appetite and energy based on leptin feedback, until feedback failure increases appetite, and allows deposition of abdominal fat, metabolic dysregulation, and metabolic syndrome. Without feedback controls, progress toward obesity is unimpeded unless diet, exercise, and/or medications provide an exit ramp.

Introduction
The worldwide obesity epidemic extends well beyond the borders of the United States or North America.
Two-thirds of US adults are overweight and 67 million (32%) are obese.\(^1,2\) The percentages of obese adults in the United States increased substantially from 1994 to 2007 as shown in Figure 1.\(^2\) Over half of Germans are overweight.\(^3\) Although the percentage of obese adults is lower in China and India, each country has twice as many obese people as the United States.\(^3\) The obesity epidemic includes less developed countries, such as Mexico, where 70% are overweight, and Egypt, where 60% are overweight.\(^3\)

**Figure 1: Changes in Percentage of Adult Obesity**

The 1994 map shows that most states had adult obesity percentages below 15% and no states had percentages over 20%. By 2001, all states except Colorado had obesity percentages over 15%, and 28 states had more than 20%. In 2007, only Colorado had less than 20% obesity and three states had increased to more than 30% adult obesity. Modified with permission from CDC website on Obesity. (http://www.cdc.gov/nccdphp/dnpa/Obesity accessed 11/10/2008)

Obesity results from a positive energy balance (PEB) and is associated with type 2 diabetes mellitus (DM2); colon, endometrial, and breast cancer; renal, gall bladder, and liver diseases; polycystic ovarian syndrome, coagulation disorders, sleep apnea, stroke, osteoarthritis, gynecological problems, ocular diseases, and development of atherosclerosis and cardiovascular disease (CVD).\(^4,11\)

Body mass index (BMI), mass (kg)/height\(^2\) (m) is commonly used to classify status: BMIs greater than 25 indicate overweight, over 30 is obese, and over 40 is considered extremely obese.\(^4,5,7,12\) People with large lean muscle mass and minimal body fat could mistakenly qualify as obese. For example, Arnold Schwarzenegger in his Mr. Universe prime, had a BMI over 30.

Molecular physiology has significantly increased our understanding of the development of obesity, yet it offers an incomplete picture. We must also consider that many dietary choices represent conscious decisions to ignore internal signals of satiety, often in futile attempts to quiet noise from a stressed-out society.\(^5,13,14\) Patients who are stressed and/or depressed may overeat because they find control and comfort in food. Children learn bad eating habits, while others overeat simply because they enjoy food. Advertising promotes economical “supersized” portions that offer large quantities of cheap calories and prolong the enjoyment of eating, but require us to ignore molecular signals of satiety. Overeating often represents addictive behavior, involving the dopamine pleasure-reward system similar to the basis of drug and alcohol addictions.\(^3,13,14\) Figure 2 illustrates many of the “entrance ramps” for the “Highway to Obesity.”

**Figure 2: Multiple Entrance Ramps Along the Highway to Obesity**

Many roads enter the obesity highway, each one delivering excessive calories or decreased caloric requirements, or both. The common characteristic is a chronic positive energy balance (PEB) that overwhelms metabolic and hormonal controls and eventually demands ectopic fat deposition. Development of leptin and insulin resistances leads inevitably to obesity if diet, exercise, and/or drugs cannot provide an exit ramp.
Genetics accounts for approximately half of the observed weight or BMI variation within a population, but the genetic contribution is polygenic and individual “obesity genes” typically make small contributions toward a person’s BMI. To date, most polymorphisms alter feedback mechanisms, and cause ineffective control of appetite and metabolism. Regardless of the underlying cause, a consistent PEB precedes obesity and destroys delicate feedback systems. Simplistically, the body ignores signals intended to suppress overeating, and excess calories are deposited as abdominal fat, resulting in lipotoxicity, inflammation, CVD, and other diseases. This review concentrates on hormonal and metabolic changes resulting in chronic overindulgence and obesity. The accompanying article focuses on metabolic syndrome and the toxic and destructive consequences of obesity. Examples of clinical case data, including lipid profiles, glucose, and hormone levels will be presented in a future article.

Methods
A comprehensive list and description of methods used to investigate the complex molecular interactions leading to obesity is beyond the scope of this article. Molecular Diagnostics, by Buckingham and Flaws, offers an excellent “primer” of molecular techniques and Tietz Fundamentals of Clinical Chemistry, edited by Burris and Ashford, provides an excellent background of clinical methodology.

Expanding Our Search for Molecular Explanations
Adipocytes: Factories, Not Just Warehouses
Traditional endocrinology described the roles of insulin, glucagon, cortisol, epinephrine, thyroxin, and the hypothalamus-pituitary axis (HPA) in metabolism, but failed to adequately explain the development of obesity. The hypothalamus, through the HPA directs three major functions associated with body weight and obesity:

1. Control of metabolism (anabolic, catabolic, and thermogenesis).
2. Sensations of hunger or satiety and food preferences (such as comfort foods).
3. Control of physical activity (for energy use or conservation).

But what signals elicit the appropriate responses by the hypothalamus, allowing it to maintain normal metabolism and avoid weight gain? Complex input from sensory and neuronal circuits are balanced with hormonal feedback to provide a remarkably effective homeostasis mechanism. When signals are ignored, as occurs with insulin resistance in DM2, homeostasis is destroyed. Questions concerning weight gain, appetite, and the frustrations of dieting remained unanswerable without additional factors. The unexpected source of these factors was adipocytes, previously visualized as primarily fat warehouses. Adipocytes are now considered major endocrine tissues, secreting products derived from 30% of their expressed genes, including the hormone leptin and several “adipokines” that contribute to the new “endocrinology of obesity.” Leptin reports energy status, or “adiposity” to the hypothalamus, which then controls metabolism, appetite, and activity.

The hypothalamus mechanisms that control metabolism and appetite are incredibly complex and are beyond the scope of this article. However, some mention of the unique neurotransmitters is helpful. For example neuropeptide Y (NPY) and melanocortins have potent effects on appetite. NPY stimulates appetite and appears to be inhibited by leptin. Melanocortins (MCH) inhibit appetite and are stimulated by leptin.

So, Why Do We Overeat?
Why do so many people overeat when they know it is unhealthy? Any comprehensive model of overeating should consider several levels of influence. One level of appetite control involves a complex balance between the hormonal and metabolic signals. This level is the focus of most cellular and molecular research (and most of this article). Level two is a sub-conscious neuronal “pleasure-reward system”, based (simplistically) on the neurotransmitter dopamine, which influences and overlaps with these hormonal interactions. This system
originally rewarded behaviors that led to physical safety and wellbeing, such as successfully avoiding a saber-toothed tiger, building a warm fire, or gathering tasty berries. Unfortunately many negative activities such as drug or alcohol abuse and overeating can subvert the dopamine reward system. In effect, negative behaviors are rewarded with the same dopamine elevations (by snorting cocaine, or consuming baskets of chips and salsa) that would ideally be reserved for behaviors that did more to secure safety and wellbeing. Another level of decision-making includes subconscious habits, such as “compulsively cleaning up one’s plate”, even though full, or anticipating a feast every time you return home for the holidays. Finally our conscious choices may override all other levels. Sometimes we simply choose to eat for enjoyment or companionship, without regard for subconscious or molecular input. Whatever the motivation, consistent overindulgence in pleasurable foods quickly results in leptin resistance and diminished appetite control. Once hormonal restrictions are removed, the neuronal systems may continually reward overeating behaviors.

