Effect of a Vegetarian-like Diet on Blood Coagulation

and Other Health Parameters in Blood Types A and O:

An Evaluation of the "Blood Type Diet"

by

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ABSTRACT

Background. Research suggests that non-O blood types are at an increased risk

of thrombosis and related health complications in cardiovascular disease (CVD).

This is due in part to higher concentrations of von Willebrand factor (VWF), an

important factor involved in blood clotting.

Objective. The purpose of this study was to examine the effects of a vegetarian-

like diet on blood coagulation and other health parameters in adults with type A

blood compared to type O blood over a four week intervention. Given the lack of

previous research on blood type and diet, it was hypothesized that no difference in

blood coagulation would be observed.

Design. This study was a randomized, parallel arm, dietary intervention using

healthy, omnivorous adults with blood types A and O. A total of 39 subjects

completed the study. Subjects were randomized into two groups: a vegetarian-

like diet group made up of 12 type As and 12 type Os and an omnivorous control

diet group made up of 11 type As and 12 type Os. At weeks 0 and 4, fasting blood

was drawn and analyzed for prothrombin time (PT), activated partial

thromboplastin time (APTT), von Willebrand factor (VWF), total cholesterol,

LDL, HDL, triglycerides, and CRP. In addition, subjects were weighed and filled

out a FFQ at weeks 0 and 4.

Results. After adhering to a vegetarian-like diet for four weeks, type Os had a

significant increase in PT ($+0.24\pm0.32$ sec/ $p=0.050$), whereas type As saw no

significant change. There was a trend of weight loss for type Os in the

vegetarian-like diet group (-1.8 ± 2.6 lb/ $p=0.092$) and significant weight loss for

type As (-0.9±2.1 lb/ p=0.037). Both blood types O and A experienced

significant decreases in BMI (-0.3±0.4/ p=0.092 and -0.2±0.3/ p=0.037,

respectively). No change was seen in APTT, VWF, total cholesterol, LDL, HDL,

triglycerides, or CRP.

Conclusion. Type Os saw an increase in PT, perhaps indicating a reduction in

risk of thrombosis and its related health complications. Type As were less

responsive to the dietary intervention and may require more rigid dietary

guidelines or a longer time on such a diet to see the benefits.

DEDICATION

This project is dedicated to my husband,

Jason,

and to my mother,

Sandy.

I am forever grateful for their support, encouragement, and love.

ACKNOWLEDGMENTS

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INTRODUCTION

Overview

More than 5 million copies of the diet book "Eat Right 4 Your Type" by naturopathic doctor Peter J. D'Adamo have been sold in over 50 languages since its publication in 1996 (1). In the book, D'Adamo purports that lectins in food interact with blood antigens and affect the state of health of the individual. He asserts that by eating a specific diet that excludes food sources of lectins that interact poorly with one's blood type, an individual will experience health benefits including weight loss, reduced disease risk, and even improved emotional outlook (2). Given the book's popularity among individuals seeking alternative dietary advice, scientific validation of these recommendations should be sought. D'Adamo has been criticized by the scientific community for his lack of scientific evidence to support his claims and his book has been labeled "pseudoscience" (3).

Although D'Adamo does not offer scientific support for his diet claims, there is some evidence that ABO blood types differ in their disease risk. Non-O blood types may be at increased risk for development of coronary atherosclerosis and increased risk for mortality during ischemic heart disease (4). Non-O blood type has also been associated with an increased risk for pulmonary embolism (5).

Thrombotic occlusion is often the cause of both myocardial infarction and embolism. Increased levels of a particular clotting factor in plasma, the von Willebrand factor (VWF), has been associated with an increased risk of arterial thrombosis, including myocardial infarction and stroke (6). Interestingly, VWF

concentrations are higher in the plasma of non-O blood types compared to the O blood type; a consequence of A and B antigen expression on VWF and the slowing of its clearance due to the presence of those antigens (6, 7). Given the higher levels of VWF in non-O blood types, perhaps there is an opportunity for personalized nutrition, e.g. a "blood type diet" for A blood type individuals to lower the risk of thrombosis.

A variety of studies have been conducted to assess the effect of dietary intervention on hemostasis, however, no such study has been conducted using D'Adamo's blood type diet. D'Adamo suggests that individuals with type A blood do best on a vegetarian-like diet that emphasizes soy proteins, grains, fruits and vegetables, with the allowance of some fish and poultry (2). A literature review identified a number of studies on plant-based foods, including soy, garlic, and purple grapes, which have demonstrated antithrombotic effects (8). Although D'Adamo never mentions VWF in his book, this may be a missing link that supports the reasoning for the type A diet. Given that non-O blood types have higher levels of VWF and that some plant-based foods have been shown to have antithrombic effects, perhaps the type A diet will lower the risk for blood clotting among type A individuals.

Statement of Purpose

The purpose of this study is to examine the effects of the type A, vegetarian-like diet on blood coagulation and other health parameters in adults with type A blood compared to type O blood over a four week intervention, and also compare the experimental group with a control group composed of both type A and O blood groups that receive no dietary intervention.

Hypothesis

Given the lack of previous research in the area of blood type diet, it is hypothesized that no difference in blood coagulation will be observed between type A and O blood groups after adhering to the type A, vegetarian-like diet for four weeks.

Definition of Terms

Antibody: a protein created by the immune system that is capable of binding to antigens and eliciting an immune response

Antigen: a substance foreign to the body that evokes a response from the immune system and is capable of binding antibodies

Embolism: the sudden obstruction of a blood vessel by an abnormal particle referred to as an embolus

Hemostasis: the process of arresting blood flow, as in blood coagulation

Thrombosis: the presence or formation of a blood clot inside a blood vessel

Lectin: a protein, usually derived from plants, that is not an antibody and is not created by the immune system, but binds carbohydrate receptors on cell surfaces (for example, those found on the surface of RBCs)

All definitions were compiled from the National Institute of Health's MedlinePlus (9).

Delimitations and Limitations

The majority of study subjects are adult male and female college students. Therefore the findings of the study are only generalizable to this population. Also, only type A and O blood types were included, so the results do not apply to other blood types (B and AB).

Limitations of this study include the reliance on subject compliance to adhere to the vegetarian-like diet and limited control over the amounts and ratios of allowed foods consumed.

Chapter 2

REVIEW OF LITERATURE

ABO Blood Type

Discovery

Until the discovery of the ABO blood groups, blood transfusions between

humans were intermittent in their success, often resulting in the death of the

patient. For this reason, they were outlawed in some countries (10). Austrian

born scientist, Karl Landsteiner, addressed this problem in his laboratory at the

turn of the 19th century by conducting experiments in which he mixed blood from

two different individuals. With some combinations he observed the red blood

cells (RBCs) forming clots, whereas other mixtures showed no effect at all. It

became clear that blood should not be transfused from a donor to a recipient that

resulted in the agglutination of RBCs. Landsteiner deduced that each individual's

serum contained antibodies that caused the agglutination of the RBCs of other

non-compatible individuals by targeting antigens on the surface of their RBCs.

However, an individual's serum did not contain antibodies for the antigens

present on his own RBCs. This basic rule of immunology, known as the

"Landsteiner's Rule," was first described by Landsteiner in his paper

"Agglutination phenomena of normal human blood" published in the Viennese

Weekly Journal of Medicine in November 1901 (11). Twenty-nine years later he

was awarded the Nobel Prize for this landmark discovery (10).

Landsteiner originally identified three blood groups, and a fourth was

described a year later by Decastello and Sturli in a study published in 1902 (12).

At first, the four blood types were known as I, II, III and IV. Later, scientists Ludwik Hirszfeld and Emil von Dungern renamed the groups as A, B, AB and O (13). The letter assigned to the blood type corresponds to the antigen present on the surface of the RBC. For example, type A has the A antigen, type B has the B antigen, and type AB has both. Type O signifies the absence of A and B antigen and was named after the German word 'ohne' meaning 'without' (14). According to Landsteiner's Rule, an individual's serum does not contain antibodies that target its own RBC antigen, but it does have antibodies against all foreign antigens. For example, type A serum has B antibody, type B serum has A antibody, and type AB serum does not contain either antibodies. Type O, on the other hand, contains both A and B antibodies (see Figure 1).
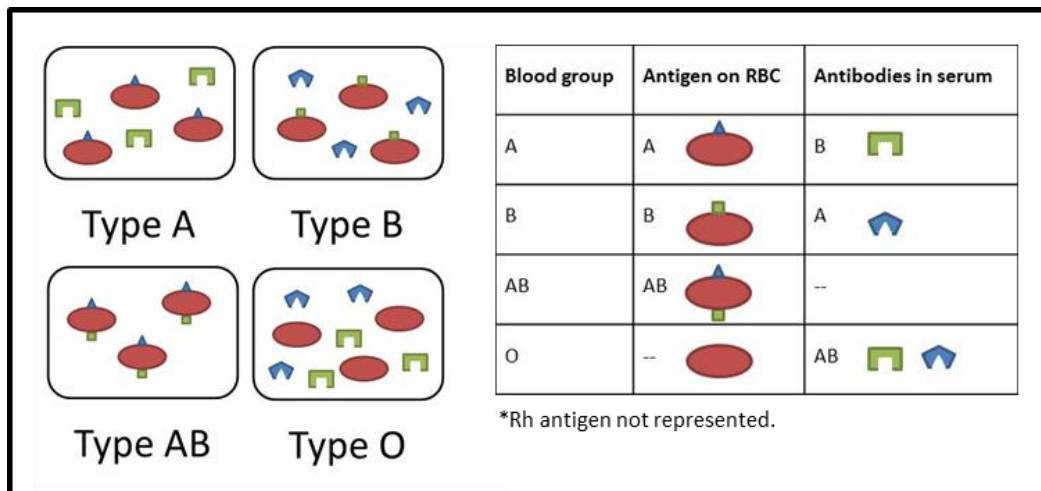
Figure 1. ABO blood group antigens and antibodies

Discovery of the ABO blood types allowed for blood transfusion to become a safe medical practice and saved many lives in the face of excessive blood loss. As knowledge of the ABO blood types expanded, many subcategories were identified based on variation in the expression of A and B antigen. For

example, in some type A individuals, the expression of the A antigen is weakened. Subcategories of the A blood group were established over time based on their reactivity to antibodies and the presence of antigen in secretory products, such a saliva. Weaker forms of the B antigen have also been identified, however to a lesser extent than A antigen subgroups. Different phenotypic subgroups are depicted using a subscript number or letter. The most common ABO phenotypes are A_1, A_2, B, A_1B, A_2B, and O (14, 15).

Inheritance

In 1910, Hirszfeld and von Dungern determined that blood type is inherited according to Mendelian laws, following a codominant autosomal design (13, 14). This means that one allele for the gene that determines blood type (located on a non-sex chromosome) is inherited from each parent, giving the offspring two alleles of the gene. These alleles are codominant, meaning they are expressed equally. For example, if a child inherits the allele for type A blood from his mother and the allele for type B blood from his father, the child will express both alleles and have both A and B antigens present on their RBCs. However, if the child receives a type B allele from his father and a type O allele from his mother, the child will be blood type B, because only the B antigen will be expressed on the RBCs (recall that type O represents the absence of antigens). A simple Punnett square illustrates the possible genotype inheritance of an offspring based upon the alleles possessed by the parents (see Figure 2). Interestingly, the inheritance of ABO blood type was one of the first human genetic markers used in forensic science and paternity testing (16).

	Father		
Mother	A	B	O
A	AA	AB	AO
B	AB	BB	BO
O	AO	BO	OO

Figure 2. ABO genotype in offspring

Biochemistry

The ABO antigens are sugars that project from the surface of RBCs. The sugar molecule L-fucose serves as a building block upon which the ABO antigen is attached. In type O individuals, no additional sugars are attached to fucose. This precursor substance is often referred to with the letter 'H'. In type A and type B individuals, a glycosyltransferase enzyme is responsible for adding the final oligosaccharide to the H antigen to form A or B antigens. The addition of N-acetyl-D-galactosamine results in the A antigen whereas the addition of D-galactose results in the B antigen. As discussed previously, some individuals present both A and B antigens (14). Beyond red blood cells, these antigens are also expressed in other various tissues including vascular endothelium, epithelial cells, and platelets. For this reason, some researchers suggest that they be called ABO "histo-blood group" antigens (17).

Molecular Genetics

In the modern age of genomics, much information has been revealed about the complexities of ABO blood type genetics. What once was a seemingly simple blood grouping system has revealed itself to be rather complex. Perhaps contrary

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to what would be expected, the antigens on the RBCs are not coded by genes directly. Instead, the ABO gene codes for enzymes (glycosyltransferases) that produce the oligosaccharide epitopes (the part of the antigen recognized by the immune system). Specifically, the A antigen is made by 3-α-N-acetylgalactosaminyltransferase (A transferase) and the B antigen is made by 3-α-N-galactosaminyltransferase (B transferase), both of which are coded from a single ABO gene located on the long arm of chromosome 9 (9q34) (14, 15). The main alleles of the ABO gene were first defined by Yamamoto and his colleagues in 1990 (18). They found that the allele that codes for A transferase versus B transferase is different by only 7 out of 1062 nucleotides, which results in a difference of only four amino acids substitutions. Blood type O was found to be the result of the deletion of a single guanosine nucleotide that shifts the reading frame of the gene, and results in the translation of a shortened protein which has no glycosyltransferase functionality.

Since the work of Yamamoto in 1990, new alleles of the ABO gene are continuously being identified. Allelic variants impact the specificity and efficacy of the resultant glycosyltransferases, and therefore produce the various phenotypic subtypes identified earlier in this paper. For example, a SNP (single nucleotide polymorphism) in the gene can result in a decreased capacity for the corresponding transferase enzyme to function normally (15). If the affinity of the enzyme for its substrate is lessened, the result would be a weaker phenotype, in which less of the corresponding RBC antigen is expressed. Numerous missense and nonsense mutations result in various alleles that ultimately alter the

phenotype of an individual. New alleles of the ABO gene will likely continue to be discovered, but as of April 2009, Storry and Olsson identified 181 alleles (65 A alleles, 47 B alleles, 58 O alleles, and 11 AB alleles) (14).

Evolution and Anthropology

It appears that ABO blood group antigens served an important function over the course of human evolution, because the frequency of blood types varies among different populations. It has been speculated that having a certain blood type over another conferred some sort of selection advantage, such as resistance to infection. Blood type O is the most common phenotype in all races, comprising 44% of Caucasians, 49% of Blacks, and 43% of Asians. Native American Indians are almost exclusively type O. Blood type A is the second most common, with its collective phenotype comprising 43% of Caucasians, 27% Blacks, and 27% of Asians. Type A is abundant in Northern and Central Europe, but is rarer in Asia. Blood type B appears in 9% of Caucasians, 20% of Blacks, and 25% of Asians. Type B is popular in Central Asia, but nearly absent in Amerindians. Finally, type AB is the rarest phenotype in all races, making up only 4% of Caucasians, 4% of Blacks, and 5% of Asians (14, 19).

The reason for the varied distribution of blood type among different populations is not well understood, and several theories about its evolution have been espoused since the discovery of ABO blood groups over 100 years ago. The current evolutionary theory has been developed from in depth statistical analysis of the numerous SNPs occurring at the ABO gene locus. In their 2008 study, Calafell et al. speculated that the oldest human DNA sequence coded for the type

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A phenotype. Based on their calculations, type B phenotype appeared next

approximately 3.5 million years ago. After that, the allele which coded for type O

blood appeared around 2.5 million years ago. Over time, various mutations in the

ABO gene gave rise to the different subtypes of the A, B, and O phenotypes that

have been mentioned previously. Calafell's study also supported earlier

speculation that interactions with pathogens in different geographical regions

likely caused certain mutations to be selected over time because they conferred

some advantage to their host (20).

Blood Group Typing

Blood group typing is the process that determines which antigens (A

and/or B) are present on the red blood cells of an individual. It should be noted

that the D antigen (also known as Rh) is usually tested for as well, but is not

relevant to the context of this paper. Blood group typing is used in blood

donation, blood transfusions, and organ transplantation to assure that the donated

blood/tissue is compatible with the receiver. Typically, a sera containing

antibodies for the ABO antigens is used to identify blood type. However, the

reverse logic can be applied, in which ABO antigens are used to spot expected

antibodies in serum – a process called reverse ABO grouping (21).