**Leptin and the “Obesity Gene”**

In 1973, Coleman demonstrated that mice with the ob/ob genotype become obese, with 5-fold more body fat than normal mice on identical diets. In 1994, Jeffrey Friedman described leptin, a 16 kd peptide hormone product of the “obesity gene” (ob), which is secreted from fat cells. Animals with the ob/ob genotype were leptin deficient. Without leptin, ob/ob mice had uncontrolled appetites (hyperphagia) and lacked thermogenesis (caloric expenditure to generate heat and without accumulation of ATP), causing development of obesity, insulin resistance, and diabetes. The incredible hyperphagia that leads leptin-deficient mice to eat until their size prevents them from reaching their food, also causes leptin resistant mice (fa/fa genotype) to do the same. Insulin resistance and diabetes in leptin-deficient mice were reversed by infusion of leptin or adenovirus-mediated insertion of the leptin gene. Leptin limits abdominal fat deposition (ectopic fat or visceral adipose tissue, VAT, see Figures 3 and 4). Insulin resistance and diabetes in leptin-deficient mice were reversed by infusion of leptin or adenovirus-mediated insertion of the leptin gene. Leptin levels increase with adipocyte mass, and elevated leptin levels provide feedback to the hypothalamus concerning energy reserves. The leptin gene, therefore was widely hailed as the “obesity gene” and millions hoped for an easy and quick solution to their weight problems. However, obese patients are rarely leptin deficient, more often exhibiting elevated leptin levels and leptin resistance. This does not diminish leptin’s impact on the subject of obesity. As with diabetes, the hormone resistant condition can be as problematic as the deficiency state.

**Leptin’s Mode of Action**

Leptin binds to cell surface receptors, activating a signaling system commonly used by cytokines (designated JAK-STAT). As shown in Figure 3A, the system branches to activate the AMP-activated protein kinase (AMPK) system and various transcriptional control elements (designated PPARs, SREBPs, among others). Thus, leptin signaling includes immediate activation/deactivation of existing enzymes and transcriptional control of the amount of enzyme present. The AMPK system is often described as the cell’s “energy gauge” because it responds to the relative amounts of AMP and ATP. Note that leptin’s activation of the AMPK signaling sequence is AMP-independent and overrides the intracellular gage in favor of the external energy status. In liver and muscle cells, leptin binding leads to an activation of the AMPK system, while leptin binding to hypothalamic receptors decreases AMPK activity and tells the hypothalamus when to suppress appetite and increase caloric expenditure.

**Leptin Limits Abdominal Fat Deposition**

Roger Unger and others suggest that leptin’s primary role is not prevention of obesity, but protection of non-adipose tissues from excessive lipid deposition. Leptin initially limits abdominal fat deposition (ectopic fat or visceral adipose tissue, VAT, see Figures 3 and 4). Notice that both AMPK activation and the transcriptional signals discussed above promote increased fatty acid oxidation and block TG synthesis in liver and muscle. Leptin sends a different message to adipocytes, where lipid synthesis and storage are promoted. Thus, by promoting β-oxidation in non-adipose tissues and the TG synthesis in fat cells, leptin...
directs fat deposition toward adipocytes and limits ectopic fat deposition.\textsuperscript{9,10}

A. Leptin binding to cell surface receptors (JAK/STAT signaling system) causes tissue specific responses at both the level of gene transcription and phosphorylation of existing enzymes. In non-adipose tissues, these actions result in increased β-oxidation and decreased triglyceride (TG) synthesis. Thus, non-adipose tissues are protected from producing too many TGs (ectopic fat). In the hypothalamus, adenosine monophosphate-activated protein kinase (AMPK) is decreased, leading to appetite suppression, thermogenesis, and increased physical activity.

B. Lipid buffering in adipocytes involves a “futile cycle” of lipolysis, followed by re-esterification of fatty acids (FAs) and glycerol to reform TGs. The glycerol liberated by lipolysis is not reused, but is exported to the liver for gluconeogenesis. Thus, glycerol must be replaced through the process of glyceroneogenesis. This multi-step process is controlled by the enzyme phosphoenolpyruvate carboxykinase (PEPCK). Approximately 40% of FAs released by lipolysis are re-esterified by this lipid buffering system. Lipid buffering prevents large amounts of free fatty acids (FFAs) from entering plasma. For additional explanation, see reference 16.

**Figure 3:** Leptin’s Signaling and the Lipid Buffering Cycle

A. Normal individual is on a good diet and healthy lifestyle. Occasional excess calories cause increased leptin and thermogenic metabolism to burn excess calories. Lean tissues have minimal ectopic fat.

B. Chronic overindulgence causes leptin resistance and massive ectopic fat deposition occurs in and around visceral organs.

**Figure 4:** Development of Obesity and Deposition of Ectopic Fat

Modified with permission from Unger RH, Biochimie 2005; 87: 57–64.

Normal rats on 60% fat diets became obese, and deposited 3-fold more fat in their liver. Leptin-deficient animals had ravenous appetites, developed insulin resistance, and deposited up to 100-fold more hepatic triglycerides (TGs), even on 6% fat diets.\textsuperscript{9,10,20} Leptin replacement controlled appetites, reversed insulin resistance, and decreased liver TGs.\textsuperscript{9,10,20,24} in deficient animals. Similarly, leptin-deficient rats fed high sucrose diets, deposited abdominal fat, with increased liver export of TGs.\textsuperscript{9,10,20} Again, leptin infusion caused rapid decreases of TGs in both liver and plasma.\textsuperscript{24}

Leptin normally limits abdominal fat and promotes expansion of subcutaneous fat tissues.\textsuperscript{27,31,32} Leptin resistance lowers metabolic rates, increases appetite, and leads to limited physical activity, ultimately causing abdominal obesity and expansion of metabolically hyperactive and toxic VAT (Figure 3).\textsuperscript{4–11}
Lipid Buffering and Re-esterification

Elevated levels of free fatty acids (FFAs) play a key role in the development of obesity and the associated lipotoxicity.\(^9,10,20\) Lipolytic release of FFAs and glycerol is catalyzed by hormone sensitive lipase (HSL) during fasting conditions (low insulin: glucagon ratio).\(^12\) Approximately 40% of FFAs released by lipolysis are resynthesized to TGs without leaving the fat cell. This futile cycle “buffers” the body from large fluctuations of serum FFA.\(^9,10,12,31,32\) Glyceroneogenesis, controlled by phosphoenolpyruvate carboxykinase, or PEPCK, produces glycerol to complete this cycle, because the glycerol released by lipolysis is exported, rather than recycled.\(^32-34\) As seen in Figure 3B, animals that overexpress PEPCK were able to effectively buffer serum lipids when on a normal diet, minimizing ectopic fat, maintaining insulin sensitivity, and having lower serum FFAs and TGs.\(^32-34\) However, on a high fat diet, these animals were ineffective at lipid buffering; they became obese, displayed insulin and leptin resistances, and had elevated FFAs and TGs.\(^33\)

Leptin Resistance Leads to “Starvation Physiology”

Fasting conditions decrease caloric requirements as metabolism “downshifts” in response to high insulin/glucagon ratios. Leptin deficiency (in ob/ob mice) or resistance (in fa/fa mice) induces “starvation physiology” that includes a dramatic shift beyond catabolic metabolism and reduced BMR, to include hyperphagia, and decreases in physical activity, body temperature, immune function, and fertility.\(^9,10,20-22\) Leptin-deficient or resistant mice exhibit these physiologic and behavioral changes that promote weight gain and obesity, even when caloric intake is matched with those of normal-weight control mice.\(^9,10,22\) These changes are eliminated by leptin infusion or insertion of functional genes.\(^9,10,20,24\)

Many Roads Converge onto the Obesity Highway

As illustrated in Figure 2, there are many “entrance ramps” onto the obesity highway. Each ramp contributes to a PEB, resulting from some combination of increased consumption and decreased caloric expenditure. Before merging onto the obesity highway, hormonal feedback systems effectively compensate for wide daily variations in food intake and exercise. However, consistent caloric excesses inevitably lead to leptin resistance and obesity. Figure 2 shows that leptin resistance often precedes insulin resistance and impaired glucose metabolism.\(^4,5,9,10\) Increasing hormonal resistance accelerates progression on the obesity highway, as the natural deterrents (leptin) to weight gain are removed, making it more difficult to exit the highway.

Where Do Adipocytes Originate?

Pre-adipocytes, which originate from the macrophage cell line, are phagocytic, express macrophage-specific cell surface antigens, and secrete high levels of pro-inflammatory adipokines, such as TNF-α and IL-6.\(^34,35\) These macrophage-like characteristics are absent or muted in mature adipocytes. Occasional overeating causes expansion of storage within pre-existing subcutaneous adipocytes and stimulates thermogenesis to eliminate excess calories. Chronic overindulgence results in leptin resistance and facilitates recruitment and maturation of pre-adipocytes into the larger adipocytes of VAT.\(^32-35\)

Cortisol, Coping, and Comfort Food

Chronic stress is manifest in many aspects of modern society, initiating the chronic stress-response network, including elevation of cortisol as shown in Figure 5.\(^5,13\)
Figure 5: Stimulation & Hormonal Feedback to Hypothalamus

Many outside stimuli are integrated by the hypothalamus with hormonal and metabolite feedback systems that provide status reports concerning internal conditions. For example, glucose and fatty acids would indicate current energy status, while insulin and leptin report on energy storage or “adiposity.”

The hypothalamus helps control appetite, metabolism, thermogenesis, and physical activity. It also releases corticotropin-releasing hormone (CRH), which signals the pituitary to release adrenocorticotropic hormone (ACTH). Finally, ACTH stimulates the release of cortisol.

Failure of these feedback systems (hormonal resistance) is a critical landmark on the obesity highway, because it perpetuates the positive energy balance by allowing increased appetite, decreased physical activity, starvation metabolism, and deposition of abdominal fat.

Studies of animal models demonstrate stress-induced hyperphagia and a preference for “comfort” foods that are high in fats and carbohydrates. In humans, high cortisol levels seem to promote compulsive or pleasurable, stress-relieving activities, such as eating sweet or fatty foods, taking drugs, and drinking alcohol. Cortisol promotes overeating and redistribution of energy reserves (toward abdominal fat) by increasing gluconeogenesis and TG synthesis, fueled by increased catabolism of muscle protein. Relieving stress by “vegging out” with a TV remote and plate of comfort food is a popular “entrance ramp” onto the highway to obesity.

Cushing disease

Cushing disease is hypercortisolemia that originates from endocrine dysfunction rather than stress. Classic symptoms of Cushing disease are the round “moon face” and “buffalo hump” accompanied by the characteristic central obesity with thin appendages. Cushing disease causes substantial muscle wasting, thinning of skin, immune suppression, and increased bruisability and bleeding, all associated with protein degradation to support gluconeogenesis.

Pleasure-Seeking Lifestyle Choices and Habits

Obesity often results from pleasure-seeking choices, reinforced by the dopamine pleasure/reward system. Many unfortunate choices result from habits learned in childhood (and unchanged in adulthood), behaviors influenced by slick advertising, lack of physical activity or exercise, and consumption of readily available, high calorie foods. Current cultural emphasis on “immediate gratification” also favors pleasurable eating over exercise or dietary restriction.

Genetic Predisposition

A strong genetic influence on the development of obesity is suggested by close similarities in BMIs found in identical twins raised apart. Analysis from family and twin studies estimates that 30–70% of body mass variation within the population results from inherited factors. However, experts agree that inheritance of a tendency toward obesity is usually not due to a single gene defect but is, instead, polygenic. Studies show that each individual gene typically exerts a modest impact on BMI. Very rare exceptions are monogenic defects, such as deficiencies of leptin, leptin receptor, or genes controlling hypothalamic feedback. In all of these cases, the result is loss of appetite suppression and early-onset obesity. Less dramatic polymorphisms within human genes for leptin, leptin receptors, adiponectin (ADN) and ADN receptors exist, but most variants are associated with minor changes in weight or BMI.

Several large population studies, using genomic and bioinformatics techniques have found specific alleles of a gene (designated FTO) that is associated with 1–3 kg
weight increases.\textsuperscript{36} Obviously, this small weight gain seldom raises a normal BMI into the obese range.

**Hypothyroidism Reduces BMR**

Thyroid hormones control our basal metabolic rate (BMR), and hypothyroidism, most commonly an autoimmune disease called Hashimoto disease, reduces the BMR by 10-15\%. Such consistent reductions in BMR make weight gain difficult to avoid. Patients with Hashimoto disease also tend to suffer from exercise and cold intolerance, lethargy, and constipation.\textsuperscript{12,37} Fortunately, thyroid hormone supplementation can moderate the effects of Hashimoto disease.\textsuperscript{11,12,37}

**Alcohol, Drugs, and Neurochemistry**

Many addictive or compulsive behaviors, including alcoholism, are often associated with dopamine. Obese patients often express fewer dopamine receptors, similar to levels found in people addicted to drugs and/or alcohol.\textsuperscript{14} Dopamine is a component of the brain’s “pleasure center”, and drugs such as cocaine potentiate dopamine activity by blocking its re-uptake. This provides a neurochemical link between obesity and obsessive-compulsive behaviors, which are 25\% more likely to occur in obese patients compared to patients of normal weight.\textsuperscript{14} Additionally, the β-adrenergic and sympathetic nervous systems are involved with thermogenic response.\textsuperscript{39,40} Mice with β-adrenergic receptor deficiencies lacked the thermogenic response to over-nutrition and became obese, compared to control mice of normal weight.\textsuperscript{40}

Some prescription drugs, such as the second-generation antipsychotics (e.g. olanzapine and clozapine) are associated with significant weight gain, abdominal fat deposition, and insulin resistance.\textsuperscript{14, 38} Interestingly, these drugs appear to affect the AMPK phosphorylation system, decreasing hypothalamic control of appetite.\textsuperscript{38}

**Lifestyles Devoid of Physical Activity**

Many jobs and many leisure activities require little or no physical activity and make a PEB difficult to avoid. Typical Americans consume roughly 100 calories/day more than in 1900, but burn several hundred fewer calories/day during physical activity.\textsuperscript{2} For example, an employee at a computer-dominated job might burn 500 work-related calories per day, compared to construction or farm workers, who burn more than 1500 work-related calories.\textsuperscript{12} Unfortunately, inconsistent vigorous workouts at the gym seldom burn enough calories to cause substantial weight loss without dietary constraints. Since progression along the obesity highway is fueled by a PEB, a long-term commitment to a combination of exercise and diet provides the best “exit ramp”.\textsuperscript{4,5,41,42}

**Yo-Yo Diets: Fighting Vainly Against Our Hypothalamus**

Dieters know the feeling of “swimming against the current” to lose weight, and the term “yo-yo diet” describes our body’s resistance to weight loss. Popular myth suggests that body weight skyrockets whenever we look away, but endocrinology indicates that our hypothalamus initially limits weight gain above our current weight or “set point”. See Table 1 for a summary of the effects of energy balance on hormones and metabolism.

Dieting decreases caloric intake and, at weights below the set point, decreased leptin levels signal the hypothalamus of diminished “adiposity” (energy storage). Fasting metabolism results in decreased caloric requirements, increased appetite, and limited physical activity.\textsuperscript{12, 39, 40, 41, 42} Another peptide hormone from the GI tract, called ghrelin (or the “hunger hormone”) also promotes increased appetite during dieting.\textsuperscript{45}

Above the set point, elevated leptin signals the hypothalamus that energy reserves are adequate. This results in appetite suppression, increased anabolic metabolism and thermogenesis, and encourages physical activity. However, chronic “over-nutrition” causes leptin/insulin resistances, negating hormonal feedback with its associated appetite and metabolic effects.\textsuperscript{12, 41,42}

**Obesity’s Effect on Lifespan**

Given the depressing list of co-morbidities associated with obesity (described in the accompanying article), it is not surprising that weight loss has a positive impact on longevity. Numerous studies have documented the metabolic and health benefits of weight loss, by either
FOCUS: OBESITY AND METABOLIC SYNDROME