A number of blood group typing tests exist, most of which use the

agglutination of red blood cells as a positive indication of antibody and antigen

interaction. One of the oldest is the slide test method, in which a drop of

antiserum is mixed with blood on a stone or glass slide. If an agglutination

reaction is observed, it means that the antibody present in the antisera reacted with

the antigen on the red blood cells, causing them to clump together. For example,

a drop of anti-A that causes agglutination of the blood would reveal that the blood

tested is type A. The main advantages of the slide test method are that it is quick

and inexpensive. It also does not require the use of a centrifuge, as do some other

tests. However, it is not as sensitive as other tests now available, including the

tube test, microplate method, and gel centrifugation assay. Nowadays, advanced

technology allows for molecular blood group typing, which involves the

genotyping of DNA and detection of SNPs to diagnose blood type (21).   At-home

kits have also been created so that individuals can easily determine their blood

type using a whole blood sample from a simple finger prick. The kits come with

a test card that is divided into separate areas that have been treated and

impregnated with dried antiserum (anti-A, anti-B, and anti-D/Rh). A small drop

of blood is gently rubbed over each antiserum site. A positive reaction of

agglutination indicates that the corresponding antigen is present on the red blood

cells (see Figure 3) (22).
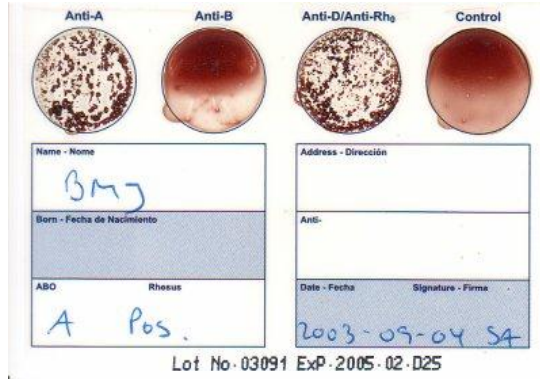
Figure 3. Sample blood typing kit[a]

ABO and Disease

When a patient receives an incompatible blood transfusion, it causes hemagglutination and can potentially result in death. This situation is due to medical intervention; however, a similar situation can occur naturally during pregnancy. Hemolytic Disease of the Newborn (HDN) takes place when the fetus and mother have different blood types. The mother's serum antibodies can attack the red blood cells of the fetus and cause symptoms of anemia, edema, and jaundice in the newborn baby. Typically symptoms are mild and do not require medical intervention, however in some cases they are severe and result in death (3, 23).

ABO blood type has also been associated with infectious disease. As discussed earlier, the evolution of blood type was likely influenced by pathogens. Pathogens often use the host's cell surface receptors, such as the sugar-based ABO antigens, for attachment. Certain ABO antigens interact with pathogenic invaders more easily than others. For example, blood type O has been found to

have a greater susceptibility to peptic ulcer caused by *Helicobacter pylori* because the bacteria has a greater affinity for the H antigen compared to the A and B antigens (24). On the other hand, there is strong epidemiological evidence that blood group O provides protection against *Plasmodium falciparum* malaria, whereas blood group A has been associated with more severe malaria. During infection, type O blood is less prone to form rosettes (aggregates of malaria infected erythrocytes with uninfected cells) (25). Surface antigens mediate the adhesion of pathogens and thus affect the progression and severity of infection.

Some forms of cancer are linked to blood type. For example, non-O blood types have a higher incidence of stomach and pancreatic cancer as compared to O blood type. Pancreatic cancer in particular has been studied extensively, and it has been concluded that polymorphisms in the ABO gene affect an individual's susceptibility to the disease, but do not cause cancer per se (3).

ABO blood type is also associated with cardiovascular disease (CVD). In a study of 4901 patients with CVD, Carpeggiani et al. found a higher prevalence of A and B blood types in those who suffered from myocardial infarction (26). Non-O blood groups were also a strong predictor of cardiac death, especially in patients younger than age 65 and in women. ABO blood type has also been associated with other diseases in which thrombosis plays a part, such as venous thromboembolism (VTE). In a prospective study of two large cohorts, Wolpin et al. examined the relationship between blood type and pulmonary embolism (PE), specifically (27). They found that non-O blood types were at a significantly increased risk for PE, compared to O blood type. Research suggests that the

increased risk for CVD and VTE observed in non-O blood types is due to increased levels of von Willebrand factor, an important intermediary protein involved in hemostasis.

Hemostasis

Under normal conditions, the endothelial walls of the blood vessels contain anticoagulant components that prevent the adhesion of particles traveling in blood. When the integrity of the vascular walls is compromised by injury, blood can quickly escape. It is imperative that bleeding is stopped as quickly as possible. Hemostasis is the physiological process that stops bleeding. It is a very complex mechanism that involves two systems: primary and secondary hemostasis.

Primary hemostasis is the body's initial reaction to vascular injury. It involves the aggregation of platelets to form a platelet plug. When blood vessels are damaged, components in the subendothelial matrix are exposed to the blood. Receptors on the surface of platelets in the blood are attracted to ligands found in the subendothelium. For example, the platelet receptor glycoprotein Ibα (GPIbα) binds to von Willebrand factor (discussed in more detail later) in the subendothelium, and receptor glycoprotein VI (GPVI) binds to collagen. Receptors GPIbα and GPVI also contribute to the activation of other platelets. When platelets are activated, their storage granules undergo exocytosis and release a number of factors that promote platelet aggregation, degranulation, and vasoconstriction such as adenosine diphosphpate (ADP), thromboxane A_2, and serotonin. As platelets adhere to each other and the site of injury, a platelet plug

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is quickly formed that temporarily seals the blood vessel until a more substantial clot can be formed (28).

In secondary hemostasis, an insoluble fibrin mesh is deposited in and around the platelet plug. This occurs via a coagulation cascade of chemical reactions that is divided into two pathways based on the activation mechanism. The extrinsic pathway is initiated by vascular injury and the release of tissue factor from damaged tissue. The intrinsic pathway is initiated by clotting factors found in blood. Both pathways converge into a common pathway, beginning with the activation of factor X. From there, factor X combines with factors III and V in the presence of platelet factor 3 and calcium to produce prothrombin activator. Prothrombin activator then converts prothrombin to thrombin, and thrombin subsequently converts fibrinogen to fibrin (with the help of other cofactors). In most cases, the extrinsic and intrinsic pathways work simultaneously to contribute to the formation of the crosslinked fibrin polymer which serves to stabilize and strengthen the blood clot (28).
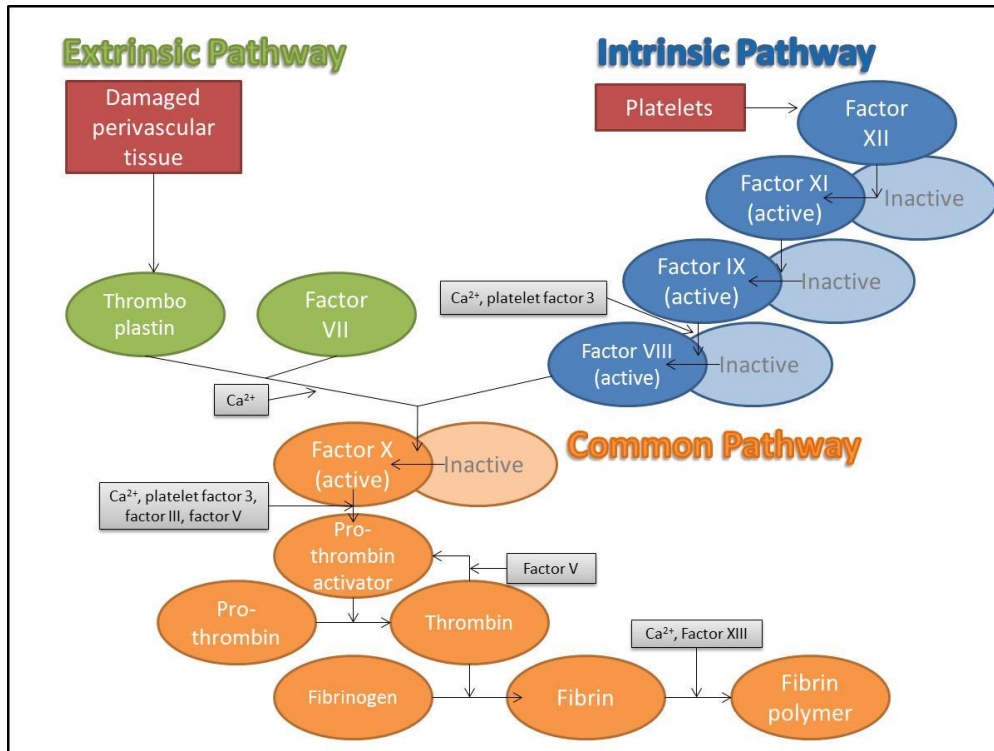
Figure 4. Coagulation pathways

Blood Coagulation Tests

The effectiveness of the extrinsic and intrinsic pathways of secondary

hemostasis, otherwise known as blood coagulation, is commonly measured by

two separate tests: prothrombin time (PT) and activated partial thromboplastin

time (aPTT). These tests are used most frequently in the medical field to assess

blood coagulation because they are both cheap to perform and accurate in their

results. They are used in a number of situations by physicians, including

preoperative assessment of hemorrhage risk, screening of blood coagulation

disorders, and monitoring of anticoagulant drug therapies. Both tests are

performed on platelet-poor plasma and measure the amount of time it takes for

blood to coagulate (reported in seconds) after the addition of some agonist (29).

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The PT test indicates the state of the extrinsic pathway and specifically measures the activity of factor VII, a clotting factor that is produced by the liver and dependent upon vitamin K. The drug warfarin is used to inhibit a key enzyme in vitamin K metabolism and therefore lessen the risk of blood clots in patients with heart problems or risk of embolism. The PT test is frequently used to determine warfarin dosage, the presence of liver dysfunction or disease, and vitamin K deficiency. A normal PT value is between 11.0 and 12.5 seconds. A value below the normal range indicates an increased risk for clotting, whereas a higher value signifies an increased risk for bleeding (29, 30).

The aPTT test evaluates the function of the intrinsic pathway and a variety of factors involved in this system. This assay is commonly used to check the efficacy of heparin, another anticoagulant drug. The normal range for an aPTT test is 30 to 40 seconds. Prolonged aPTT times are associated with a number of disorders including congenital bleeding disorders, liver disease, Von Willebrand Disease, and leukemia (30). Shortened aPTT times have been shown to indicate hypercoagulability and an increased risk for venous thromboembolism (31).

Thrombosis

Thrombosis, the formation of a blood clot within a blood vessel, is responsible for a large portion of morbidity and mortality in the Western world. It is linked to arterial diseases, such as stroke, myocardial infarction and peripheral occlusive disease. It is also related to disorders involved with venous thromboembolism, such as deep vein thromboembolism (DVT) and pulmonary embolism. In all of these disease states, a traveling blood clot can potentially

occlude smaller blood vessels, eventually leading to an ischemic event that may result in death. Hypercoagulability of the blood is a risk factor for both arterial diseases and venous thrombosis (32).

The steps involved in hemostasis can be suppressed in order to lessen the risk of thrombosis. This can occur through a few different mechanisms: a decrease in cell adhesion molecule expression (such as von Willebrand factor), a decrease in molecules that stimulate platelet aggregation (such as thromboxane A_2), an increase in aggregation inhibitors, the clearance of activated coagulation factors, and the inhibition of platelet aggregation (33). The prevention of thrombosis is a fine balance between lessening the risk of clot formation while avoiding an increased risk of hemorrhage. Using diet may offer subtle protection against thrombosis without interfering with hemostasis (32).

Von Willebrand Factor

Structure and Function

Von Willebrand factor (VWF) is a glycoprotein that plays a key role in blood hemostasis. VWF can exist in very large, multimeric forms, making it the largest protein in human plasma. A pro-VWF monomer consists of 2050 amino acids, up to 22 carbohydrate side chains, and discrete domains that exhibit specific functions. VWF is produced by the endothelial cells of the blood vessels and the megakaryocytes (bone marrow cells) from which platelets are formed. In the endoplasmic reticulum of the cells, two pro-VWF monomers combine to form a dimer via a disulphide bond at their C-terminal region. Then the pro-VWF dimers move to the Golgi apparatus to be packaged as very large multimers via

disulphide bonds at the N-terminal end of the dimer. Once formed, VWF

multimers are packed in storage organelles called α-granules in platelets and

Weibel-Palade bodies in endothelial cells. VWF is also secreted into plasma

constitutively by endothelial cells and is present in the subendothelial connective

tissue of blood vessels bound to collagen. When stimulated by an agonist, such as

thrombin, epinephrine, or ADP, the VWF multimers are released from the storage

granules. Eventually, VWF is cleared from plasma by the liver. VWF serves two

main functions: 1) it functions as an intermediary adhesive molecule in primary

hemostasis, and 2) it binds and protects factor VIII (FVIII) in plasma (34-36).

In its role in primary hemostasis, VWF serves as an adhesive molecule

that allows exposed collagen in damaged blood vessels to bind to platelets, as

discussed above. Large VWF multimers have an affinity for collagen and

activated platelets 100-times higher than that of VWF monomers. This allows

platelets to aggregate under conditions of high shear rates (36). Shear rate is the

difference in blood flow velocity as a function of distance from the vascular wall.

The velocity of blood at the center of the vessel is faster than it is at the wall,

which creates a shearing effect that is greatest at the wall. The highest shear rates

(up to 5000 s^{-1}) are observed at the luminal surface of small arterioles with a

diameter less than 50 μm. Shear rates are also significantly higher in stenosed

arteries, as in the case of progressed atherosclerosis. VWF is essential for platelet

aggregation at high shear rates, but not necessarily at lower shear rates (35).

Storage of large multimer VWF and subsequent secretion upon vascular

injury allows for maximal function when rapid platelet aggregation and adhesion

is necessary. Adhesion occurs at the A1 domain of the VWF protein, which can bind to both collagen and the GPIbα receptor on platelets. The interaction of the A1 domain on VWF and the GPIbα receptor on platelets is also important for platelet-to-platelet interactions because it activates integrin αIIbβ3 receptors on platelets, which can subsequently bind to the C1 domain of VWF and instigate platelet aggregation (see Figure 5). Once released from storage, VWF undergoes reduction in multimer size by the action of plasma proteases, such as ADAMTS-13 (a disintegrin and metalloproteinase with thrombospondin type-1 repeats). ADAMTS-13 protects against uncontrolled platelet adhesion by free VWF multimers. As mentioned above, it is thought that VWF is ultimately cleared from plasma by the liver (34, 35).
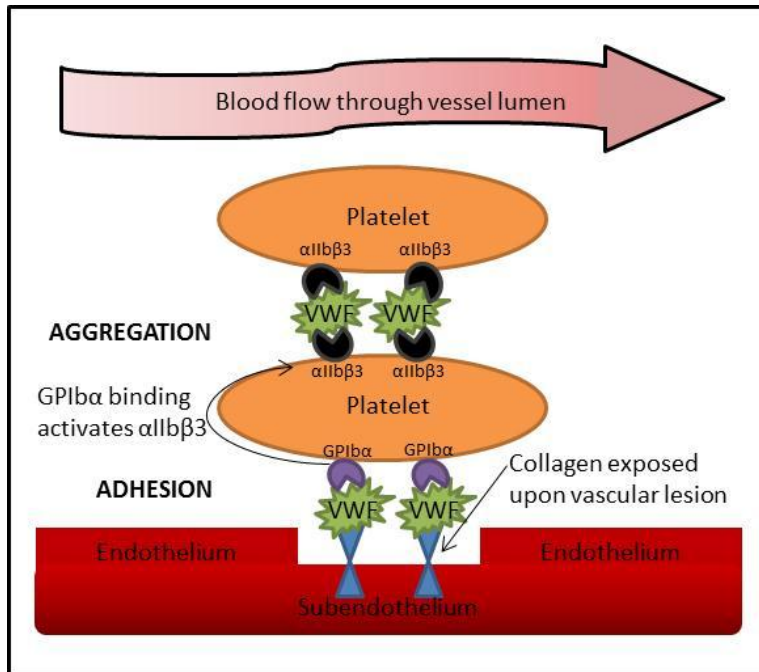
Figure 5. Function of VWF in platelet adhesion and aggregation

In its second function of binding FVIII, VWF protects the clotting factor from proteolytic degradation in plasma. VWF can bind to a FVIII molecule at two different binding domains: D´ and D3. As its carrier, VWF prolongs the half-life of FVIII and helps to localize it to the site of vascular lesion. FVIII is responsible for activating factor X in the intrinsic pathway of blood coagulation. A deficiency in FVIII leads to classical hemophilia (hemophilia A), a serious bleeding disorder (28, 34, 37).