Table 1: Interaction of Hormones and Metabolism Resulting From Dietary Changes

<table>
<thead>
<tr>
<th>Status of Energy Stores, Weight Change</th>
<th>Hormonal Signals</th>
<th>Metabolic Rate Storage or Starvation</th>
<th>Appetite or Satiety</th>
<th>Physical Activity</th>
<th>Net Weight Gain or Loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative energy balance (diet); weight loss</td>
<td>Leptin (\uparrow)</td>
<td>Starvation metabolism (\downarrow) BMR</td>
<td>Appetite (\uparrow)</td>
<td>Physical activity conserve energy</td>
<td>Promotes weight gain</td>
</tr>
<tr>
<td>Neutral energy balance; No weight change</td>
<td>⇦ Leptin (\Rightarrow) Insulin</td>
<td>Normal metabolism (\Rightarrow) Normal appetite</td>
<td>Normal activity (\Rightarrow)</td>
<td>Promotes current weight</td>
<td></td>
</tr>
<tr>
<td>Positive energy balance; (occasional feasting) little weight gain</td>
<td>Leptin (\uparrow) Insulin (\Rightarrow)</td>
<td>Storage metabolism (\uparrow) BMR</td>
<td>Appetite (\uparrow)</td>
<td>Physical activity (\Rightarrow)</td>
<td>Promotes current weight or minimizes weight gain</td>
</tr>
<tr>
<td>Chronic positive energy balance; obesity</td>
<td>Leptin (resist) (\uparrow)</td>
<td>Starvation metabolism (\downarrow) BMR</td>
<td>Appetite (\uparrow)</td>
<td>Physical activity conserve energy</td>
<td>Promotes weight gain</td>
</tr>
</tbody>
</table>

diet or exercise. Experts now predict a general decrease in lifespan for the first time in centuries, as obesity and metabolic syndrome overwhelm medical advances. As described in the accompanying article, surplus lipids lead to lipotoxicity and apoptosis, while adipokines promote inflammation, atherosclerosis, and CVD. Unfortunately, given the complexities in development of obesity and the cellular damage caused by lipotoxicity and atherosclerosis, dietary “magic bullets” seem unlikely. While the new “endocrinology of obesity” offers hope for new prevention strategies, it also reaffirms the benefits of diet and exercise in controlling stress and reversing obesity and hormone resistance. Caloric restriction, even to levels slightly below accepted “requirements”, tends to extend lifespan.

Benefits of Diet and Exercise
Since obesity results from chronic over-nutrition, successful weight loss requires a “healthy” negative energy balance. Although exercise options are often limited for obese patients, finding appropriate ones is an important lifestyle change, with benefits beyond simply burning calories. When combined with diet, an exercise regimen helps counteract the usual “metabolic downshift” and decreased physical activity directed by the hypothalamus. Exercise promotes anabolic metabolism, increases BMR, reduces stress, and promotes hormonal sensitivity by up-regulating receptors and signaling pathways. Additionally, exercise can provide an alternate method of stimulating the brain’s pleasure center, which may have previously been dominated by unhealthy eating behaviors. A healthy diet and exercise can help decelerate progress along the obesity highway and even provide an exit ramp.

Approaches to Drug Treatments
Drugs such as sibutramine and rimonabant are appetite suppressants that modify our perceptions of hunger in the brain. Orlistat (sold as Alli) is an FDA-cleared drug that blocks intestinal fat absorption. Our knowledge of signaling pathways for control of appetite and metabolism enables the design of specific inhibitors or agonists. For example, neuropeptide Y (NPY) is a potent stimulator of appetite. Leptin inhibition of NPY...
explains part of its role in appetite suppression. Melanocortins also cause neuronal inhibition of appetite. Animal models with defective melanocortin-4 receptors become obese. Other appetite-signaling pathways include obvious targets, such as ghrelin, the “hunger hormone”.

The thiazolidinediones (TZDs) are specific activators of peroxisome proliferator–activated receptor (PPAR) and have been shown to improve insulin sensitivity in type 2 diabetic patients. TZDs have a direct antidiabetic effect on glucose metabolism in skeletal muscle and liver. In addition, TZDs increase insulin sensitivity by increasing lipid storage capacity of adipose tissue and reducing circulating FFA and triglyceride levels. TZDs decrease FA release from adipose tissue by increasing FFA re-esterification via the induction of both phosphoenolpyruvate carboxykinase (PEPCK), a regulatory enzyme of glyceroneogenesis, and glycerol kinase.

Other approaches include inhibition of adipocyte enzymes or transporters to prevent uptake or synthesis of TGs. Finally, stimulation of “fat burning” mechanisms, by “uncoupler proteins,” may enable burn-off of unwanted calories. Although many dietary supplements and over-the-counter formulations already claim success, availability of drugs that have cleared clinical trials and obtained FDA approval is several years away.

**Conclusion**

Leptin normally provides energy status reports to the hypothalamus, which then controls appetite and energy expenditure. Obesity generally involves a PEB that exceeds the adipocyte’s capacity to store fat and results in resistance to leptin. Once leptin resistance occurs, appetite suppression and control of energy expenditure are lost and starvation physiology defaults to conditions that actually promote continued weight gain (see Figure 6). The alarming list of physiologic changes includes: decreased metabolism, hyperphagia, chronic inflammation, deposition of abdominal fat, dysregulation of carbohydrate and lipid metabolism (and elevated glucose and FFAs), lipotoxicity, development of atherosclerosis, and CVD. All of these outcomes result in acceleration along the obesity highway toward metabolic syndrome.

![Figure 6: Progression Along the Obesity Highway](image)

*Clin Lab Sci* encourages readers to respond with thoughts, questions, or comments regarding this Focus section. Email responses to westminsterpublishers@comcast.net. In the subject line, please type “CLIN LAB SCI 23(1) FOCUS: OBESITY AND METABOLIC SYNDROME”. Selected responses will appear in the Dialogue and Discussion section in a future issue. Responses may be edited for length and clarity. We look forward to hearing from you.

**REFERENCES**

FOCUS: OBESITY AND METABOLIC SYNDROME

LEARNING OBJECTIVES
After reading the following article, the reader will be able to answer the following:

1. Identify the major contribution to our understanding of obesity and metabolic syndrome by each of the following researchers: Gerald Reaven, Norman Kaplan, Richard Unger, and Jeffery Friedman.
2. Identify the criteria used for diagnosis of metabolic syndrome (MSX).
3. Identify and describe three outcomes of normal leptin feedback to hypothalamus and three outcomes related to leptin resistance.
4. Describe the relationship between free fatty acids (FFAs) and ceramide and their significance in lipotoxicity and apoptosis.
5. Describe four destructive outcomes of elevated FFAs leading to disease and apoptosis.
6. Identify a proposed physiologic role for leptin that is independent of hypothalamus.
7. Describe “lipid buffering” and how the development of leptin resistance allows fat deposition into non-adipocytes (ectopic fat).
8. Describe why glyceroneogenesis and PEPCK activity are required for “lipid buffering” or fatty acid re-esterification.
9. Describe the signal transduction resulting from leptin binding (JAK/STAT system), which exerts both transcriptional level control (PPARs) and control of pre-existing enzyme activities through the AMPK system.
10. Discuss the importance of adiponectin (ADN) as an “adipokine” in relation to obesity and metabolic syndrome.
11. Describe the development of atherosclerosis including the inflammatory process that recruits macrophages and list three ways that ADN helps prevent this process.

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ABSTRACT
Metabolic syndrome (MSX) identifies clinical symptoms and lab results, including abdominal obesity, insulin resistance, hyperglycemia, hyperlipidemia, and hypertension, that lead to an increased risk of cardiovascular disease (CVD). Obesity typically results in insulin and leptin resistance and a shift from expansion of subcutaneous fat to deposition of abdominal and ectopic fat. These conditions cause metabolic dysregulation, elevated fatty acids (FFA), and increased secretion of pro-inflammatory “adipokines”. Left untreated, these conditions cause lipotoxicity, chronic inflammation, hypertension, atherosclerosis, and CVD.