Von Willebrand Disease

A deficiency or defect in VWF leads to a bleeding disorder known as Von Willebrand Disease (VWD). VWD is the most frequently inherited hemostatic disorder, but it can also be acquired over one's lifetime due to other health issues. The inherited forms are categorized into three types (type 1, 2, and 3) and type 2

is further subdivided into 4 categories (2A, 2B, 2M, and 2N). Categorization is based on mutations in the genes that code for VWF and the cellular machinery responsible for assembling and processing it. Type 1 VWD is the most common form of the disease and comprises 60% to 80% of all cases. In type 1, VWF concentration in plasma is low, causing symptoms such as mild bleeding in skin and mucous membranes (e.g. easy bruising, bleeding gums, and nose bleeds). In type 2 VWD, plasma VWF concentration is normal but there are structural and functional issues with the clotting factor that result in minor bleeding issues. Individuals with type 1 or type 2 VWD lead relatively normal lives. On the other hand, Type 3 VWD is associated with a complete absence of VWF in plasma, due to a recessive genetic mutation. Individuals with type 3 VWD suffer from severe mucosal bleeding from the time they are born. Unlike the three inherited forms of VWD, the disease can also come about because of other disease states. For example, individuals suffering from aortic stenosis can develop acquired VWD and associated intestinal bleeding. VWD treatments include the use of antifibrinolytic drugs to inhibit the premature breakdown of clots, intravenous VWF replacement therapy, and desmopressin (a synthetic derivative of vasopressin which stimulates endothelial cells to release VWF) (37).

VWF and ABO Blood Type

Interestingly, the most important determinant of VWF level in plasma is ABO blood type. Like red blood cells, VWF molecules also present A and B blood group antigens attached to N-linked oligosaccharide side chains. Current evidence suggests that the presence of these antigens slows the clearance of VWF,

so non-O blood group individuals have higher concentrations. It has been speculated that non-O blood types have 25% more VWF in their plasma when compared to O blood type (36). In a large study of twins, Orstavik et al. found that 66% of the variation in plasma levels of VWF was genetically determined, and 30% of that was contributable to ABO blood group (38). In normal individuals, blood group O has VWF antigen levels of 65.4 to 102.8 IU/dl, with most levels falling below 90 IU/dl. On the other hand, group A, B and AB individuals have an average of 90 to 139 IU/dl of VWF antigen (39).

Some studies have investigated the difference in VWF levels among ABO blood group subcategories. For example, Souto et al. found that individuals with a homozygous genotype for blood group O (OO) demonstrated the lowest level of VWF. Also, individuals who were heterozygous for the O allele (AO and BO) had significantly lower levels of VWF in plasma compared to individuals without an O allele (AA, BB, and AB) (40). Given the increased levels of VWF seen in non-O blood types, a good deal of research has investigated the associated risk of thrombosis.

VWF, ABO Blood Group, and Risk of Thrombosis

Beginning in the 1990s, researchers began to study the relationship between high plasma concentrations of VWF and risk of arterial and venous thrombosis. A larger portion of the research has focused on arterial thrombosis, specifically. One of the more recent studies conducted was the Prospective Epidemiological Study of Myocardial Infarction (PRIME), published in 2004 (41). It followed 9,758 healthy men aged 50 to 59 years old for a total of five

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years. Men with VWF levels in the top 25% experienced a risk of coronary heart disease (CHD) three times higher compared to men with VWF levels in the lowest 25% of study subjects. Even after adjusting for inflammatory markers such as C-reactive protein, interleukin-6, and fibrinogen, the value of VWF as an independent risk factor for CHD remained the same. Likewise, in a meta-analysis conducted by Whincup et al. in 2002, previous prospective studies looking at VWF and CHD were analyzed. They found that subjects who fell in the top third of baseline VWF values had a combined odds ratio for CHD of 1.5 compared to subjects in the bottom third (95% confidence interval, 1.1 to 2.0) (42).

In another meta-analysis published by Wu et al. in 2008, investigators tried to elucidate the relationship between ABO blood group, VWF levels, and risk of arterial thrombotic complications, such as myocardial infarction (MI) (39). They found a significant association between non-O blood types and risk of MI (pooled OR of 1.25, 95% confidence interval, 1.14 to 1.36); however this association was only seen in retrospective studies. Wu and his colleagues also found an increased risk in non-O blood types for peripheral vascular disease (PVD) (pooled OR of 1.45, 95% confidence interval, 1.35 to 1.56) and cerebral ischemia of arterial origin (CIAO) (pooled OR of 1.14, 95% confidence interval, 1.01 to 1.27). Although a causal relationship between VWF, ABO blood group, and arterial thrombosis seems plausible, the current body of research lacks well-designed prospective studies to confirm the nature of the relationship. It may be that VWF levels are simply a reflection of preexisting endothelial dysfunction, which leads to atherosclerosis and ensuing arterial thrombosis (6).

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Raised levels of VWF have also been linked to venous thrombosis, although fewer studies have looked at this connection when compared to arterial thrombosis (34). In 1995 Koster et al. studied 301 subjects over 70 years old who suffered from a primary episode of VTE compared to 301 controls (43). A univariate analysis showed that high concentrations of VWF and FVIII, along with non-O blood group, were all risk factors related to DVT. However, in a multivariate analysis, only FVIII remained a significant risk factor. Their study suggests that VWF and ABO blood group may confer risk for VTE, but their effects are mediated through the function of FVIII in the coagulation pathway (recall that VWF is the carrier for FVIII and serves to lengthen the clotting factor's half-life). In another study named the Longitudinal Investigation of Thromboembolism Etiology (LITE) published in 2002, Tsai et al. followed 19,237 healthy individuals over the age of 45 for an average of 7.8 years. Of the 159 VTE events that occurred over this time period, both VWF and FVIII were found to be independently linked in a dose-dependent manner to an increased risk for development of VTE. Individuals in the highest quartile of VWF concentrations saw a hazards ratio of 4.6 (95% confidence interval, 2.2 to 9.2). Furthermore, in the systematic review and meta-analysis by Wu et al. mentioned above, researchers also analyzed 21 studies on blood group and VTE (39). Although there was significant heterogeneity between studies, all but three found that non-O blood groups bore a significantly increased risk for VTE, with an odds ratio of 1.75 (95% confidence interval, 1.51 to 2.03).

Data from numerous studies point towards a possible causal relationship between VWF, blood group, and thrombosis, particularly with venous thrombosis. This has raised the question of whether there would be value in assessing blood type when screening patients for risk of thrombosis, but further research is required to establish the particular risk associated with various blood group genotypes and phenotypes (44). Whereas universal screening may not be clinically or cost effective, there is some suggestion that selective screening of ABO blood type, in addition to routine thrombophilia tests, may prove useful in identifying individuals at risk (particularly AA, BB, and AB genotypes) (39).

Other Factors that Raise VWF Concentration

Although ABO blood group has proven to be the most influential factor on blood concentration of VWF, it is not the only one. For example, genetic mutations in the enzymes that process VWF, such as metalloprotease ADAMTS-13, can significantly affect the amount of VWF and the size of its multimers in plasma. A congenital or acquired deficiency of ADAMTS-13 results in very large VWF multimers that form platelet-rich thrombi in small blood vessels, leading to a disease known as thrombotic thrombocytopenic purpura (TTP). In this disease, the constant formation of platelet-rich clots leads to low platelet counts in plasma. The lack of available platelets can lead to bleeding in other parts of the body (such as the skin, leading to small purple spots called "purpura"). Because ADAMTS-13 does not function properly in TTP, VWF levels are unusually high in those with the disease (34).

Chronically high concentrations of VWF have also been seen in older age. This may be caused by arterial rigidity, but the precise mechanism is not well understood. Certain chronic health conditions also raise levels of VWF including obesity, hypertension, diabetes, chronic inflammation, rheumatoid arthritis, cancer, liver disease, and renal disease. Temporary health conditions, such as pregnancy, surgery, and exercise have also been shown to raise VWF concentration. Some hormones and their synthetic forms can also raise VWF, such as epinephrine, vasopressin, and desmopressin (6, 34).

Dietary Intervention and Hemostasis

The relationship between diet and hemostasis has been explored by a number of studies. In some, a complete dietary approach has been used as the independent variable, such as vegetarians versus meat eaters. In other studies, singular foods have been looked at or even particular food components such as fatty acids, vitamins, and phytochemicals. The following section reviews the literature available on dietary intervention and its effect on blood clotting.

Whole Diets

Mediterranean Diet

The Mediterranean diet is modeled after the style of eating practiced in the countries surrounding the Mediterranean Sea. The main components of the diet are healthy fats (such as olive oil), fish, fruits and vegetables, and wine. The benefits of this way of eating on heart health have been widely studied. For instance, in a study published by Passaro et al. in 2008, middle-aged women with moderate risk for CVD followed a Mediterranean-like diet. The study began with

a washout period of three weeks which had the subjects adhere to an isocaloric diet. After this phase, they began a diet that emphasized dry white wine, extra virgin olive oil, salmon, nuts and carbohydrates low on the glycemic index. After following the diet for three weeks, there was another washout period of three weeks during which the subjects returned to the isocaloric diet. The researchers measured various hemostatic factors in addition to other know markers for CVD risk. Most notably, they saw factor VII and factor VIII levels decrease in those on the diet, which in turn reduced levels of tissue factors. Thrombin generation times were also extended after dietary intervention. However, levels of fibrinogen and VWF were not significantly changed (45).

Vegetarian Diet

Plant based diets have been shown in some cases to improve hemostatic measures. For example, a few cross-sectional studies have been conducted over the past thirty years that showed favorable hemostatic conditions for vegetarians versus non-vegetarians. The earliest, conducted by Haines et al. in 1980, found that vegetarian participants had significantly lower levels of clotting factor VII in both males and females (46). A later study in 1999 by Famodu et al. looked at black, vegetarian Seventh-Day Adventists compared to non-vegetarians in nearby communities. In vegetarian subjects, they found fibrinogen levels to be significantly lower, suggesting a lower risk for arterial thrombosis. Also, the vegetarians had significantly higher fibrinolytic activity, which the authors suggested was due to their higher fiber intake (47). Likewise, in a study by Mezzano et al. in 1999, the researchers found that factor VII and fibrinogen levels

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were lower in vegetarians compared to non-vegetarians. On the other hand, the

vegetarians had increased platelet aggregation times. The authors suggested this

was due to the low consumption of anti-inflammatory eicosapentaenoic acid

(EPA) and docosahexaenoic acid (DHA) in vegetarians, two fatty acids that

displace arachidonic acid (AA) in platelet membranes. AA is a precursor to

thromboxane A_2, which is a key factor in platelet aggregation (48).

Not all studies have shown a difference in clotting measures between

vegetarians and meat eaters. For example, in a cross-sectional comparison

performed by Li et al. in 1999, the researchers compared thrombotic risk factors

in vegetarian men with their meat-eating counterparts. The researchers

hypothesized that the meat eaters would have higher levels of blood markers that

indicate increased thrombotic risk, due to the higher intake of AA from eggs and

meat. However, they did not see an increased risk for platelet aggregation in the

meat eaters, perhaps due to their higher intake of EPA and DHA also suggested

by Mezzano et al. (49).

Whole Foods

<u>Fish</u>

See "Fatty Acid" section below.

<u>Fruits and Vegetables</u>

Although diets high in fruits and vegetables have been recognized to lower the risk of CVD, the possibility that this occurs due to antithrombotic effects of fruits and vegetables has not been studied extensively. In 2004, Freese et al. published a study which explored the antithrombic effects of vegetables, berries and apples. The study included healthy men and women divided into four groups: two who consumed a diet high in vegetables, berries and apples, and two with a diet poor in those foods. In addition, the two experimental groups were further divided into diets rich in linoleic acid or oleic acid, as were the two control groups. The groups followed their prescribed diet for six weeks. The researchers performed many hemostatic measures, including platelet aggregation, but found no difference in platelet and endothelium markers between the four groups (50).

<u>Garlic and Onion</u>

Garlic and onion are members of the *allium* plant species, and contain a number of compounds with high sulfur content. Research has shown that some of these compounds inhibit platelet aggregation. These effects are greatest when the sulfur levels are high and the garlic and/or onion is served raw. Onion and garlic preparations appear to inhibit the production of thromboxane from its AA precursor, an important factor in platelet aggregation discussed above (32). In addition, a component of garlic called ajoene appears to interfere with the binding

of VWF and fibrinogen to platelets by reducing the number of GPIIb/IIIa receptors sites on the platelet membrane (51).

Ginger

Ginger and its extracts have been shown to inhibit platelet aggregation. It is believed that the components in ginger exhibiting these effects are the gingerols and shogaols that form during storage and cooking (32). These pungent constituents appear to inhibit AA metabolism, as do the compounds in garlic and onions discussed above. However, most experimentation has been performed in vitro, and results from human in vivo studies are less conclusive. Experiments on rats that administered high levels of ginger extract (500 mg/kg) reduced thromboxane B_2 levels (52), however, experiments with humans consuming raw (5-15 g/day) and cooked (40 g/day) ginger for one to two weeks had no effect of thromboxane B_2 concentration in serum (53).

Soy

The effect of soy on hemostasis and thrombosis has not been studied much in human subjects. In an animal study published in 2006, Sawashita et al. fed apolipoprotein E (apoE)-deficient mice an eight week diet of varying protein powders derived from soy, pork, egg, chicken, or fish. They found that the mice fed soy protein exhibited a significant antithrombic effect, although they contributed it to the hindrance of atherosclerosis instead of the inhibition of platelets. At the end of the eight weeks, they also found that pork protein had a prothrombic effect (54).

Soy contains a polyphenol named genistein that has been studied in relation to thrombosis. Refer to the "Genistein" section below for further detail.

Tomatoes

A link between tomatoes and lower risk of heart disease has been reported in epidemiological studies. Note that tomatoes are a major ingredient in the Mediterranean Diet discussed previously. Tomatoes are the major dietary source of the carotenoid lycopene. Although blood levels of lycopene have been inversely correlated with the early development of atherosclerosis, coronary events and CVD mortality, it is not known exactly how tomatoes are conferring this protective effect (32). Research suggests that these benefits may be via inhibition of platelet aggregation, among other mechanisms. For example, in a study by Lazarus et al. in 2004, individuals with type 2 diabetes who had hyperactive platelets were given a tomato extract supplement that significantly reduced platelet aggregation. Contrary to expected reasoning, the researchers reported that lycopene was not the responsible component (55).

Wine

See "Resveratrol" section below.

Nutrients

Carbohydrates

The intake of carbohydrates is the principle driver of postprandial insulin concentration. It has been shown that insulin stimulates the production of plasminogen activator inhibitor (PAI-1). PAI-1 inhibits fibrinolysis, an important step in the breakdown of clots. Therefore, increased insulin levels have been associated with the development of atherothrombosis (56). Replacing high glycemic index (GI) foods (such as white bread) with low GI foods (such as multi-grain bread) may reduce the risk of thrombogenesis (57).

Fatty Acids

Monounsaturated fatty acids (MUFA) that are present in plant-based oils such as canola oil and olive oil may have a favorable impact on thrombotic and hemostatic measures. For example, Larsen et al. conducted a randomized crossover trial in 1999 during which healthy adults ate a diet rich in rapeseed oil, sunflower oil, or olive oil for a total of three weeks. The oils vary in their fatty acid composition: rapeseed oil is high in both MUFA and omega-6 (n-6) polyunsaturated fatty acid (PUFA); sunflower oil is high in n-6 PUFA; and olive oil is high in MUFA. Subjects who followed the olive oil diet had significantly lower levels of factor VII compared to the sunflower oil group. A similar trend was seen when compared with the rapeseed oil group, although the difference did not reach significance (58). The Mediterranean Diet, discussed above, is known to have high levels of olive oil and has also been shown to lower factor VII levels (45, 59).

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The effect of omega-3 (n-3) PUFAs on hemostasis has been studied extensively. N-3 PUFAs are present in fatty fish, such as salmon, along with EPA and DHA. The consumption of fish is inversely related to the incidence of CVD (60). Supplementation of fish oil increases the ratio of n-3 to n-6 fatty acids in human tissue, including platelets, and has been shown to inhibit platelet aggregation (32). EPA displaces AA in the platelet membrane, and therefore inhibits the production of active thromboxane, as discussed previously.

Vitamin E

Increased vitamin E intake has been associated with a decreased incidence of coronary events. Among other mechanisms, the inhibition of platelet adhesion and aggregation has been suggested as a reason behind this phenomenon. Studies performed in vitro have demonstrated that lipid peroxidation is inhibited by α-tocopherol (a form of vitamin E) and therefore reduces the release and aggregation of platelets (61). However, in a study conducted by Dereska et al. in 2005, healthy adult subjects showed no change in platelet aggregation or bleeding times in vivo (62). They speculated that the effects of α-tocopherol in vitro on platelet aggregation may not be reproducible in human subjects.

Vitamin K

Various proteins in the body need vitamin K to convert to their active forms, including blood clotting factors such as prothrombin, factor VII, factor IX, and factor X. Anticoagulant medications, such as warfarin, interfere with the recycling of vitamin K and therefore inhibit blood clotting. Patients on warfarin are often instructed to avoid eating high vitamin K foods in excess, such as dark

leafy greens. However, oral supplementation of vitamin K has been shown to be an effective and safe way to ameliorate excessive hemorrhagic effects from warfarin (63, 64). The important role of vitamin K in thrombogenesis has been well established (32).

Polyphenols

Polyphenols are a broad class of molecules that share the structural commonality of containing more than one phenolic hydroxyl group. Although polyphenols are not considered nutrients, they are molecules that play beneficial roles in the human body through various mechanisms. High consumption of foods rich in polyphenols is inversely associated with death caused by thrombosis and IHD (8).

Flavanols and Proanthocyanidins

Flavanols are a type of polyphenol found in plant foods such as cocoa, red wine, grape seeds, and green tea. Flavanols can exist as single compounds, known as catechins, or oligomeric compounds, known as proanthocyanidins. In an epidemiological study of elderly men by Arts et al. in 2001, intake of catechin rich foods (such as black tea, apples, and chocolate) was inversely related to mortality from IHD (65). Besides exhibiting antioxidant and anti-inflammatory benefits, polyphenols have been shown to inhibit platelet activation and aggregation. For example, in a crossover study by Pearson et al. in 2002, healthy adult subjects consumed cocoa (rich in flavanols and proanthocyanidins), aspirin, or a combination of the two. The researchers performed tests on platelet activation and function at two and six hours after ingestion. The cocoa was found

to significantly inhibit platelet function, although the effects were less pronounced than those of aspirin (66).

Genistein

Genistein is an isoflavonoid found in soy foods. In vitro studies have shown that genistein interferes with platelet function and the action of thrombin. Via these mechanisms, it has been suggested that genistein may have the capability of altering blood coagulation and subsequently mitigating the progression of atherosclerosis (67). Human experimental trials on soy and genistein are limited, but effects have been studied in animals. For example, in 2002 Kondo et al. tested the ability of genistein to inhibit femoral artery occlusion in mice, in vivo. In addition, they measured in vitro platelet aggregation. They found that genistein prolonged the time to vessel occlusion and inhibited platelet aggregation. These results suggest that eating soy foods can moderate the progression of thrombosis and atherosclerosis (68).

Resveratrol

Resveratrol is a polyphenol found in the skin of red grapes and in red wine. It has been the topic of much recent study, due to the link between red wine consumption and a reduced risk of CVD. This has been described in the literature as the "French paradox" because the French consume relatively high amounts of saturated fat but have a low incidence of CVD. A protective effect was attributed to their high consumption of red wine (69). Resveratrol in red wine and purple grape juice has been found to interfere with platelet function. For example, in a randomized cross-over study by Keevil et al. in 2000, healthy adults consumed

either purple grape juice, orange juice, or grapefruit juice daily for 7 to 10 days. They found that the purple grape juice significantly reduced platelet aggregation, while there was no effect in the other groups. The purple grape fruit juice had three times the polyphenol concentration of the other juices (70).

The Blood Type Diet

In 1996, naturopathic doctor Peter J. D'Adamo published the diet book "Eat Right 4 Your Type" and has since sold over 5 million copies in more than 50 languages (1). In the book, D'Adamo suggests that people should be eating specialized diets based on their blood type. This idea was first set forth by his father, who was also a naturopathic doctor. The two developed their theory on blood type diets based on anecdotal evidence collected from patients. D'Adamo claims that patients who have followed his diets have experienced more energy, weight loss, improved digestion, and relief from chronic ailments such as heartburn, asthma and headaches. He states that the diets are "designed for optimal performance" (2).

Lectins

Lectins are proteins that have an affinity for binding carbohydrates. They are common in plant foods such as beans and grains. They have the ability to agglutinate human cells, and have been show to resist protein breakdown initiated by heat from cooking or digestive enzymes (71). The theory behind the blood type diet is that lectins interact with ABO blood cell antigens and can potentially cause the cells to agglutinate, resulting in various adverse health effects. Therefore, foods with lectins that would cause this interaction with a particular ABO antigen should be avoided by individuals of that type. D'Adamo writes that he has performed numerous tests with lectins from various foods in which he mixed them with blood samples and observed the results under a microscope. In a reactive blood type, D'Adamo observed the RBCs agglutinating (2). D'Adamo's findings have not been published in peer-reviewed journals.

However, research has been published on the interaction of dietary lectins and blood cell antigens. For example, in 2002 Evans et al. published a case-control study looking at intake of galactose (found in fruit and vegetable fiber) and risk for colorectal cancer. The researchers hypothesized that galactose conferred a reduction in risk by binding a blood cell antigen known as Thomsen Friedenreich (TF), which is expressed by precancerous and cancerous epithelial cells in the colon and rectum. As the theory goes, the binding of galactose to the TF antigen may inhibit the binding of dietary or microbial lectins that would cause the cancerous cells to proliferate (72). In a previous study, researchers were able to show that a peanut lectin caused such proliferation (73). Indeed, Evans et

al. found that subjects in the highest quartile of galactose consumption saw a significantly lower odds ratio for colorectal cancer.

Interestingly, in an associated study, whole peanut lectins were found in the peripheral venous blood of subjects who ingested peanuts one to four hours before the blood draw (71). This demonstrates that some lectins can avoid digestion and go past the gut into circulation, completely intact. Other research has also shown that lectins from cereals and legumes can interact with the gut to increase its permeability, allowing bacterial and dietary antigens to enter into circulation (74). These finding are in line with D'Adamo's statement that lectins can enter the blood stream and affect cells beyond the digestive tract. However, research indicates that lectins very rarely have a high affinity for one singular ABO blood cell antigen, with the exception of some beans (3).

A commentary on dietary lectins and disease published in the British Medical Journal by allergist David Freed points out associations between dietary lectins and diseases such as celiac disease, type 1 diabetes, rheumatoid arthritis, and peptic ulcer. He described how the attachment of certain dietary lectins to various cells can elicit an autoimmune response that leads to the aforementioned conditions (75). However, much of the research he reported on was speculative and further investigation is warranted.

Individualized Diets

Each blood type – O, A, B, and AB – has its own chapter in the book in which D'Adamo prescribes what foods should and should not be eaten. He divides each of the food groups into three categories: "highly beneficial," "neutral," and "avoid." He instructs the reader to eat mostly from the highly beneficial foods, occasionally from the neutral foods, and never from the foods to be avoided. Foods in the "avoid" category supposedly contain the lectins harmful to that particular blood type. Each diet is described in general terms below:

- Type Os are the meat eaters. They do well with plenty of animal protein, but should avoid dairy and most grains.

- Type As are the vegetarians. They thrive on plant-based protein such as soy, and are more tolerant of a wide range of grains. Like type Os, they should also avoid dairy.

- Type Bs have the most flexibility in their diet and can consume a variety of items. For example, they can consume dairy products and meat, although there are some odd restrictions, such as chicken and pork.

- Type ABs are considered a combination of types A and B. Many of their dietary restrictions are the same as those of the previous two types.

Dr. D'Adamo's diet is regarded as "pseudo-science" in the literature and has not been properly corroborated by science (3, 74), but the popularity of his book warrants scientific investigation so that the public may be better informed before taking his advice to heart.

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Chapter 3

METHODS

Study Design and Subjects

This study was a randomized, parallel arm, dietary intervention using

healthy, omnivorous adults with blood types A and O. The study was approved

by the Arizona State University Institutional Review Board (see Appendix A for

IRB approval). Study volunteers were recruited largely from the ASU downtown

campus via email invitation distributed on the ASU nutrition listserv and

recruitment flyers posted on campus. The goal was to enroll 60 subjects in hopes

of retaining at least 40 by the end of the study, based on a review of sample size

in similar studies. Potential subjects were initially screened by an online survey

posted on Survey Monkey™ to eliminate candidates that did not meet inclusion

criteria. Out of 104 people who responded to the online survey, 24 did not qualify

because they met one or more of the exclusion criteria listed in the following

paragraph. The remaining 80 people were invited via email to an in-person

screening at ASU's downtown campus. Forty-eight individuals attended the in-

person screening and were admitted into the study. Informed written consent was

obtained from each subject after the study objective, benefits, and risks were

thoroughly explained (see Consent Form in Appendix A).

Participants were over 18 years old and in good health. Exclusion criteria

included smoking habit, previous history of bleeding diathesis, such as

hemophilia; hypertension (blood pressure greater than 140/90 mmHg); a

previously recorded abnormal bleeding time; abnormally high or low platelet

count; ingestion of over-the-counter anticoagulant medication such as aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) within two weeks of the start of the trial; ingestion of prescription anticoagulant medications such as Coumadin, sulfinpyrazone or dipyridamole; injection of heparin or dextran in the two weeks prior to the study; recent liver disease; collagen vascular diseases such as lupus; cancer; current pregnancy; antibiotic use within the two weeks prior to the study or history of chronic ingestion of antibiotics; and known vitamin K deficiency (62). Furthermore, study subjects could not practice vegetarianism and confirmed consumption of meat and dairy products daily via a food frequency questionnaire at the in-person screening. Participants were willing to omit meats (with the exception of chicken, turkey, and fish) and all dairy products from their diet, and consume soy products every day for four weeks.

One of the individuals who attended the in-person screening and signed the consent form dropped out (citing a lack of time) before the researchers randomized subjects into study groups. A total of 47 subjects (23 with blood type A and 24 with blood type O) were stratified by blood type and weight and subsequently randomized into two groups: an experimental group made up of 12 type A individuals and 12 type O individuals that adhered to the type A, vegetarian-like diet for four weeks, and a control group made up of 11 type A individuals and 12 type O individuals that continued their normal, omnivorous diet for the duration of the study (see Figure 6).

Eight more subjects withdrew over the course of the study. Five individuals cited scheduling conflicts and lack of time and three women found out

they were pregnant and were therefore excluded. A total of 39 subjects completed the study.
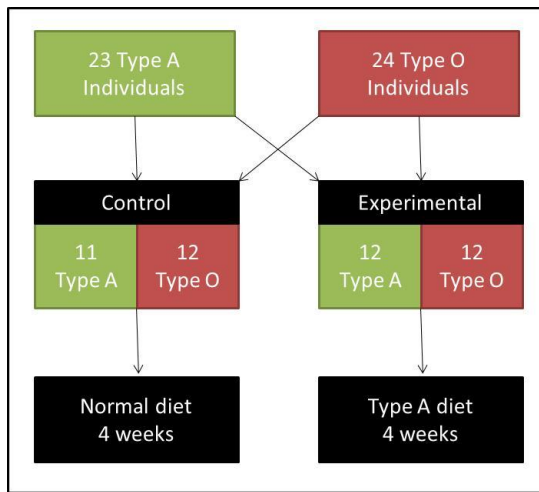
Figure 6. Study design

Diet

Half of the type A subjects and half of the type O subjects comprised the intervention group that followed the type A diet plan as described in D'Adamo's book, for four weeks (2). This is a vegetarian-like diet that focuses on soy proteins, vegetables, and grains. However, it is not completely meatless because some fish and poultry are allowed. Food sources should also be consumed in as natural a state as possible, avoiding processed and refined foods. For a complete description of the diet, see the Diet Guidelines handout provided to subjects in Appendix B. The control group followed their normal, omnivorous diet during the four week study period. Subjects in the vegetarian-like diet group were provided with 28 servings of soy milk packaged in 8 oz aseptic containers, to be consumed once a day. The remaining soy milk was distributed to the control diet group at the conclusion of the study.

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Study Protocol

   After initial screening by online survey, potential subjects visited the ASU downtown campus for an in-person screening. After their consent was obtained, they filled out questionnaires regarding medical history and food frequency to confirm meat and dairy consumption (see Appendix B for copies of the questionnaires). If they could not provide a validated blood donor card or other official documentation of blood type, a finger prick test was performed to assess blood type. Age, weight, height, and blood pressure were also recorded.

   All blood type A and O individuals who qualified for the study were stratified by blood type and weight and then randomized into the vegetarian-like diet group or control diet group. Participants were asked to abstain from the use of prescription and over the counter medications, including aspirin and NSAIDs, for two weeks following the in-person screening, per Dereska et al. (62). During this time, subjects continued to eat their normal diet. Both study groups returned to ASU after two weeks to have their blood drawn at week 0 of the study.

   In addition to a blood draw, subjects also were weighed again and given a $10 Target gift card. Those in the vegetarian-like diet group received detailed instruction on the type A diet and were given a 28 day supply of 8 oz vanilla soy milk. In addition to a detailed Diet Guidelines handout and a Meal Replacement Guide, experimental subjects were also given a Compliance Calendar and asked to place an 'X' on the dates they complied and to note non-compliant foods and serving size on days they did not. All of these handouts can be found in Appendix B.

After this visit, the vegetarian-like diet group began the type A diet while the control diet group maintained their usual, omnivorous diet. Weekly follow up emails were sent to the vegetarian-like diet group to encourage compliance. At week 4 both groups returned to ASU to have their blood drawn again. In addition, their weight was recorded, a food frequency questionnaire was completed, and they received another $10 Target gift card. Leftover soy milk was divided and distributed among the returning control diet group subjects.

For more detail on study protocol, see the timeline in Appendix C.

Laboratory Analysis

Blood type was determined from whole blood obtained from a finger prick using an EldonCard™ 2511-1 ABO-Rh Home Blood Typing Test Kit (Craig Medical Distribution Inc., Vista, CA). Venous whole blood was drawn from all study subjects at week 0 and week 4 of the study. Blood was collected in tubes that contain an anticoagulant, such as citrate, and gently inverted by hand four times to ensure proper mixing (62). In addition to the clotting tests detailed below, blood was analyzed for c-reactive protein (CRP) concentration and lipid profile using the cobas c 111 analyzer (F. Hoffmann-La Roche Ltd, Switzerland). Detailed procedures for all analytical tests performed can be found in Appendix D.

Von Willebrand Factor

Von Willebrand factor was analyzed using the von Willebrand factor - IMUBIND® vWF ELISA (product number 828, American Diagnostica Inc., Stamford, CT).

Citrated Whole Blood Coagulation Testing

Prothrombin time (PT) and activated partial thromboplastin time (APTT) were used to assess the extrinsic and intrinsic blood coagulation pathways, as they have been in other dietary intervention studies (62, 76-79). Both tests were run through Sonora Quest Laboratories in Phoenix, AZ on a Sysmex CA-7000 analyzer (Siemens Healthcare Diagnostics Inc., Tarrytown, NY). The Sysmex CA-7000 has been evaluated by Fischer et al. and demonstrated an intra- and inter-assay coefficient of variation below 5% for most parameters, demonstrating satisfactory precision (80).

Statistical Analysis

Statistical analysis was performed using SPSS Statistical Analysis system 19.0 (Chicago, IL). Results are expressed as mean ± standard deviation. Statistical differences between the experimental and control groups, as well as blood type groups, were calculated by two-way, between-group analysis of variance (ANOVA). Attempts were made to normalize values that did not conform to a normal distribution. Differences were considered significant at $P < 0.05$.

Chapter 4

RESULTS

Baseline Data

Of the 39 subjects who completed the study, 19 were blood type O and 20
were blood type A. There were 34 women and 5 men, with an average age of
27.5 ± 8.7 years and an average BMI of 24.0 ± 3.7, which is classified as normal.
There were 27 Caucasians (69%), 6 Hispanics (15%), 3 Asians (8%), 1 African
American (3%), and 2 subjects who reported their race as "other" or left the
question blank. Baseline data (study week 0) were collected at the subjects'
second visit, approximately two weeks after their initial in-person screening.
During that two week interim, subjects abstained from the use of NSAIDs and
continued their normal eating habits. In addition to height and weight, a venous
blood sample was taken at study week 0 and tested for PT, APTT, VWF, CRP,
and lipid profile. Week 0 characteristics of the four study groups are presented in
Table 1. No significant differences were found between the four groups, with the
exception of VWF. Blood type O subjects had significantly lower blood levels of
VWF compared to blood type A subjects; 690.4 ± 189.7 mU/ml and 1012.1 ± 298.5
mU/ml, respectively ($p<0.001$) (see Figure 7).