INTRODUCTION
Abdominal obesity is the most obvious symptom of metabolic syndrome, the term used to describe a cluster of symptoms that increase risk of cardiovascular disease (CVD). In 1988, Reaven’s landmark paper included type 2 diabetes (DM2), insulin resistance, hyperglycemia, hyperlipidemia, and hypertension as risk factors for atherosclerosis and CVD.1, 2 A year later, Kaplan included abdominal obesity, glucose intolerance, hypertension, and hyperlipidemia as the “deadly quartet,” thereby formalizing the connection between obesity, metabolic syndrome, and cardiovascular risk.3 Thousands of papers have discussed various aspects of the syndrome and proposed many names. Metabolic syndrome is the regrettable generic term used most often.4–11

Other medical conditions associated with obesity include DM2, several forms of cancer, renal and liver diseases, polycystic ovarian syndrome, sleep apnea, chronic inflammation, and atherosclerosis.4–14 In 1983, researchers reported that women with visceral (android) fat were 10-fold more likely to develop diabetes than women with similar amounts of lower body (gynoid) fat.15 Leptin, a hormone secreted by fat cells, directs storage of excess calories into subcutaneous deposits, but as obese patients develop leptin resistance, fat is deposited between visceral organs and inside non-adipocytes (ectopic fat).9, 10–13 Visceral adipose tissue (VAT) consists of larger cells with increased lipolysis (releasing FFAs) and greater secretion of pro-inflammatory factors than subcutaneous adipocyte tissues.9, 10–13

Enzyme or hormone deficiencies cause many metabolic diseases, such as type I diabetes, phenylketonuria (PKU), or von Gierke’s disease (impaired glycogen storage).16 Genetic analysis from family and twin studies of obesity produces estimates of “heritability” ranging from 30–70%, meaning that approximately half of the variation in body mass within a population is a result of inherited factors.17–20 Most experts assume a polygenic component to obesity and that individual “obesity genes” typically make only modest contributions toward a person’s BMI.17, 19, 20

In most cases, the underlying cause of MSX is acquired hormone resistance caused by “overnutrition” that overwhelms previously normal endocrine function.4, 5 Leptin resistance releases appetite suppression, decreases caloric expenditure, and permits storage of excess calories as VAT. Abdominal fat and hormonal resistances lead to dysfunctional lipid and carbohydrate metabolism, lipotoxicity, and apoptosis (programmed cell death).9–12 Chronic inflammation is promoted by excessive secretion of “adipokines” (cytokines secreted by fat cells) that lead to “hyperresponsiveness” of the arterial endothelium, increased leukocyte recruitment, oxidative damage, plaque formation, hypertension, vascular hypertrophy, atherosclerosis, and CVD.5, 6, 9, 21 Physiological connections between the diverse symptoms were initially unclear, but molecular explanations are resolving the complex relationships and bringing many causes of heart disease into sharper focus. Researchers recognize that many factors contribute to obesity, including genetics, stress, poor lifestyle habits, compulsive or depressive disorders, and many more. The preceding article discusses hormonal feedback systems that originally curb appetite, increase metabolism and promote physical activity to prevent rapid weight gain.22 However hormonal resistance occurs, development of obesity becomes more likely. This review focuses on the consequences of obesity, including metabolic syndrome and cardiovascular disease.
Diagnostic Criteria for Metabolic Syndrome

Many organizations worldwide have proposed diagnostic criteria for MSX; Table 1 summarizes criteria from three prominent organizations, the World Health Organization (WHO), the National Cholesterol Education Program (NCEP, also known as the Adult Treatment Panel III), and the American Association of Clinical Endocrinologists (AACE).5 A 1998 WHO report defined MSX assuming insulin resistance and hyperglycemia, along with two or more of the following: obesity (high waist to hip ratio or BMI > 30), a poor lipid profile with high TGs and low HDLs, hypertension, and renal damage (indicated by microalbuminuria).14

Both NCEP and AACE criteria specifically excluded diabetic patients (as a separate diagnosis), but assumed insulin resistance and the “pre-diabetic” hyperglycemic conditions of impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT), along with hyperlipidemia, high cholesterol, hypertension, and abdominal obesity.5,23 The NCEP and AACE criteria are typically used by physicians for diagnosis of MSX (designated dysmetabolic syndrome X, ICD-9 diagnostic code 277.7). The AACE criteria also include a family history of CVD, diabetes, or hypertension.5,23

Note that all definitions combine easy physical measurements (estimates of BMI, waist to hip ratios, etc) with routine lab analyses, such as elevated glucose, TGs, total cholesterol, HDLs and LDLs. Thus, diagnosis of MSX is possible in the absence of obesity if non-obese patients have dyslipidemia, hyperglycemia, and hypertension (see Table 1). Although measurements of hormones such as insulin, cortisol, and leptin would help define underlying abnormalities, they would require complex and expensive lab measurements that are not required for the diagnosis. The diagnostic goal is to identify patients with increased cardiovascular risk.5,14,23

Pathophysiology of Metabolic Syndrome

Human obesity is far more often related to “overnutrition” and resistances to insulin, leptin and cortisol than to genetic deficiencies.5–8,17–20,24,25 The accompanying article acknowledges many different causes of weight gain and describes leptin and insulin resistances as landmarks along the “highway to obesity”.22 The present article continues along the obesity highway toward the MSX pathologies of lipotoxicity, apoptosis, chronic inflammation, hypertension, atherosclerosis, and CVD.

Cortisol Promotes Appetite and Abdominal Fat

Various forms of stress cause the hypothalamus to signal the pituitary to release adrenocorticotropic hormone (ACTH), which stimulates the adrenal cortex to release cortisol.16 Cortisol, a steroid hormonal, alters metabolism and also stimulates an appetite for high calorie “comfort” foods and deposition of abdominal fat.16,24,25 Cortisol helps redistribute energy reserves by promoting release of amino acids from protein catabolism and gluconeogenesis and lipid synthesis.16 Glucocorticoid hormone levels vary widely in a diurnal pattern, respond to environmental influences, and exist as both an inactive (cortisone) and active (cortisol) form in plasma. Interconversion between these forms by the enzyme 11-β-hydroxysteroid dehydrogenase type I (11-HSD) is tissue-specific and is the subject of intense research.24,25

Leptin Helps Restrict Abdominal Fat Deposition

Leptin levels initially increase with increasing fat cell mass and provide feedback to the hypothalamus of adequate “adiposity” or energy reserves. The hypothalamus then suppresses appetite, stimulates physical activity, and promotes thermogenesis to utilize excess calories.5,7 Therefore, leptin plays a critical role in directing fat deposition and control of body weight.27,28

Leptin normally directs deposition of fat to preexisting subcutaneous fat cells, while restricting ectopic TG deposition and expansion of deposition within VAT.9,11–13,26–30 Rapid or excessive weight gain leads to dyslipidemia and leptin resistance, causing the hypothalamus to become unresponsive, no longer suppressing hyperphagia.11–13,27,28 Infusion of leptin into leptin-deficient animals corrects diet-induced steatosis and dyslipidemia.31
FOCUS: OBESITY AND METABOLIC SYNDROME

Table 1: A Comparison of the Diagnostic Criteria for Metabolic Syndrome

<table>
<thead>
<tr>
<th>Symptom</th>
<th>WHO</th>
<th>NCPE (ATP III)</th>
<th>AACE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperglycemia</td>
<td>IFG or DM2 as initiating factor</td>
<td>IFG (FG &gt; 110 mg/dL)</td>
<td>IFG (FG &gt; 110-125 mg/dL) 2 hr OGTT glucose level &gt; 140 mg/dL</td>
</tr>
<tr>
<td>Insulin resistance</td>
<td>DM2 is considered the “initiating” factor</td>
<td>IFG, insulin resistance is assumed, but DM2 not included in definition</td>
<td>IFG or IGT, Insulin resistance is, assumed but DM2 is not included</td>
</tr>
<tr>
<td>Abdominal Obesity</td>
<td>Waist to hip ratio</td>
<td>Waist circumference: BMI &gt; 25 kg/m² or waist: BMI &gt; 25 kg/m² or waist: circumference &gt; 40 in. ♀; &gt; 35 in. ♂</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt; 0.90 in ♀; &gt; 0.85 in ♀ or BMI &gt; 30 kg/m²</td>
<td>&gt; 40 in. ♀; &gt; 35 in. ♀</td>
<td></td>
</tr>
<tr>
<td>Hypertriglyceridemia</td>
<td>TGs &gt; 150 mg/dL</td>
<td>TGs &gt; 150 mg/dL</td>
<td>TGs ≥ 150 mg/dL</td>
</tr>
<tr>
<td>Hypercholesterolemia (with decreased HDL)</td>
<td>HDL &lt; 35 mg/dL ♀; HDL &lt; 35 mg/dL ♀</td>
<td>HDL &lt; 40 mg/dL ♀; HDL &lt; 40 mg/dL ♀</td>
<td>HDL &lt; 40 mg/dL ♀; HDL &lt; 50 mg/dL ♀</td>
</tr>
<tr>
<td>Hypertension</td>
<td>&gt; 140/90 mm Hg</td>
<td>&gt; 130/85 mm Hg</td>
<td>&gt; 130/85 mm Hg</td>
</tr>
<tr>
<td>Other factors: family history, age, sex, ethnicity, proteinuria</td>
<td>&gt; 20 µg/min microalbumin or albumin/creatine &gt; 20 mg/g</td>
<td>Not included in definition</td>
<td>Sedentary lifestyle, age, ethnicity, family history</td>
</tr>
<tr>
<td>Diagnostic Requirements</td>
<td>Diabetes, IGT, IFG, or IRS and two or more of above</td>
<td>Three or more of the above factors included</td>
<td>Insulin resistance assumed but seldom measured. Final diagnosis is left to discretion of physician</td>
</tr>
</tbody>
</table>

As seen in Figure 1A, leptin binding to a non-adipose cell, activates a signaling pathway (designated JAK/STAT) that produces a transcription factor (designated PPAR-α), which promotes transcription of genes needed for β-oxidation of FFAs. However, in fat cells, TG storage is promoted over β-oxidation (not shown). Diet-induced obesity (DIO) leads to leptin resistance and a different transcription factor (designated PPAR-γ) promotes enzymes involved with lipid synthesis and storage. Since the cells depicted in Figure 1B are non-adipose cells, storage of significant amounts of TGs would constitute ectopic fat deposition. Unfortunately one outcome of the excessive FA synthesis is the production of the lipotoxic compound ceramide.

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FOCUS: OBESITY AND METABOLIC SYNDROME

A. Normal non-adipocytes respond to leptin by activating the signaling pathways and producing the transcription element designated PPAR-α (peroxisome proliferator-activated receptor-α). The element promotes β-oxidation (solid line) and decreases triglyceride storage (TG, dashed line).

B. Leptin resistant non-adipocytes do not respond to leptin and a different transcription element designated PPAR-γ (peroxisome proliferator-activated receptor-γ) is produced. This element promotes TG storage (solid line) and decreases β-oxidation (dashed line). Elevated free fatty acids (FFAs), are often seen in leptin resistance and are also combined with the amino acid serine to form cytotoxic ceramide.

Figure 1: Toxic Effects of FFAs and Leptin Signaling in Non-Adipocytes

Fatty liver, frequently associated with MSX, is often differentiated into alcoholic and non-alcoholic steatosis. Normal hepatic metabolism supplies fasting glucose and secretes the majority of plasma proteins, including albumin, lipoproteins, and coagulation factors. Fatty liver disorders reduce protein secretion, leading to clotting disorders, dyslipidemia, and edema. Alcoholic fatty liver results from ethanol's high caloric content, disrupted glucose metabolism, and ethanol's easy conversion to fatty acids. Nonalcoholic steatohepatitis (NASH) is very common among obese patients and is now recognized as the most common cause of elevated liver enzymes among asymptomatic patients. Exposure of cultured hepatocytes to high levels of FFAs resulted in uncontrolled gluconeogenesis and excessive export of glucose. NASH results in elevated AST and ALT levels, hyperlipidemia, decreased secretion of plasma proteins, and altered hepatic metabolism.

Ectopic fat results in increased secretion of pro-inflammatory adipokines, hepatomegaly, and hepatocellular damage caused by toxic ceramide, apoptosis, and replacement of hepatocytes with fibroblasts.

Ectopic fat deposited within the pancreas results in a similar dysfunction, as illustrated in Figure 2. Hypersecretion of insulin initially coincides with TG deposition in pancreatic tissue, but, eventually, lipotoxic cell damage decreases insulin secretion and causes insulinopenia similar to that seen in DM1. Cultured β-cells exposed to chronic high FFA levels secrete less insulin. These changes result in decreased glucose uptake and increased gluconeogenesis, observed in IFG, IGT, and DM2.

Ventricular hypertrophy often results from leptin resistance and ectopic fat deposition within myocardial tissue. Again, lipotoxicity and chronic inflammatory conditions promote cellular damage, inefficient contractions, and dysfunctions such as hypertension and congestive heart failure.
In skeletal muscle, fatty deposits decrease the efficiency of contractions, adding resistance to cardiovascular function and hypertension.9

Although abdominal obesity is not an absolute prerequisite for diagnosis of MSX, ectopic fat is extremely common and correlates with increased risk of CVD. However, as figure 3 illustrates, obese patients with less abdominal fat have better metabolic profiles and healthier outcomes than patients with similar BMIs, but more abdominal obesity.

**Metabolic Changes and Hormone Resistances**

Insulin promotes glucose uptake by muscle and fat cells and the storage of carbohydrates (as glycogen), lipids (as TGs) and amino acids (as proteins), while inhibiting glycogenolysis, lipolysis, and protein catabolism16. Insulin resistance diminishes these functions, leading to dysregulation of carbohydrate and lipid metabolism. As a result, obese patients with DM2 and MSX typically exhibit hyperglycemia and elevated FFAs, TGs, and total and LDL cholesterol (See Table 2).4–13

As described in the previous article, glyceroneogenesis is critical to “lipid buffering”, involving lipolysis, followed by re-esterification back to TGs, all within the same cell. Overexpression of PEPCK leads to obesity in rabbits on a normal diet. However, because of effective lipid buffering, the obesity occurs without significant ectopic fat deposition, insulin or leptin resistance, or excessive release of FFAs.35 On a high fat diet, however, these animals deposit ectopic fat, develop insulin resistance, and metabolic dysregulation 35. When excess dietary lipids are available, lipid buffering is overwhelmed and the excess calories accumulate within non-adipose tissues.

**AMPK, Transcription, and Obesity Metabolism**

Leptin provides feedback of energy status through the
Table 2: Typical Clinical Data* for Patients With and Without Metabolic Syndrome

<table>
<thead>
<tr>
<th>Clinical Analyte</th>
<th>Obese Non-MSX Patient Data</th>
<th>Obese MSX Patient Data</th>
<th>Reference Range or Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting glucose</td>
<td>5.3</td>
<td>6.0</td>
<td>&lt; 6.1 mmol/L</td>
</tr>
<tr>
<td>2 hr glucose OGTT</td>
<td>6.9</td>
<td>13.9</td>
<td>&lt; 11.1 mmol/L</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.4</td>
<td>3.2</td>
<td>0.6-3.7 mmol/L</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>4.9</td>
<td>6.5</td>
<td>&lt; 5.2 mmol/L</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>1.5</td>
<td>1.1</td>
<td>0.7-1.7 mmol/L</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>3.5</td>
<td>5.0</td>
<td>2.2-5.0 mmol/L</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>0.5</td>
<td>0.6</td>
<td>0.3-0.9 mmol/L</td>
</tr>
<tr>
<td>Fasting insulin</td>
<td>57</td>
<td>168</td>
<td>&lt; 120 pmol/L</td>
</tr>
<tr>
<td>Insulin resistance**</td>
<td>3.6</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td>Leptin</td>
<td>3.11</td>
<td>2.96</td>
<td>µg/L</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>10.4</td>
<td>7.6</td>
<td>8.0-12.0 mg/L</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>4.4</td>
<td>5.0</td>
<td>0.2-6.1 mg/L</td>
</tr>
<tr>
<td>TNF-α</td>
<td>2.8</td>
<td>3.3</td>
<td>2.0-3.1 µg/L</td>
</tr>
</tbody>
</table>


** Insulin resistance calculated from HOMA (homeostasis model assessment (IR = fasting glucose (mg/dL) x fasting insulin (µU/mL) / 22.5)

AMP-activated protein kinase (AMPK) phosphorylation system. The AMPK system integrates this extracellular information with the intracellular energy status, via the AMP:ATP ratio. Leptin binding to hypothalamic receptors communicates, through the AMPK system, that energy status is adequate and signals for appetite control, thermogenesis, and increased activity. In liver and muscle, leptin binding activates AMPK, thereby stimulating β-oxidation of fatty acids and inhibiting synthesis of TG and FA. This restricts ectopic fat deposition in these tissues. In adipocytes, AMPK mediates leptin inhibition of lipolysis and decreases the release of FFAs, promoting accumulation of TGs in fat cells.

As shown in Figure 1A, binding of leptin to its receptor also stimulates the JAK/STAT system to control metabolism at the transcriptional level.