Table 1. Baseline data (study week 0) collected from four study groups[a]

| Variable	Control Type O (n=9)	Control Type A (n=9)	Experimental Type O (n=10)	Experimental Type A (n=11)	p Value[b]
Age[c] (yr)	26.4±5.8	27.8±10.3	28.0±10.9	27.8±8.1	0.981
Height (in)	67.3±4.2	64.6±1.7	66.3±2.9	65.6±3.0	0.298
Weight (lb)	162.2±42.5	138.2±14.7	145.7±20.8	150.1±29.1	0.354
BMI	25.0±5.3	23.3±2.7	23.3±3.3	24.3±3.3	0.719
PT (sec)	10.8±0.9	10.7±0.3	10.7±0.3	10.9±0.6	0.739
APTT (sec)	30.1±1.3	28.8±2.3	30.3±2.0	29.1±2.7	0.344
VWF (mU/ml)	627.7±152.6	991.3±374.1	746.8±209.3	1029.1±237.9	0.003[d]
Total Chol (mg/dl)	165.6±40.6	152.6±29.3	146.8±22.0	152.7±18.6	0.536
HDL (mg/dl)	53.9±21.4	69.8±16.0	65.6±12.2	60.5±13.1	0.186
LDL (mg/dl)	105.8±32.6	81.6±24.1	77.1±19.3	89.8±16.6	0.061
Triglycerides[e] (mg/dl)	104.5±35.2	69.7±27.0	78.9±35.4	77.1±24.1	0.134
hsCRP[c] (mg/L)	3.95±4.28	0.94±0.86	1.94±2.84	1.47±1.51	0.219
Kcal/day	1734±615	1678±480	1581±539	1652±625	0.908

[a]Data presented as mean±SD
[b]p value represents one-way, between-group ANOVA
[c]Data transformed to log for normality
[d]LSD post hoc test shows significant difference between type O and type A subjects across both diet groups
[e]Data transformed to square root for normality
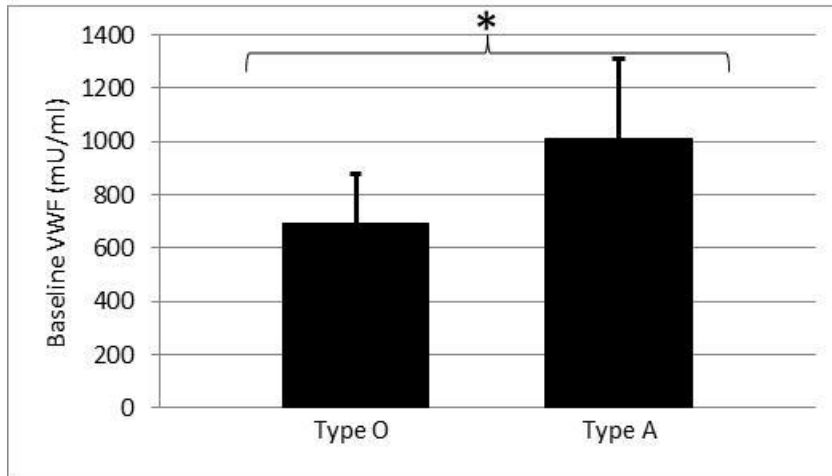
Figure 7. Baseline values for Von Willebrand Factor in blood type O and blood type A subjects. P value derived from independent samples t-test. *Denotes significant difference between blood types (p<0.001).

Compliance

In order to assess compliance with the vegetarian-like diet, a food frequency questionnaire (FFQ) was designed to isolate foods permitted and restricted on the diet. Subjects recorded the number of servings of each food item they ate per week, then stated whether the serving size was small, average, or large. To score the FFQs, the number of servings for each food item was multiplied by a number assigned to serving size (small = 0.5, average = 1, and large = 1.5), then these scores were added together for the permitted and prohibited food categories. All subjects filled out the FFQ at week 0 and week 4.

At week 0, the control diet group had an average score of 50.2±23.1 in the category of vegetarian-like diet permitted foods and a score of 52.3±21.0 in the restricted foods category. At week 4, the control group had an average score of 45.6±20.8 in the permitted foods category and 47.3±18.3 in the restricted foods category. There was no significant difference between the scores at week 0 and

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week 4 in the control diet group, indicating that the group did not change their eating patterns.

At week 0, the vegetarian-like diet group had an average score of 49.9±18.1 in the permitted foods category and a score of 48.4±19.0 in the restricted foods category. At week 4, the vegetarian-like diet group had an average score of 80.0±39.0 for permitted foods and 5.2±5.0 for restricted foods. There was a significant difference between week 0 and week 4 scores among the vegetarian-like diet group (p<0.001), as well as a significant difference between permitted and restricted food scores at week 4 (p<0.001), indicating compliance to the experimental diet.
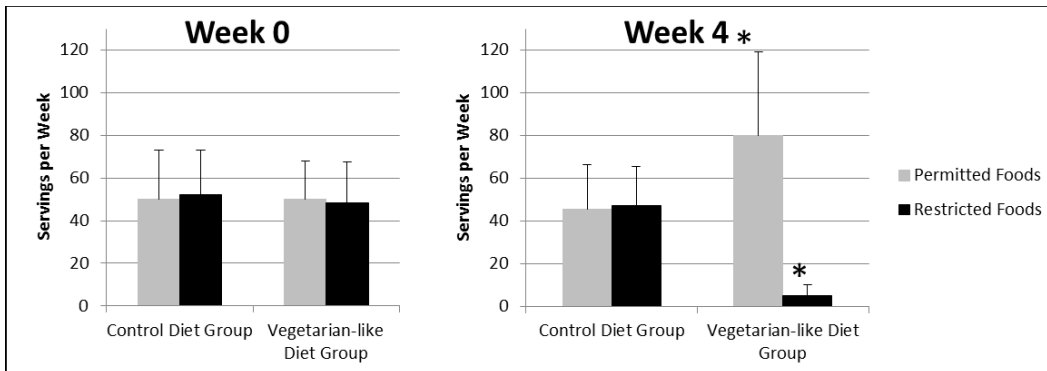
Figure 8. FFQ scores of permitted and restricted foods on vegetarian-like diet by omnivorous control diet group and vegetarian-like diet group at week 0 and week 4. *Denotes significant increase in permitted foods and decrease in restricted foods from week 0 to week 4 in the vegetarian-like diet group (p<0.001).

Finally, all subjects in the vegetarian-like diet group filled out a daily compliance calendar for four weeks on which they put an 'X' for all days they complied, or recorded the restricted foods they consumed on days of noncompliance. The number of days they complied was divided by 28 to calculate a compliance percentage. Blood type Os complied 89±9% of all study

days whereas blood type As complied 87±7% of all study days. There was no significant difference between type O's and type A's compliance percentages.

Post-intervention Data

A table containing all mean data for weeks 0 and 4 can be found in Appendix E.

Clotting Variables

A medium negative correlation (Pearson r of -0.350/ p=0.029) existed between the change in PT and age. No other significant relationships between descriptors and outcome variables were identified. Therefore, age was controlled for when running the two-way, between-group ANOVA for change in PT. Subjects in the experimental group (who followed a vegetarian-like diet for four weeks) experienced a significant increase in PT, +0.12±0.32 seconds, whereas the control group (who maintained their omnivorous diet for four weeks) saw a decrease of -0.06±0.33 seconds (p=0.054). The vegetarian-like dietary intervention had a medium effect size (10.5%) for change in PT. Furthermore, there was a significant difference between the change in PT for type Os in the vegetarian-like diet group (+0.24±0.32 seconds) compared to type Os in the control diet group (-0.01±0.30 seconds) (p=0.050). On the other hand, there was no significant difference between the change in PT for type As in the vegetarian-like diet group compared to type As in the control diet group. Change in PT over the course of the four week trial is represented in Figure 8 and mean change±SD is reported in Table 2.

No significant change was observed in APTT: the vegetarian-like diet group collectively saw an increase of 0.46±1.07 seconds compared to an increase of 0.28±1.06 seconds in the control diet group. Likewise, there was no significant change in VWF concentration. See Table 2 for details.

Table 2. Change in values for blood clotting measures over four weeks in four study groups[a]

Variable	Control Type O (n=9)	Control Type A (n=9)	Experimental Type O (n=10)	Experimental Type A (n=11)	p Value[b]
PT (sec)	-0.01±0.30	-0.10±0.37	0.24±0.32*	0.01±0.28	0.054[c]
APTT (sec)	-0.18±0.85	0.74±1.09	0.45±1.41	0.47±0.70	0.599
VWF (mU/ml)	260.7±172.4	85.3±245.7	148.3±304.7	215.5±156.7	0.931

[a]Data presented as mean±SD
[b]p value represents group effect analyzed by two-way, between-group ANOVA
[c]Controlled for age; *differs significantly from control type O
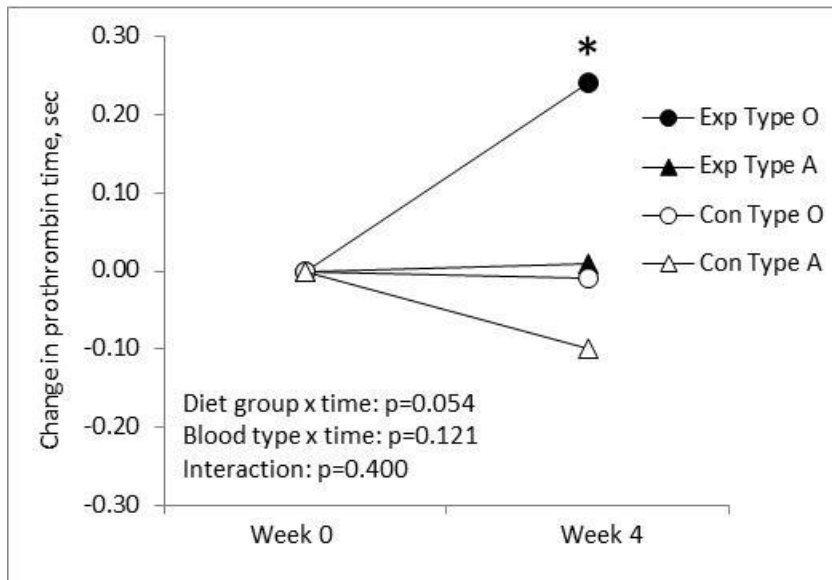
Figure 9. Change in prothrombin time in participants randomized to continue their normal omnivorous diet (Con) or adopt a vegetarian-like diet (Exp) for four weeks. Participants are also defined by blood type O or A. P values derived from univariate analysis, controlling for age. *Denotes significant diet group effect for blood type O (p=0.050)

Lipids and CRP

Overall, dietary intervention did not result in significant change in the subjects' lipid profiles or CRP levels. Mean values for change in total cholesterol were -7.19±20.14 mg/dl for the vegetarian-like diet group and -3.25±13.40 mg/dl for the control diet group. Mean values for change in HDL cholesterols were -0.83±5.91 mg/dl for the vegetarian-like diet group and -2.92±7.41 mg/dl for the control diet group. Mean values for change in LDL cholesterol were -4.71±18.09 mg/dl for the vegetarian-like diet group and -2.04±8.21 mg/dl for the control diet group. Mean values for change in triglycerides were -2.87±20.31 mg/dl for the vegetarian-like diet group and 3.58±20.97 mg/dl for the control diet group. Mean values for change in CRP were -0.13±1.81 mg/L in the vegetarian-like diet group and -0.53±2.44 mg/L in the control diet group. For the CRP analyses, two

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outliers were removed from the data (values >3 SD from mean). Also, data for

change in CRP did not fall on a normal curve and was therefore assessed via a

nonparametric Mann-Whitney U test. Mean values for study groups reported by

blood type are listed in Table 3.

Table 3. Change in values for lipid and CRP measures over four weeks in four study groups[a]

Variable	Control Type O (n=9)	Control Type A (n=9)	Experimental Type O (n=10)	Experimental Type A (n=11)	p Value[b]
Total Chol (mg/dl)	-3.89±10.83	-2.61±16.23	-0.70±17.99	-13.1±21.0	0.514
HDL (mg/dl)	-5.22±7.58	-0.61±6.88	2.10±5.52	-3.50±5.11	0.278
LDL (mg/dl)	-1.12±5.42	-2.97±10.58	-0.26±16.64	-8.75±19.17	0.599
Triglycerides (mg/dl)	-1.79±20.53	8.96±21.16	-2.92±27.56	-2.82±11.88	0.296[c]
hsCRP[d] (mg/L)	-0.97±3.16	0.04±0.94	-0.59±2.45	0.30±0.83	0.530[e]

[a]Data presented as mean±SD
[b]p value represents group effect analyzed by two-way, between-group ANOVA
[c]Controlled for baseline weight
[d]Two outliers removed because values were out of range
[e]Data unable to normalize; nonparametric Mann-Whitney U Test used

Weight and BMI

Subjects in the vegetarian-like diet group lost significantly more weight

than those in the control diet group, with mean values of -1.4±2.3 pounds and

+0.7±2.3 pounds respectively (p=0.01). The vegetarian-like dietary intervention

had a large effect size for change in weight (17.5%). Among subjects with blood

type A, there was significant weight loss in the vegetarian-like diet group

compared to the control diet group (p=0.037). Among subjects with blood type

O, there was a trend for weight loss in the vegetarian-like diet group compared to the control diet group (p=0.092). See Table 4 and Figure 9 below for details on weight loss values.

Subjects in the vegetarian-like diet group also experienced a significant decrease in BMI compared to the control diet group (-0.2±0.4 and +0.1±0.3 respectively; p<0.01). The vegetarian-like dietary intervention had a large effect size for change in BMI (20.8%). For those who followed the vegetarian-like diet, both blood types O and A experienced significant decreases in BMI compared to their counterparts in the control diet group (p=0.092 and p=0.037, respectively). Details on BMI are represented in Table 4 and Figure 10.

Although the vegetarian-like diet group lost weight over the four week study, based on computations from the FFQs, the amount of kilocalories consumed per day did not change from week 0 to week 4. A two-way, between-group ANOVA revealed that diet group effect on change in kilocalories did not reach significance (one outlier was removed). However, change in weight was highly correlated to change in kilocalories (r=0.418/ p=0.008), although, when the same outlier was removed, significance was lost.

Table 4. Change in values for weight, BMI, and Kcals over four weeks in four study groups[a]

Variable	Control Type O (n=9)	Control Type A (n=9)	Experimental Type O (n=10)	Experimental Type A (n=11)	p Value[b]
Weight[c] (lb)	0.5±3.2	0.8±1.2	-1.8±2.6[+]	-0.9±2.1*	0.011
BMI[c]	0.1±0.5	0.1±0.2	-0.3±0.4*	-0.2±0.3*	0.005
Kcal/day	-143±451	-105±476	-221±426	-353±493	0.284

[a]Data presented as mean±SD
[b]p value represents group effect analyzed by two-way, between-group ANOVA
[c]One outlier removed; *differs significantly from control; [+]trend of difference from control
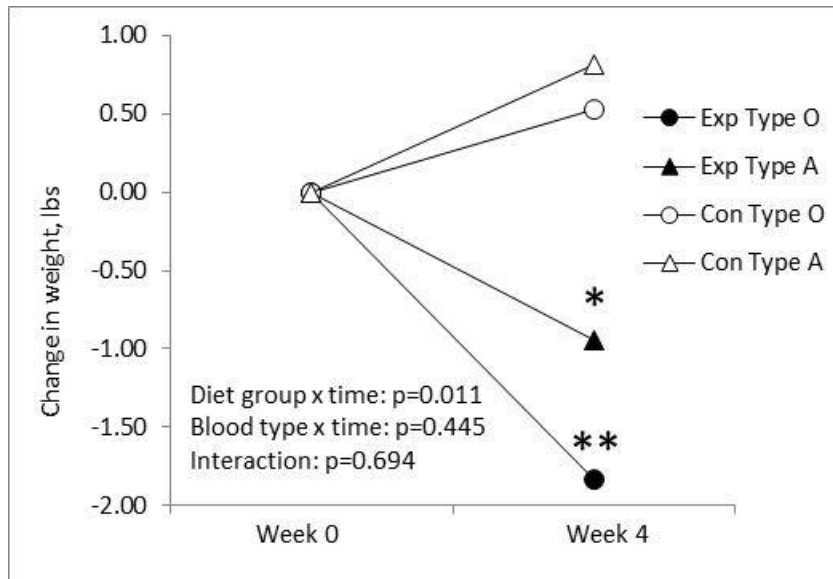
Figure 10. Change in weight in participants randomized to continue their normal omnivorous diet (Con) or adopt a vegetarian-like diet (Exp) for four weeks. Participants are also defined by blood type O or A. P values derived from univariate analysis, excluding one outlier. *Denotes significant diet group effect for blood type A (p=0.037). **Denotes a trend for diet group effect for blood type O (p=0.092).
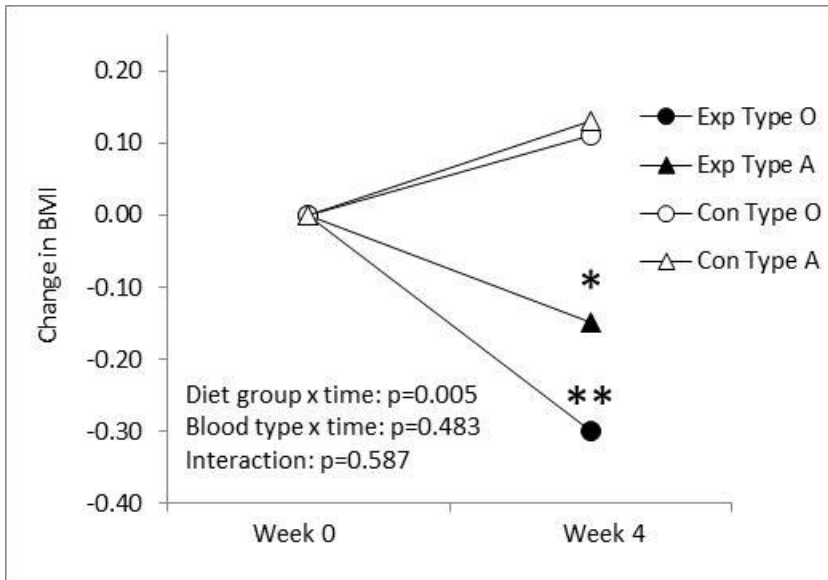
Figure 11. Change in BMI in participants randomized to continue their normal omnivorous diet (Con) or adopt a vegetarian-like diet (Exp) for four weeks. Participants are also defined by blood type O or A. P values derived from univariate analysis, excluding one outlier. *Denotes significant diet group effect for blood type A (p=0.036). **Denotes significant diet group effect for blood type O (p=0.052).

Chapter 5

DISCUSSION

Clotting Variables

We originally hypothesized that there would be no difference in blood coagulation between type A and type O blood groups after adhering to a vegetarian-like diet for four weeks, given the lack of published research on blood type and diet. Contrary to this hypothesis, there was a significant diet group effect for blood type O. Type Os in the vegetarian-like diet group had an increased PT of $+0.24 \pm 0.32$ seconds (p=0.050). However, type As in the vegetarian-like diet group saw relatively no change in PT.

A prolonged PT is typically evidential of a possible bleeding disorder, but a shortened PT may be associated with an increased risk of thrombosis. A reduction in PT of 0.2 seconds may represent an important change in terms of risk for CVD. Freedman et al. conducted a cross-sectional study of male US Army veterans in which they examined associations between PT and traits linked to CVD (81). They found that mean PT was 0.2 seconds shorter among cigarette smokers compared to nonsmokers. Cigarette smoking is an established risk factor for CVD. The type Os in the vegetarian-like diet group saw a reduction in PT on par with nonsmokers versus smokers. Freedman et al. also noted several other inverse associations between PT and CVD risk factors including weight, total cholesterol, and triglycerides.

APTT is another blood coagulation test often performed in conjunction with PT. Whereas both tests measure the common coagulation pathway, APTT

specifically tests the intrinsic pathway. When taken alongside a normal PT, APTT can point to a clotting factor deficiency in the intrinsic pathway. The reverse situation, in which PT is prolonged and APTT is normal, indicates a deficiency in the extrinsic pathway. When both test results are prolonged, it points to an inhibition or deficiency in the common coagulation pathway (refer back to Figure 4 in chapter 2) (82). Change in PT and change in APTT over our four week trial had a medium positive correlation (r=0.331/ p=0.039).

Beyond using APTT as a test to indicate bleeding disorders, shorter APTT has been linked in research to risk for thrombotic disorders such as VTE. In a 13 year prospective study, Zakai et al. studied the relationship between baseline APTT and future incidence of VTE (83). They found that baseline APTT values that fell below the median were associated with an increased risk of future VTE, even after adjusting for demographic and hemostatic factors. When compared to subjects in the fourth quartile (with the longest APTTs), those in the first and second quartiles had a 2.4-fold and 1.9-fold higher risk for VTE. The median value in Zakai's study was 28.75 whereas in our study the median value for baseline APTT was 29.6 seconds. Using Zakai's median APTT of 28.75, 15 subjects in our study fell below this value (9 of which were in the vegetarian-like diet group) and would be associated with an increased risk for VTE. After the four week dietary intervention, one subject from the vegetarian-like diet group and one from the control diet group had an increased APTT time that was above 28.75, suggesting the vegetarian-like diet did not make a difference on this

measure. Indeed, we saw no significant change in this value when analyzed by a two-way, between-group ANOVA.

Interestingly, Zakai et al. also found an association between non-O blood groups and a lower APTT. In the first quartile 85% were non-O blood types whereas only 60% were non-O in the fourth quartile. Similarly, in our study, a one-way ANOVA performed on baseline APTT values using blood type as a factor revealed a trend for type As to have a shorter APTT (29.0 ± 2.4 sec) than type Os (30.2 ± 1.6 sec) ($p=0.069$).

It is not clear why we saw a significant increase in PT for type Os in the vegetarian-like diet group, but no significant increase in APTT. Perhaps the diet affected some factor in the extrinsic clotting pathway for type Os, but not in the intrinsic or common pathways. The possibility also exists that there was a preanalytical cause of inaccuracy. For example, prolonged time lapses between collection and measurement by the lab may produce erroneous results (82). Finally, the significant finding may have been due to chance.

As for VWF, plasma concentration at week 0 was significantly higher in blood type A than blood type O. This difference corresponds with previous research discussed in the literature review of this thesis. VWF concentration did not change after our four week dietary intervention. VWF and PT were not correlated in our study, but VWF and APTT shared a strong negative correlation ($r=-0.595$/ $p<0.001$). This would be expected given VWF's role in protecting FVIII, one of the clotting factors involved in the intrinsic pathway of coagulation. Actually, APTT is a commonly used test to help diagnose Von Willebrand

Disease (84). Based on the link between higher VWF levels and increased risk for CVD, it is of interest to pursue further studies that look at dietary interventions that lower risk of thrombosis. The addition of other hemostatic measures, such as platelet aggregation, would give a broader picture of how diet affects hemostasis.

Lipids and CRP

There were no significant changes in the study subjects' lipid profiles or CRP levels after the four week vegetarian-like dietary intervention. However, the study subjects were healthy, young adults with lipid and CRP values that fell within normal ranges. The average total cholesterol for all study subjects at week 0 was 154.1 ± 27.9 mg/dl, which is well below the "desirable" category of <200 mg/dl recommended by the National Heart, Lung, and Blood Institute (NHLBI) (85). Average HDL for all study subjects at week 0 was 62.4 ± 16.3 mg/dl, which falls into the "best" category of HDL levels at >60 mg/dl, recommended by NHLBI. Average LDL for all study subjects at week 0 was 88.3 ± 25.0 mg/dl, which falls within the <100 mg/dl recommendation by NHLBI for people with risk of heart disease, and well below the recommendation of <130 mg/dl for healthy adults. Average triglyceride level for all study subjects at week 0 was 82.2 ± 32.1 mg/dl, which falls well below the NHLBI recommendation of <150 mg/dl. Finally, average CRP for all study subjects at week 0 was 2.0 ± 2.8 mg/L. When CRP is used as a biomarker for heart disease risk, 2.0 mg/L is classified as "medium" risk (low <1.0 mg/L; medium 1.0 to 3.0 mg/L; high >3.0 mg/L) (86). Being that the study subjects started with very healthy cholesterol, triglyceride, and CRP levels, the dietary intervention was not expected to have a large impact

on these measures. If the study were to be repeated, picking subjects from a population with increased lipid and CRP levels, such as those at risk for heart disease, may better reveal how the vegetarian-like diet impacts these measures.

Weight and BMI

Subjects who followed the vegetarian-like diet for four weeks lost nearly a pound and a half. Based on calculations from FFQs, both blood types O and A in the vegetarian-like diet group reduced their caloric intake, however, because controls also reported a slight reduction in calories, the difference did not reach significance. Beyond the fact that reduction in calories leads to weight loss, research by Canfi et al. suggests that alterations in consumption from specific food groups affects weight loss (87). They observed that an increase in vegetables consumed and a decrease in cookies and cakes were universal weight loss predictors, regardless of diet type (low fat, low carbohydrate, or Mediterranean diet). In our study, subjects in the vegetarian-like diet saw a trend towards an increase in fruit and vegetable consumption from 21±8 weekly servings at week 0 to 28±17 weekly servings at week 4 (p=0.086). The control diet group saw no change in fruit and vegetable consumption at all. Also, the vegetarian-like diet group significantly decreased their consumption of sweets from 7±6 weekly servings at week 0 to 2±3 weekly servings at week 4 (p=0.003), whereas the control diet group's consumption of sweets remained the same. See Figure 11 for further detail.
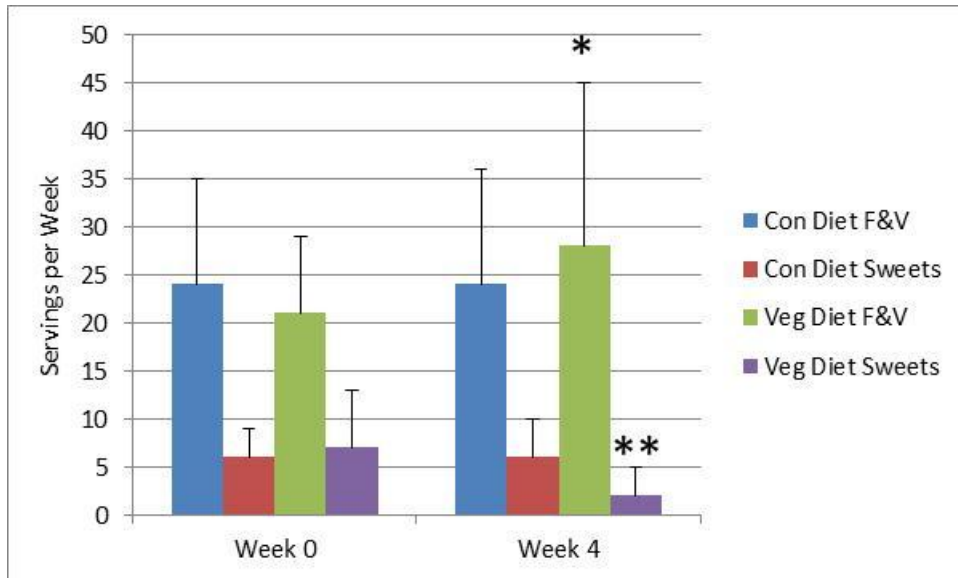
Figure 12. Reported weekly consumption of fruits & vegetables and sweets by vegetarian-like diet group and omnivorous control diet group after four weeks. *Denotes a trend for increase in F&V consumption in vegetarian-like diet group at week 4 (p=0.086). **Denotes significant decrease in sweets consumption in vegetarian-like diet group at week 4 (p=0.003).

One of the main dietary changes those in the vegetarian-like diet group underwent was the elimination of red meat. Research is inconsistent when it comes to the effect of red meat on weight and BMI. Vergnaud et al. noted a significant positive association between red meat consumption (as well as total meat, poultry and processed meats) and weight gain in a five year prospective study of adults aged 25 to 70 years (88). However, in a subsequent study by Gilsing et al., red meat consumption was not associated with changes in BMI over a 14 year follow-up of older adults aged 55 to 69 years (89). Yet they did note significant associations between increased BMI and pork consumption in women and chicken consumption in both women and men. In our study, the vegetarian-like diet group significantly reduced their BMI by 0.2, suggesting that eliminating red meat may contribute to a reduction in BMI.

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Limitations

Our study was powered for our primary outcome measures of PT and APTT, and therefore was underpowered to find significant change in caloric consumption. In order to power for change in caloric intake at 80%, a total of 246 subjects would have to be enrolled in this parallel-arm study. Future studies should include more subjects. Also, the dietary intervention in this study occurred outside of a controlled setting, and subjects were responsible for adhering to the dietary guidelines themselves. Because their compliance and food intake was self-reported, the possibility of inaccuracies exists.

The Evidence-Based Blood Type Diet

The outcome of this study is contradictory to D'Adamo's recommendations for blood type A. It was not the type As, but the type Os who faired best on the vegetarian-like diet in our study. Type Os saw an increase in PT indicating that their blood clotting times were lengthened, perhaps reducing the risk of thrombosis and its related health complications. It seems that blood type A is less responsive to the dietary intervention and may require more rigid dietary guidelines or a longer time on such a diet to see the benefits. While some of D'Adamo's recommendations are backed by research, others are not.

In order to overcome an increased risk of CVD linked to higher levels of VWF, blood type As should emphasize those foods that have been established as antithrombic and heart healthy by scientific research. Referring back to the foods discussed in the literature review section, type As would likely benefit from the Mediterranean diet, emphasizing olive oil, fish (such as salmon), red wine, and

fruits and vegetables. Type As should cook often with garlic, onion, and ginger.

Tomatoes are also a large component of the Mediterranean diet that have been

shown to inhibit platelet aggregation. This is contrary to Dr. D'Adamo's advice

that type As should avoid tomatoes because they agglutinate blood cells. When

choosing carbohydrate-rich foods, type As should avoid those high on the

glycemic index and focus on whole grain products. Plant-based foods high in

polyphenols are also very healthy for type As, such as black tea, apples, soy, dark

chocolate, and red wine.

Conclusion

Results from this study suggest that a vegetarian-like diet may improve

coagulation measures, especially in individuals with blood type O. However,

because blood type As are at an increased risk of CVD due to their higher levels

of VWF, future studies are warranted to explore dietary interventions that can

reduce risk in type As. Further exploration is also needed into how PT and APTT

relate to risk of CVD. They may prove to be a useful screening measure in

addition to LDL and triglycerides, among others.

REFERENCES

1. Welcome to personalized nutrition. Available at: http://www.dadamo.com/program_welcome2.htm. Accessed 11/14, 2011.

2. D'Adamo P. *Eat Right 4 Your Type*. New York, NY: Penguin Group; 1996.

3. Yamamoto F, Cid E, Yamamoto M, Blancher A. ABO research in the modern era of genomics. *Transfus Med Rev*. 2011;26(2):103-18.

4. Carpeggiani C, Coceani M, Landi P, Michelassi C, L'Abbate A. ABO blood group alleles: A risk factor for coronary artery disease. an angiographic study. *Atherosclerosis*. 2010;211(2):461-466.

5. Wolpin BM, Kabrhel C, Varraso R, Kraft P, Rimm EB, Goldhaber SZ, Camargo Jr CA, Fuchs CS. Prospective study of ABO blood type and the risk of pulmonary embolism in two large cohort studies. *Thromb Haemost*. 2010;104(5):962.

6. van Schie MC, van Loon JE, de Maat M, Leebeek FWG. Genetic determinants of von willebrand factor levels and activity in relation to the risk of cardiovascular disease: A review. *J Thromb Haemost*. 2011;9(5):899-908.

7. Clark P, Wu O. ABO blood groups and thrombosis: A causal association, but is there value in screening? *Future Cardiology*. 2011;7(2):191-201.

8. Rajaram S. The effect of vegetarian diet, plant foods, and phytochemicals on hemostasis and thrombosis. *Am J Clin Nutr*. 2003;78(3):552S-558S.

9. MedlinePlus. Available at: http://www.nlm.nih.gov/medlineplus/mplusdictionary.html. Accessed November 14, 2011.

10. Figl M, Pelinka LE. Karl landsteiner, the discoverer of blood groups. *Resuscitation*. 2004;63(3):251-254.

11. Landsteiner K. Ueber agglutinationserscheinungen normalen menschlichen blutes. *Wien Kli Wchnschr*. 1901;14:1132-1134.

12. Decastello A, Sturli A. Ueber die isoagglutinine im gesunder und kranker menschen. *Munch Med Wochenschrift*. 1902;49:1090-1095.

13. Okroi M, McCarthy LJ. The original blood group pioneers: The hirszfelds. *Transfus Med Rev*. 2010;24(3):244-246.

14. Storry J, Olsson M. The ABO blood group system revisited: A review and update. *25Celebrating*. 2009;25(2):48-59.

15. Yazer MH. What a difference 2 nucleotides make: A short review of ABO genetics. *Transfus Med Rev*. 2005;19(3):200-209.

16. Crow JF. Felix bernstein and the first human marker locus. *Genetics*. 1993;133(1):4.

17. Greenwell P. Blood group antigens: Molecules seeking a function? *Glycoconj J*. 1997;14(2):159-173.

18. Yamamoto F, Clausen H, White T, Marken J, Hakomori S. Molecular genetic basis of the histo-blood group ABO system. *Nature*. 1990;345(6272):229-233.

19. Reid ME, Lomas-Francis C. *The Blood Group Antigen: Factsbook*. Academic Pr; 2004.

20. Calafell F, Roubinet F, Ramírez-Soriano A, Saitou N, Bertranpetit J, Blancher A. Evolutionary dynamics of the human ABO gene. *Hum Genet*. 2008;124(2):123-135.