Hormone Resistances Cause Vascular Damage and Hypertension
Resistance to insulin and leptin typically results in elevated plasma glucose, FFAs and TGs. Among the consequences of hyperglycemia is the non-enzymatic reaction of glucose with protein amino groups. Hemoglobin A1C assays monitor this reaction with hemoglobin to assess chronic hyperglycemia, but similar glycation reactions occur with amino groups on many proteins. Diabetic complications such as hypertension, retinal damage and blindness, decreased peripheral circulation and infections, heart and kidney disease can be attributed, in part, to protein glycation.

Dyslipidemia refers to a poor lipid profile, involving high TGs, high total cholesterol, and a low HDL-to-LDL ratio. This condition is typically found in obese patients with insulin and leptin resistance, and frequently results in lipotoxicity and atherosclerosis (discussed below). Elevated FFAs contribute significantly to lipotoxicity and athero-sclerosis, but their levels are seldom measured. Leptin resistance also affects vascular remodeling, as suggested by the development of ventricular hypertrophy by leptin deficient or resistant mice.

FFAs, Ceramide, Bcl-2, and Apoptosis
As illustrated in Figure 1, serum FFAs and leptin binding affect production of transcription factors (designated PPAR-α and PPAR-γ) and alter lipid metabolism in non-adipose cells. When FFA levels are low, the catabolic processes of β-oxidation dominate and a minimal lipid pool exists for immediate energy requirements. Leptin resistance and high FFA levels cause TG synthesis to dominate the lipid buffering cycle and ectopic fat begins to accumulate (figure 1). Unger and coworkers suggest that among leptin’s primary role is the control of ectopic fat deposition.

Ceramides are a class of compounds that play an important role in lipotoxicity and initiation of apoptosis (programmed cell death). Sphingosine is first derived from a reaction of a fatty acid, palmitate, and an amino acid, serine. The sphingosine backbone has various
FOCUS: OBESITY AND METABOLIC SYNDROME

groups added (similar to the additions made to the glycerol backbone in phospholipids) to form the ceramides.\textsuperscript{12}

\[
\text{serine + palmitate} \rightarrow \text{sphingosine} \rightarrow \text{ceramide} \rightarrow \text{induction of iNOS} \rightarrow \uparrow \text{NO} \rightarrow \text{apoptosis}
\]

The above sequence shows ceramide compounds activating the inducible nitric oxide synthase (iNOS), which produces toxic levels of NO and promotes apoptosis.\textsuperscript{9, 11, 12}

The Bcl-2 gene family helps suppress apoptosis, but exposure of pancreatic cells to high FFA levels down regulates Bcl-2m resulting in rapid cell death.\textsuperscript{9, 12} Leptin reverses this downregulation and increases cell survival. In leptin-resistant fa/fa rodents (lacking leptin receptors), leptin cannot reverse Bcl-2 suppression and islet cells still die quickly. Reinsertion of a functional receptor gene “cures” the deficiency, enabling leptin to block FFA-related apoptosis so that cells achieve a normal life span.\textsuperscript{9, 11–13} Leptin prevents both ceramide-related cell damage and apoptotic effects resulting from high FFAs. Leptin resistance, common in obese patients, allows for these forms of lipotoxicity.\textsuperscript{9, 11–13}

Inflammation & High LDLs Promote Atherosclerosis

Mature VAT adipocytes secrete a wide array of proinflammatory adipokines (cytokines secreted by fat cells), which promote inflammation, recruit macrophages, and stimulate smooth muscle and fibroblast expansion. Proinflammatory adipokines and cytokines include: CRP, $\alpha$-TNF, IL-1, IL-6, IL-8, IL-10, resistin, and two chemokines.\textsuperscript{28–30}

$\alpha$-TNF is one of the primary inflammatory and lipotoxic factors stimulating the acute phase response, including secretion of CRP and other proinflammatory factors.\textsuperscript{28, 29} In conjunction with IL-6, $\alpha$-TNF also stimulates phagocytosis, promotes adhesion of WBCs to endothelial cells, and is a potent chemoattractant for neutrophils. $\alpha$-TNF also contributes to insulin resistance by inhibiting insulin receptor signaling pathways.\textsuperscript{28–30}

RT-PCR experiments (using reverse transcriptase to copy mRNAs, followed by PCR amplification) demonstrated CRP synthesis by adipocytes.\textsuperscript{39} CRP is an opsonin and marker of inflammation that correlates with increased cardiac risk. Resistin is an adipokine that promotes insulin resistance, expression of adhesion molecules on human endothelial cells, and increased CVD risk.\textsuperscript{40} Angiotensinogen and ACE (angiotensin converting enzyme) are also secreted by adipocytes and promote hypertension, another characteristic of MSX.\textsuperscript{37} Together, these secretory products promote what Ross called a “hyperresponsive healing” process resulting in destruction of vascular tissue, fibrosis, necrosis, and calcification that may ultimately rupture and cause thrombosis.\textsuperscript{21}

Plaque formation and the resulting atherosclerosis are well documented consequences of dyslipidemia.\textsuperscript{11, 12} Oxidized LDLs are engulfed by foam cells, which intercalate between the endothelial and smooth muscle layers of the arteries, contributing to plaque formation.\textsuperscript{11, 12, 41}

**Adiponectin (ADN) is Anti-inflammatory and Anti-atherogenic**

As illustrated by Figure 4, ADN is an anti-inflammatory adipokine that help protect against atherosclerosis by inhibiting endothelial adhesions, recruitment of macrophages, endothelial dysfunction, inflammation, and plaque formation.\textsuperscript{41} Ouchi found that ADN is lower and CRP is higher in patients with coronary artery disease.\textsuperscript{40} Similarly, ADN-deficient mice had higher CRP levels than mice with normal ADN levels.\textsuperscript{40}

ADN reduces expression of vascular adhesion molecules and inhibits adhesion of monocytes to endothelial cells.\textsuperscript{21, 33, 41} ADN also inhibits expression of “LDL-scavenger receptors” on macrophages, reducing LDL uptake and producing fewer plaque-forming foam cells.\textsuperscript{21, 33, 41} Excessive endothelial injuries and vascular remodeling were also seen in ADN deficient mice. Adiponectin affects the AMPK system by altering uptake, metabolism, and storage of both lipids and glucose.\textsuperscript{27, 37}
Figure 4: Summary of the Pathophysiology of Metabolic Syndrome

Metabolic syndrome develops as failed hormonal feedback (resistance to leptin and insulin) results in deposition of ectopic and visceral fat, release of adipokines tumor necrosis factor (TNF-α), C-reactive protein (CRP), interleukin 6 (IL-6) and elevated free fatty acid (FFA) levels. These lead to metabolic dysregulation, lipotoxicity, inflammation, and promotion of changes resulting in atherosclerosis and heart disease. Adiponectin (ADN) counteracts many of these proinflammatory effects and fights atherosclerosis.

**ADN Promotes Insulin Sensitivity**
Plasma ADN promotes insulin sensitivity and increased β-oxidation, and its level is inversely related to levels of VAT.4,5,41,42 Mice lacking ADN developed marked hyperglycemia and insulin resistance, even without obesity. Both conditions were reversed with administration of ADN,33 ADN counters many of the negative outcomes of obesity and MSX, including inflammation, atherosclerosis, hyperglycemia, and insulin resistance.

Some obese patients inherit lower ADN levels and become leptin resistant, have increased VAT, and become insulin resistant. Japanese families with histories of DM2 had single nucleotide polymorphisms (SNPs) in their ADN gene, resulting in lower ADN levels (10.4 µg/mL, compared to the normal 16.6 µg/mL).40, 41,45 Similarly, the Pima Indians have a high prevalence of obesity, with lower ADN levels, insulin resistance, DM2, ischemia, hypertension, and CVD.33, 40, 41, 43 In one study of nine patients with inherited low ADN levels, all nine had hyperglycemia, eight of the patients had hypertension or hyperlipidemia, and six had already developed CVD.41,44

**Metabolic Syndrome’s Overall Effects**
Several factors, relating to obesity and MSX, have been shown to affect an individual’s risk of cardiovascular disease. Among the most powerful of these factors are leptin and ADN. Leptin exerts its influence primarily during the development of obesity, signaling the hypothalamus concerning “adiposity,” or the status of energy reserves. Adequate reserves (moderate leptin
levels) result in appetite suppression and thermogenesis, eliminating excess calories while preventing abdominal fat deposition. Chronic overindulgence results in leptin resistance, increased appetite, deposition of abdominal fat, and the development of obesity. ADN retards the many negative consequences of obesity and MSX, such as chronic inflammation and development of atherosclerosis.

Two common metabolites central to the development of MSX are glucose and FFAs. The devastating effects of chronic hyperglycemia are known to cause many sequelae of diabetes. The toxic effects of elevated FFAs have recently become appreciated as major contributors to the development of MSX, lipotoxicity, and CVD.

The most devastating consequences of abdominal obesity include: 1) resistance to insulin and leptin, 2) dyslipidemia related to increased VAT, 3) chronic inflammation leading to atherosclerosis, and 4) lipotoxic damage to tissues and vasculature. Together, these result in a dramatically increased risk of CVD.

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Continuing Education Questions

WINTER 2010

To receive 2.5 contact hours of basic level P.A.C.E.® credit for the Focus: Obesity and Metabolic Syndrome questions, insert your answers in the appropriate spots on the answer sheet that follows; then complete and mail the form as directed.

Questions

1. Obesity is defined as a BMI of greater than:
   A. > 15%
   B. > 25%
   C. > 30%
   D. > 35%

2. Which of the following hormones is a component of the HPA system?
   A. leptin
   B. insulin
   C. TSH
   D. ghrelin

3. After leptin feedback indicates adequate adiposity, which of the following is not among the three primary ways that the hypothalamus helps regulate body weight?
   A. stimulation of thermogenesis
   B. suppression of appetite
   C. stimulation of physical activity
   D. restriction of thermogenesis

4. Which of the following statements correctly describes the molecular types for leptin, insulin and cortisol?
   A. Cortisol is a steroid, the other two are peptide hormones.
   B. Cortisol and leptin are steroids, insulin is a peptide hormone.
   C. Cortisol and insulin are steroids, leptin is a peptide hormone.
   D. All three hormones are peptides.

5. Which of the following is least likely to be considered an “entrance ramp” onto the “Highway to Obesity”?
   A. Grave’s disease
   B. Stressed lifestyle or obsessive personality
   C. Cushing’s disease
   D. Lifestyle devoid of physical activity

6. Leptin resistance results in each of the following, except:
   A. hyperphagia
   B. appetite suppression
   C. loss of ectopic fat restriction
   D. decreased thermogenesis

7. Which of the following best describes the concept of “lipid buffering” as it restricts ectopic fat deposition?
   A. Maximal re-esterification of fatty acids is stimulated in all tissues by leptin binding to its receptor.
   B. Maximal re-esterification of fatty acids is stimulated by the hypothalamus.
   C. Adipocytes are stimulated to increase lipolysis and decreased re-esterification, whereas the opposite is true for non-adipocytes.
   D. Non-adipocytes are stimulated to increase lipolysis and decreased re-esterification, whereas the opposite is true for adipocytes.

8. Which of the following does not involve the dopamine “pleasure/reward system”?
   A. alcohol abuse
   B. cocaine abuse
   C. overeating
   D. genetics
9. Which of the following best describes inheritance on an individual patient’s tendency to become obese?
   A. Inheritance of obesity is generally determined by the monogenic \( ob/ob \) genotype.
   B. Inheritance of the tendency to become obese is a complex polygenic involving multiple alleles, each with a modest impact on BMI.
   C. A complete deficiency of leptin or leptin receptor usually causes human obesity.
   D. Complete deficiencies of multiple genes (or gene products) are required to produce the additive effect of obesity.

10. Which of the following analytes are least likely to be elevated in serum from an obese patient?
    A. glucose
    B. free fatty acids
    C. adiponectin
    D. leptin

11. Identify the two researchers usually credited with originally defining the concept of metabolic syndrome:
    A. Reaven and Friedman
    B. Kaplan and Reaven
    C. Unger and Friedman
    D. Unger and Kaplan

12. Which of the following best describes the origin of plasma FFAs (free or non-esterified fatty acids) and the significance of elevated plasma FFA levels?
    A. FFAs are released from the gut following a fatty meal and elevated FFAs tend to be toxic to many cell types.
    B. FFAs are released from adipocytes following a big meal and elevated FFAs provide an excellent energy source for muscle cells.
    C. FFAs are released from adipocytes during fasting conditions and elevated FFAs tend to be toxic to many cell types.
    D. FFAs are released from the liver following a big meal and elevated FFAs tend to be toxic to many cell types.

13. Researchers (including Dr. Richard Unger) have investigated several possible physiologic roles for leptin. Indicate which role of leptin that appears to be independent of the hypothalamus.
    A. preventing ectopic fat deposition
    B. stimulate thermogenesis
    C. appetite suppression
    D. activate the pleasure/reward system

14. Which of the following pairs is not listed among the criteria for diagnosis of metabolic syndrome?
    A. elevated hsCRP and high FFAs
    B. hyperglycemia and hypercholesterolemia (with low HDLs)
    C. abdominal obesity and hypertriglyceridemia
    D. hypertension and abdominal obesity

15. Which of the following does not occur as a result of elevated FFAs?
    A. downregulation \( Bcl-2 \) resulting in rapid cell death
    B. TG synthesis dominates the lipid buffering cycle and ectopic fat begins to accumulate
    C. most FFAs are engulfed by foam cells to form plaque
    D. ceramides activate the nitric oxide synthase system and promote apoptosis

16. Leptin resistance results in the loss of all of the following, except:
    A. stimulation of thermogenesis
    B. suppression of appetite
    C. stimulation of physical activity
    D. stimulation of lipolysis
17. Leptin normally promotes lipid buffering in fat cells but inhibits re-esterification in non-adipocytes. Which of the following best describes the changes that allow ectopic fat deposition when leptin resistance occurs?
   A. Leptin no longer promotes re-esterification in adipocytes
   B. Leptin no longer inhibit re-esterification in non-adipocytes
   C. Leptin resistance promotes re-esterification in both adipocytes and non-adipocytes
   D. Leptin resistance inhibits re-esterification in both adipocytes and non-adipocytes

18. Ceramide is a toxic compound formed from the reaction of which of the following pairs of compounds
   A. serine and cholesterol
   B. alanine and lysine
   C. serine and palmitate
   D. fatty acids and glycerol

19. Metabolically healthy obese individuals would tend to have each of the following characteristics (compared obese individuals with metabolic syndrome) except:
   A. lower LDLs
   B. lower triglycerides
   C. increased insulin resistance
   D. increased sensitivity to leptin

20. Which of the following best describes how leptin binding to its receptor can activate metabolism at both transcriptional level and activation of pre-existing enzymes.
   A. The AMPK system controls transcription, while PPAR-α causes phosphorylation of existing enzymes.
   B. Leptin binds directly to AMPK, causing activation of existing enzymes, PPAR-α promotes transcription of specific genes.
   C. Leptin binds to an intracellular receptor that recognizes promoter sequences and affects transcription and the activation of existing enzyme.
   D. The PPAR-α system controls transcription, while AMPK causes phosphorylation of existing enzymes.

21. Adiponectin (ADN) is an “adipokine” with all of the following characteristics, except:
   A. ADN promotes insulin sensitivity
   B. ADN has anti-inflammatory effects
   C. ADN deficiency is related to hyperglycemia
   D. ADN levels are directly proportional to fat mass

22. ADN inhibits the development of atherosclerosis by limiting all of the following effects except:
   A. Expression of endothelial adhesion molecules
   B. Increased oxidation of LDLs
   C. Expression of chemotactic molecules
   D. Expression of LDL “scavenger receptors” on macrophages

23. Which of the following best explains why glycerogenesis and the activation of the PEPCK enzyme must occur during lipid buffering?
   A. The glycerol released during lipolysis is exported and must be replaced by new synthesis of glycerol, using the enzyme PEPCK.
   B. Lipolysis of triglycerides (requiring PEPCK) from lipoprotein fractions, such as chylomicrons and VLDLs, is the source of the glycerol for adipocytes.
   C. Glyceroneogenesis uses the glycolysis pathway and the enzyme PEPCK to produce glycerol from glucose.
   D. Lipid buffering refers to glycerol’s acid-base properties, and glycerol must be synthesized from fatty acids using the enzyme PEPCK.
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