21. Malomgré W, Neumeister B. Recent and future trends in blood group typing. *Anal Bioanal Chem*. 2009;393(5):1443-1451.

22. EldonCard™ 2511-1 ABO-Rh Home Blood Typing Test Kit. Available at: http://www.craigmedical.com/Blood_typing_kit.htm. Accessed 11/29, 2011.

23. Gruslin AM MT. Erythroblastosis fetalis and hydrops fetalis. In: *Pathology of the Human Placenta*. 5th ed. New York, NY: Springer Science; 2006:519.

24. Boren T, Falk P, Roth KA, Larson G, Normark S. Attachment of *helicobacter pylori* to human gastric epithelium mediated by blood group antigens. *Science*. 1993;262(5141):1892.

25. Weatherall D. Genetic variation and susceptibility to infection: The red cell and malaria. *Br J Haematol*. 2008;141(3):276-286.

26. Carpeggiani C, Coceani M, Landi P, Michelassi C, L'Abbate A. ABO blood group alleles: A risk factor for coronary artery disease. an angiographic study. *Atherosclerosis*. 2010;211(2):461-466.

27. Wolpin BM, Kabrhel C, Varraso R, Kraft P, Rimm EB, Goldhaber SZ, Camargo Jr CA, Fuchs CS. Prospective study of ABO blood type and the risk of pulmonary embolism in two large cohort studies. *Thromb Haemost*. 2010;104(5):962.

28. Gale AJ. Continuing education course# 2: Current understanding of hemostasis. *Toxicol Pathol*. 2011;39(1):273.

29. Suchman A GP. Diagnostic uses of the activated partial thromboplastin time and prothrombin time. *Ann Intern Med*. 1986;104:810-816.

30. King M. Blood Coagulation Tests and Interpretations. Available at: http://themedicalbiochemistrypage.org/blood-coagulation.html. Accessed 06/17, 2012.

31. Tripodi A, Chantarangkul V, Martinelli I, Bucciarelli P, Mannucci PM. A shortened activated partial thromboplastin time is associated with the risk of venous thromboembolism. *Blood*. 2004;104(12):3631.

32. Phang M, Lazarus S, Wood LG, Garg M. Diet and thrombosis risk: Nutrients for prevention of thrombotic disease. *Semin Thromb Hemost*. 2011;37(3):199-208.

33. Jackson SP. The growing complexity of platelet aggregation. *Blood*. 2007;109(12):5087-5095.

34. Franchini M, Lippi G. Von willebrand factor and thrombosis. *Ann Hematol*. 2006;85(7):415-423.

35. Ruggeri ZM. The role of von willebrand factor in thrombus formation. *Thromb Res*. 2007;120:S5-S9.

36. Jenkins PV, O'Donnell JS. ABO blood group determines plasma von willebrand factor levels: A biologic function after all? *Transfusion*. 2006;46(10):1836-1844.

37. Bharati KP, Prashanth UR. Von willebrand disease: An overview. *Indian J. Pharm. Sci.* 2011;73(1):7.

38. Orstavik K, Magnus P, Reisner H, Berg K, Graham J, Nance W. Factor VIII and factor IX in a twin population. evidence for a major effect of ABO locus on factor VIII level. *Am J Hum Genet*. 1985;37(1):89.

39. Wu O, Bayoumi N, Vickers M, Clark P. ABO (H) blood groups and vascular disease: A systematic review and meta-analysis. *J Thromb Haemost*. 2008;6(1):62-69.

40. Souto JC, Almasy L, Muñiz-Diaz E, Soria JM, Borrell M, Bayén L, Mateo J, Madoz P, Stone W, Blangero J. Functional effects of the ABO locus polymorphism on plasma levels of von willebrand factor, factor VIII, and activated partial thromboplastin time. *Arterioscler Thromb Vasc Biol*. 2000;20(8):2024-2028.

41. Morange P, Simon C, Alessi M, Luc G, Arveiler D, Ferrieres J, Amouyel P, Evans A, Ducimetiere P, Juhan-Vague I. Endothelial cell markers and the risk of coronary heart disease. *Circulation*. 2004;109(11):1343-1348.

42. Whincup P, Danesh J, Walker M, Lennon L, Thomson A, Appleby P, Rumley A, Lowe G. Von willebrand factor and coronary heart disease. prospective study and meta-analysis. *Eur Heart J*. 2002;23(22):1764.

43. Koster T, Vandenbroucke J, Rosendaal F, Briët E, Blann A. Role of clotting factor VIII in effect of von willebrand factor on occurrence of deep-vein thrombosis. *Lancet*. 1995;345(8943):152-155.

44. Clark P, Wu O. ABO blood groups and thrombosis: A causal association, but is there value in screening? *Future Cardiol*. 2011;7(2):191-201.

45. Passaro A, Calzavarini S, Volpato S, Caruso P, Poli A, Fellin R, Bernardi F. Reduced factor VII and factor VIII levels and prolonged thrombin-generation times during a healthy diet in middle-aged women with mild to moderate cardiovascular disease risk. *J Thromb Haemost*. 2008;6(12):2088-2094.

46. Haines A, Chakrabarti R, Fisher D, Meade T, North W, Stirling Y. Haemostatic variables in vegetarians and non-vegetarians. *Thromb Res*. 1980;19(1-2):139-148.

47. Famodu A, Osilesi O, Makinde Y, Osonuga O, Fakoya T, Ogunyemi E, Egbenehkhuere I. The influence of a vegetarian diet on haemostatic risk factors for cardiovascular disease in africans. *Thromb Res*. 1999;95(1):31-36.

48. Mezzano D, Martinez C, Cueras A, Panes O, Aranda E, Guasch V, Strobel P, Rodrtguez S. Vegetarians and cardiovascular risk factors: Hemostasis, inflammatory markers and plasma homocysteine. *J Thromb Haemost* . 1999;81:913-917.

49. Li D, Sinclair A, Mann N, Turner A, Ball M, Kelly F, Abedin L, Wilson A. The association of diet and thrombotic risk factors in healthy male vegetarians and meat-eaters. *Eur J Clin Nutr*. 1999;53(8):612-619.

50. Freese R, Vaarala O, Turpeinen AM, Mutanen M. No difference in platelet activation or inflammation markers after diets rich or poor in vegetables, berries and apple in healthy subjects. *Eur J Nutr*. 2004;43(3):175-182.

51. Apitz-Castro R, Ledezma E, Escalante J, Jain MK. The molecular basis of the antiplatelet action of ajoene: Direct interaction with the fibrinogen receptor. *Biochem Biophys Res Commun*. 1986;141(1):145-150.

52. Thomson M, Al-Qattan K, Al-Sawan S, Alnaqeeb M, Khan I, Ali M. The use of ginger (zingiber officinale rosc.) as a potential anti-inflammatory and antithrombotic agent. *Prostag Leukotr Ess*. 2002;67(6):475-478.

53. Srivastava K. Effect of onion and ginger consumption on platelet thromboxane production in humans. *Prostag Leukotr Ess*. 1989;35(3):183-185.

54. Sawashita N, Naemura A, Shimizu M, Morimatsu F, Ijiri Y, Yamamoto J. Effect of dietary vegetable and animal proteins on atherothrombosis in mice. *Nutrition*. 2006;22(6):661-667.

55. Lazarus SA, Bowen K, Garg ML. Tomato juice and platelet aggregation in type 2 diabetes. *JAMA*. 2004;292(7):805-806.

56. Griel AE, Ruder EH, Kris-Etherton PM. The changing roles of dietary carbohydrates. *Arterioscler Thromb Vasc Biol*. 2006;26(9):1958-1965.

57. Brand-Miller J, Dickinson S, Barclay A, Allman-Farinelli M. Glycemic index, glycemic load, and thrombogenesis. *Semin Thromb Hemost*. 2009;35(1):111.

58. Larsen LF, Jespersen J, Marckmann P. Are olive oil diets antithrombotic? diets enriched with olive, rapeseed, or sunflower oil affect postprandial factor VII differently. *Am J Clin Nutr*. 1999;70(6):976-982.

59. Passaro A, Calzavarini S, Volpato S, Caruso P, Poli A, Fellin R, Bernardi F. Reduced factor VII and factor VIII levels and prolonged thrombin-generation times during a healthy diet in middle-aged women with mild to moderate cardiovascular disease risk. *J Thromb Haemost*. 2008;6(12):2088-2094.

60. Grundt H, Nilsen DWT. n-3 fatty acids and cardiovascular disease. *Haematologica*. 2008;93(6):807-812.

61. Steiner M, Anastasi J. Vitamin E. an inhibitor of the platelet release reaction. *J Clin Invest*. 1976;57(3):732.

62. Dereska NH, McLemore EC, Tessier DJ, Bash DS, Brophy CM. Short-term, moderate dosage vitamin E supplementation may have no effect on platelet aggregation, coagulation profile, and bleeding time in healthy individuals. *J Surg Res*. 2006;132(1):121-129.

63. Gunther KE, Conway G, Leibach L, Crowther MA. Low-dose oral vitamin K is safe and effective for outpatient management of patients with an INR> 10. *Thromb Res*. 2004;113(3):205-209.

64. Crowther MA, Ageno W, Garcia D, Wang L, Witt DM, Clark NP, Blostein MD, Kahn SR, Vesely SK, Schulman S. Oral vitamin K versus placebo to correct excessive anticoagulation in patients receiving warfarin. *Ann Intern Med*. 2009;150(5):293-300.

65. Arts ICW, Hollman PCH, Feskens EJM, De Mesquita HBB, Kromhout D. Catechin intake might explain the inverse relation between tea consumption and ischemic heart disease: The zutphen elderly study. *Am J Clin Nutr*. 2001;74(2):227-232.

66. Pearson DA, Paglieroni TG, Rein D, Wun T, Schramm DD, Wang JF, Holt RR, Gosselin R, Schmitz HH, Keen CL. The effects of flavanol-rich cocoa and aspirin on ex vivo platelet function. *Thromb Res*. 2002;106(4-5):191-197.

67. Wilcox JN, Blumenthal BF. Thrombotic mechanisms in atherosclerosis: Potential impact of soy proteins. *J Nutr*. 1995;125(3):631S-638S.

68. Kondo K, Suzuki Y, Ikeda Y, Umemura K. Genistein, an isoflavone included in soy, inhibits thrombotic vessel occlusion in the mouse femoral artery and in vitro platelet aggregation. *Eur J Pharmacol*. 2002;455(1):53-57.

69. Lippi G, Franchini M, Favaloro EJ, Targher G. Moderate red wine consumption and cardiovascular disease risk: Beyond the "French paradox." *Semin Thromb Hemost*. 2010;36(1):59-70.

70. Keevil JG, Osman HE, Reed JD, Folts JD. Grape juice, but not orange juice or grapefruit juice, inhibits human platelet aggregation. *J Nutr*. 2000;130(1):53-56.

71. Wang Q, Yu LG, Campbell BJ, Milton JD, Rhodes JM. Identification of intact peanut lectin in peripheral venous blood. *Lancet*. 1998;352(9143):1831-1832.

72. Evans RC, Fear S, Ashby D, Hackett A, Williams E, van der Vliet M, Dunstan FDJ, Rhodes JM. Diet and colorectal cancer: An investigation of the lectin/galactose hypothesis. *Gastroenterology*. 2002;122(7):1784-1792.

73. Ryder SD, Jacyna MR, Levi AJ, Rizzi PM, Rhodes JM. Peanut ingestion increases rectal proliferation in individuals with mucosal expression of peanut lectin receptor. *Gastroenterology*. 1998;114(1):44-49.

74. Pusztai A, Bardocz S, Ewen SW. Uses of plant lectins in bioscience and biomedicine. *Front Biosci*. 2008;13:1130-1140.

75. Freed DLJ. Do dietary lectins cause disease? *BMJ*. 1999;318(7190):1023-1024.

76. Nelson GJ, Schmidt PC, Corash L. The effect of a salmon diet on blood clotting, platelet aggregation and fatty acids in normal adult men. *Lipids*. 1991;26(2):87-96.

77. Nelson G, Schmidt P, Bartolini G, Kelley D, Kyle D. The effect of dietary docosahexaenoic acid on plasma lipoproteins and tissue fatty acid composition in humans. *Lipids*. 1997;32(11):1137-1146.

78. Benito P, Nelson G, Kelley D, Bartolini G, Schmidt P, Simon V. The effect of conjugated linoleic acid on plasma lipoproteins and tissue fatty acid composition in humans. *Lipids*. 2001;36(3):229-236.

79. Kelly F, Sinclair A, Mann N, Turner A, Abedin L, Li D. A stearic acid-rich diet improves thrombogenic and atherogenic risk factor profiles in healthy males. *Eur J Clin Nutr*. 2001;55(2):88-96.

80. Fischer F, Appert-Flory A, Jambou D, Toulon P. Evaluation of the automated coagulation analyzer Sysmex® CA-7000. *Thromb Res*. 2006;117(6):721-730.

81. Freedman DS, Byers T, Barboriak JJ, Flanders WD, Duncan A, Yip R, Meilahn EN. The relation of prothrombin times to coronary heart disease risk factors among men aged 31–45 years. *Am J Epidemiol*. 1992;136(5):513-524.

82. Hood JL, Eby CS. Evaluation of a prolonged prothrombin time. *Clin Chem*. 2008;54(4):765-768.

83. Zakai NA, Ohira T, White R, Folsom AR, Cushman M. Activated partial thromboplastin time and risk of future venous thromboembolism. *Am J Med*. 2008;121(3):231-238.

84. Lusher JM. Screening and diagnosis of coagulation disorders. *Obstet Gynecol*. 1996;175(3):778-783.

85. National Heart, Lung, and Blood Institute. Executive summary of the third report of the national cholesterol education program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel III). National Institutes of Health; 2001;01-3670.

86. Arcari A, Zito F, Di Castelnuovo A, De Curtis A, Dirckx C, Arnout J, Cappuccio F, Van Dongen M, De Lorgeril M, Krogh V. C reactive protein and its determinants in healthy men and women from european regions at different risk of coronary disease: The IMMIDIET project. *J Thromb Haemost*. 2008;6(3):436-443.

87. Canfi A, Gepner Y, Schwarzfuchs D, Golan R, Shahar DR, Fraser D, Witkow S, Greenberg I, Sarusi B, Vardi H. Effect of changes in the intake of weight of specific food groups on successful body weight loss during a Multi–Dietary strategy intervention trial. *J Am Coll Nutr*. 2011;30(6):491-501.

88. Vergnaud AC, Norat T, Romaguera D, Mouw T, May AM, Travier N, Luan J, Wareham N, Slimani N, Rinaldi S. Meat consumption and prospective weight change in participants of the EPIC-PANACEA study. *Am J Clin Nutr*. 2010;92(2):398-407.

89. Gilsing AMJ, Weijenberg MP, Hughes LAE, Ambergen T, Dagnelie PC, Goldbohm RA, van den Brandt PA, Schouten LJ. Longitudinal changes in BMI in older adults are associated with meat consumption differentially, by type of meat consumed. *J Nutr*. 2012;142(2):340-349.

APPENDIX A

IRB APPROVAL AND CONSENT FORM

ASU Knowledge Enterprise
Development
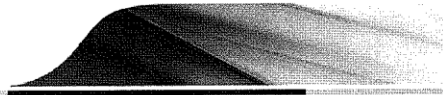
Office of Research Integrity and Assurance

To: Carol Johnston

From: Shannon Ringenbach
                     Biosci IRB

Date: 01/31/2012

Committee Action: **Expedited Approval**

Approval Date: 01/31/2012

Review Type: Expedited F2 F7

IRB Protocol #:   1201007333

Study Title: Effect of a vegetarian-like diet on blood coagulation and other health parameters in blood types A and O: An evaluation of the "Blood Type Diet"

Expiration Date: 01/30/2013

The above-referenced protocol was approved following expedited review by the Institutional Review Board.

It is the Principal Investigator's responsibility to obtain review and continued approval before the expiration date. You may not continue any research activity beyond the expiration date without approval by the Institutional Review Board.

Adverse Reactions: If any untoward incidents or severe reactions should develop as a result of this study, you are required to notify the Biosci IRB immediately. If necessary a member of the IRB will be assigned to look into the matter. If the problem is serious, approval may be withdrawn pending IRB review.

Amendments: If you wish to change any aspect of this study, such as the procedures, the consent forms, or the investigators, please communicate your requested changes to the Biosci IRB. The new procedure is not to be initiated until the IRB approval has been given.

Please retain a copy of this letter with your approved protocol.

CONSENT FORM
Diet and Blood Type

INTRODUCTION
The purposes of this form are to provide you (as a prospective research study participant) information that may affect your decision as to whether or not to participate in this research and to record the consent of those who agree to be involved in the study.

RESEARCHERS
Carol Johnston, PhD, RD of the School of Nutrition and Health Promotion at Arizona State University, along with nutrition graduate student Jennifer Brown, have invited your participation in a research study.

STUDY PURPOSE
This study will investigate whether blood type and diet interact to affect health parameters. Specifically, the researchers are testing the health effects of the "Eat Right 4 Your Type" diet by naturopathic doctor Peter J. D'Adamo in individuals with blood types A and O.

DESCRIPTION OF RESEARCH STUDY
If you decide to participate, a finger prick will be performed to confirm your ABO blood type (only blood types A and O will be invited to participate). Once accepted into the study, you will be asked to complete health and food questionnaires, and your height, weight, and blood pressure will be recorded. Participants are then randomly assigned to the experimental group or the control group. Experimental group members will be asked to alter their diet, including the restriction of dairy products and red meats. Control group members will continue to follow their typical diet patterns. Blood samples will be collected from all study participants at the initiation of the 4-week trial and at trial completion. Participants will be asked to refrain from using aspirin and other over-the-counter non-steroidal anti-inflammatory medications for six weeks following the in-person screening. (Participants are able to use Tylenol for pain relief during the study as needed).

If you say YES, then your participation will last for six weeks at ASU's downtown campus. Approximately 60 subjects will be participating in this study.

RISKS
Blood draws may cause light-headedness, dizziness, fainting, headaches, and/or temporary bruising. A research nurse will be performing the blood draws. As with any research, there is some possibility that you may be subject to risks that have not yet been identified.

BENEFITS
Although there may be no direct benefits to you, the possible benefit of your participation is that you will be able to experience what it is like to be a part of a research study that may provide new evidence to support a blood type specific diet for health improvement.

NEW INFORMATION
If the researchers find new information during the study that would reasonably change your decision about participating, then they will provide this information to you.

CONFIDENTIALITY
All information obtained in this study is strictly confidential unless disclosure is required by law. The results of this research study may be used in reports, presentations, and publications, but the researchers will not identify you. Your name will not be associated with any data related to the study. In order to maintain confidentiality of your records, you will be assigned to a subject number, which will be used throughout the course of the study to identify you. Only the investigators will have access to subject names and their corresponding codes.

ASU IRB
Approved
Sign _____ for Shannon Ringenbach
Date 1-31-12 to 1-30-13

WITHDRAWAL PRIVILEGE
It is ok for you to say no. Even if you say yes now, you are free to say no later, and withdraw from the study at any time. Your decision will not affect your relationship with Arizona State University or otherwise cause a loss of benefits to which you might otherwise be entitled.

COSTS AND PAYMENTS
The researchers want your decision about participating in the study to be absolutely voluntary, yet they recognize that your participation may pose some costs such as inconvenience and a small time commitment. In order to help defray these costs, you will receive a $10 Target gift card at the second and third study visits ($20 total). You will also receive food vouchers either during the study and/or after the study is completed.

COMPENSATION FOR ILLNESS AND INJURY
If you agree to participate in the study, then your consent does not waive any of your legal rights. However, no funds have been set aside to compensate you in the event of injury.

VOLUNTARY CONSENT
Any questions you have concerning the research study or your participation in the study, before or after your consent, will be answered by Dr. Carol Johnston, Principal Investigator and Professor of Nutrition at ASU (602-827-2265), or Jennifer Brown, Graduate Student (623-332-3335).

If you have questions about your rights as a subject/participant in this research, or if you feel you have been placed at risk; you can contact the Chair of the Human Subjects Institutional Review Board, through the ASU Office of Research Integrity and Assurance, at 480-965 6788.

This form explains the nature, demands, benefits and any risk of the project. By signing this form you agree knowingly to assume any risks involved. Remember, your participation is voluntary. You may choose not to participate or to withdraw your consent and discontinue participation at any time without penalty or loss of benefit. In signing this consent form, you are not waiving any legal claims, rights, or remedies. A copy of this consent form will be given (offered) to you.

Your signature below indicates that you consent to participate in the above study.

_____   _____   _____
Subject's Signature   Printed Name   Date

Preferred contact: phone and/or email:

_____

INVESTIGATOR'S STATEMENT
"I certify that I have explained to the above individual the nature and purpose, the potential benefits and possible risks associated with participation in this research study, have answered any questions that have been raised, and have witnessed the above signature. These elements of Informed Consent conform to the Assurance given by Arizona State University to the Office for Human Research Protections to protect the rights of human subjects. I have provided (offered) the subject/participant a copy of this signed consent document."

Signature of Investigator_____   Date_____

```
ASU IRB
Approved
Sign_____ for ⟨Shannon Ringenbach⟩
Date_1-31-12_ to _1-30-13
```

APPENDIX B

QUESTIONNAIRES AND HANDOUTS:

MEDICAL HISTORY QUESTIONNAIRE

FOOD FREQUENCY QUESTIONNAIRE

TYPE A DIET GUIDELINES

MEAL REPLACEMENT GUIDE

COMPLIANCE CALENDAR

HEALTH / MEDICAL HISTORY QUESTIONNAIRE   ID#_____

1. (To be completed by researchers):   Height _____   Weight _____

                                         Percent body fat _____   BMI _____

2. Age: _____   Gender: _____

3. Have you lost or gained <u>more than</u> 5 lbs in the last 12 months?   Yes   No

     If yes, how much lost or gained? _____   How long ago? _____

4. College Status (please circle)   Fresh.   Soph.   Jr.   Sr.   Grad.   Not Applicable

5. Ethnicity: (please circle) Native American   African-American   Caucasian   Hispanic   Asian   Other

6. Do you smoke?   No, never _____

                        Yes   _____   # Cigarettes per day = _____

                        I used to, but I quit _____ months/years (circle) ago

7. Do you take any medications regularly?   Yes   No   *If yes, list type and frequency:*

<u>Medication</u>   <u>Dosage</u>   <u>Frequency</u>
_____
_____
_____
_____
_____
_____
_____
_____
_____
_____

8. Do you currently take supplements (vitamins, minerals, herbs, etc.) ?   Yes   No   *If yes, list type and frequency:*

<u>Supplement</u>   <u>Dosage</u>   <u>Frequency</u>
_____
_____
_____
_____
_____
_____

9. Have you ever been hospitalized? _____ If yes, for what? _____

┌─────────────┐
│ OVER →   │
└─────────────┘

10. Please ANSWER (YES/NO) if you **currently have** or if you **have ever** been diagnosed with any of the following diseases or symptoms:

	YES	NO		YES	NO
Coronary Heart Disease			Chest Pain		
High Blood Pressure			Shortness of Breath		
Heart Murmur			Heart Palpitations		
Rheumatic Fever			Any Heart Problems		
Irregular Heart Beat			Coughing of Blood		
Varicose Veins			Feeling Faint or Dizzy		
Stroke			Lung Disease		
Diabetes			Liver Disease		
Low Blood Sugar			Kidney Disease		
Bronchial Asthma			Thyroid Disease		
Hay Fever			Anemia		
Leg or Ankle Swelling			Hormone Imbalances		
Eating Disorders			Emotional Problems		
Hemophilia			Lupus		
Other bleeding disorder			Cancer		
Vitamin K deficiency					

Please elaborate on any condition listed above._____
_____

11. How would you rate your lifestyle?

                          Not active _____   Active _____

                          Somewhat active _____   Very Active _____

12. Please circle the total time you spend in each category for an average **week.**

       Light activities such as:
       Slow walking, golf, slow cycling, doubles tennis, easy swimming, gardening
       Hours per week: 0 1 2 3 4 5 6 7 8 9 10+

       Moderate activities such as:
       Mod. Walking, mod. cycling, singles tennis, mod. swimming, mod. weight lifting
       Hours per week: 0 1 2 3 4 5 6 7 8 9 10+

       Vigorous activities such as:
       Fast walking/jogging, fast cycling, court sports, fast swimming, heavy/intense
       weight lifting
       Hours per week: 0 1 2 3 4 5 6 7 8 9 10+

13. How much alcohol do you drink? (average # drinks per day) _____

14. Do you have any food allergies?   Yes   No   If yes, explain:_____

15. Do you follow a special diet? (weight gain/loss, vegetarian, low-fat, etc.)   Yes   No

   If yes, explain: _____

ID Number: _____   Date: _____

Food Frequency Questionnaire

This questionnaire will give us information about your eating habits. There are no "right" or "wrong" answers. Accurate and thoughtful responses are appreciated!

- Use the **past month** as your standard for how you eat.
- Recall the times during the day when you ate, and what you had.
- Include snacks as well as meals and beverages.
- If you ate out regularly or traveled, remember to include those foods too.
- Please answer every item on this form. If you did not eat a food listed below, or ate it less than once a week, write a '0' in the space provided. Please do not leave blanks.

For each of the foods listed below, please indicate how many servings **per week** you **usually** ate in the past month. Where indicated, check whether your servings are large, small, or about average in size.

Food Item	Average Weekly Servings	Serving Size:			Size of average serving
		Small	Average	Large	
Red meat (beef, pork and ham, veal, lamb)	_____	☐	☐	☐	4 ounces (size of deck of cards)
Meat dishes (casseroles, tacos, pizza, meat sauce)	_____	☐	☐	☐	1 cup casserole, 1 taco or pizza slice
Chicken or turkey	_____	☐	☐	☐	1 large or 2 small pieces
Fish (such as salmon or tuna, including fish canned in water)	_____	☐	☐	☐	4 ounces, ½ can
Shellfish (such as lobster, shrimp, clams, oysters, scallops, crab, and squid/calamari)	_____	☐	☐	☐	4 ounces
Bacon, sausage	_____	☐	☐	☐	2 pieces
Hot dogs	_____	☐	☐	☐	1 hot dog
Luncheon meats: salami, bologna, ham, roast beef	_____	☐	☐	☐	1 piece
Luncheon meats: chicken, turkey	_____	☐	☐	☐	1 piece
How many of the above servings are from fast food outlets (McDonald's, Taco Bell, etc.)?	_____				

Food Item	Average Weekly Servings	Serving Size:			Size of average serving
		Small	Average	Large	
Eggs (scrambled, hard boiled, over easy, etc.)	_____	☐	☐	☐	1 egg
Cow's milk	_____	☐	☐	☐	1 cup (8 ounces)
Yogurt (derived from cow's milk)	_____	☐	☐	☐	1 cup (8 ounces)
Cottage cheese	_____	☐	☐	☐	1 cup (8 ounces)
Hard cheese (cheddar, Monterey jack, Swiss, provolone, etc.)	_____	☐	☐	☐	1 ounce/slice
Cream cheese	_____	☐	☐	☐	1 ounce
Ice cream	_____	☐	☐	☐	½ cup (1 scoop)
Frozen yogurt	_____	☐	☐	☐	½ cup (1 scoop)
Soy milk	_____	☐	☐	☐	1 cup (8 ounces)
Soy yogurt	_____	☐	☐	☐	1 cup (8 ounces)
Soy cheese	_____	☐	☐	☐	1 ounce/slice
Olive, flaxseed, or walnut oil	_____	☐	☐	☐	1 tablespoon
Coconut, corn, or peanut oil	_____	☐	☐	☐	1 tablespoon
Peanuts and peanut butter	_____	☐	☐	☐	2 tablespoons
Walnuts	_____	☐	☐	☐	2 tablespoons
Cashews	_____	☐	☐	☐	2 tablespoons
Pistachios	_____	☐	☐	☐	2 tablespoons

Food Item	Average Weekly Servings	Serving Size:			Size of average serving
		Small	Average	Large	
Black beans	_____	☐	☐	☐	¾ cups (cooked)
Pinto beans	_____	☐	☐	☐	¾ cups (cooked)
Lentils	_____	☐	☐	☐	¾ cups (cooked)
Garbanzo beans (chickpeas); including hummus	_____	☐	☐	☐	¾ cups (cooked)
Kidney beans	_____	☐	☐	☐	¾ cups (cooked)
Oatmeal	_____	☐	☐	☐	1 medium bowl
Shredded wheat cereal	_____	☐	☐	☐	1 medium bowl
Sprouted wheat bread (eg. Ezekiel)	_____	☐	☐	☐	1 slice
English muffin	_____	☐	☐	☐	1 muffin
Whole wheat bread	_____	☐	☐	☐	1 slice
White bread	_____	☐	☐	☐	1 slice
Pasta (from durum or semolina)	_____	☐	☐	☐	1 cup
Pasta (from whole wheat flour)	_____	☐	☐	☐	1 cup
Quinoa	_____	☐	☐	☐	¾ cups (cooked)
Rice (all varieties)	_____	☐	☐	☐	¾ cups (cooked)
Chips or French fries	_____	☐	☐	☐	1 cup
Nightshade vegetables (Potatoes, tomatoes, sweet and hot peppers, eggplant, tomatillos, tamarios, pepinos, and pimientos)	_____	☐	☐	☐	1 cup raw or ½ cup cooked

Food Item	Average Weekly Servings	Serving Size:			Size of average serving
		Small	Average	Large	
All other vegetables (non-nightshades)	_____	☐	☐	☐	1 cup raw or ½ cup cooked
Berries (blackberries, blueberries, cranberries, etc.)	_____	☐	☐	☐	1 cup raw
Bananas	_____	☐	☐	☐	1 medium banana
Orange	_____	☐	☐	☐	1 medium orange
Grapefruit	_____	☐	☐	☐	1 medium grapefruit
All other fruit (not listed above)	_____	☐	☐	☐	1 piece
Mustard	_____	☐	☐	☐	1 tablespoon
Soy sauce	_____	☐	☐	☐	1 tablespoon
Mayonnaise	_____	☐	☐	☐	1 tablespoon
Ketchup	_____	☐	☐	☐	1 tablespoon
Salad dressing	_____	☐	☐	☐	2 tablespoons
Vinegar (balsamic and other varieties)	_____	☐	☐	☐	1 tablespoon
Baked desserts and pastries (cake, cookies, etc.)	_____	☐	☐	☐	1 slice or 2 cookies
Donuts or sweet rolls	_____	☐	☐	☐	1 piece
Chocolate or candy bars	_____	☐	☐	☐	1 regular size candy bar
Soda (including regular and diet)	_____	☐	☐	☐	1 large glass/ 1 12 oz can
Coffee	_____	☐	☐	☐	1 cup/ 8 ounces

Food Item	Average Weekly Servings	Serving Size:			Size of average serving
		Small	Average	Large	
Green tea	_____	☐	☐	☐	1 cup/ 8 ounces
Black tea	_____	☐	☐	☐	1 cup/ 8 ounces
Fruit juice	_____	☐	☐	☐	1 cup/ 8 ounces
Red wine	_____	☐	☐	☐	6 ounces
Beer	_____	☐	☐	☐	12 ounces
Liquor	_____	☐	☐	☐	1.5 ounces

Please look over this form to be sure you answered every question. Do not leave any items blank.

Thank you!

This FFQ has been adopted from Kaiser Permanente's FFQ available at
http://www.permanente.net/homepage/kaiser/pdf/6116.pdf (accessed 1/24/2012).

Category	Most beneficial	Food allowed	Food not allowed
Meats and Poultry		Chicken, turkey	All other meats: pork, beef, duck, lamb, etc.
Seafood	Cod, mackerel, red snapper, salmon, sardines, trout	Sea bass, mahi-mahi, swordfish, tilapia, tuna, yellowtail	Anchovies, striped bass, catfish, clams, crab, eel, flounder, grouper, halibut, herring, lobster, lox, mussel, oysters, scallops, shrimp, sole, squid (calamari)
Dairy and Eggs		Eggs, feta cheese, goat cheese, kefir, mozzarella cheese, ricotta cheese, yogurt	American cheese, blue cheese, brie, butter, cheddar, colby, cottage cheese, cream cheese, half & half, ice cream, cow's milk, Monterey jack, muenster, parmesan, provolone, string cheese, Swiss cheese
Oils	Olive, flaxseed, walnut	Almond, canola, safflower, sesame, soy, sunflower	Coconut, corn, peanut
Nuts and Seeds	Flaxseed, peanut, peanut butter, pumpkin seed, walnut	Almond, almond butter, hazelnut, macadamia, pecan, poppy seed, sesame butter (tahini), sesame seed, sunflower seed	Brazil, cashew, pistachio
Beans and Legumes	Black bean, black-eyed pea, fava, green/string/snap bean, lentil, pinto bean, soybean and all related products (soy milk, soy cheese, miso, tempeh, tofu)	Cannellini, mung bean, northern bean, green pea, white bean	Garbanzo bean (chickpea), hummus, kidney bean, lima bean, navy bean, tamarind
Cereals	Amaranth, buckwheat/kasha, oat bran, oatmeal	Barley, cornflakes, grits, millet, quinoa, rice	Cream of wheat, farina, Grape Nuts, wheat germ, seven-grain, shredded wheat, wheat bran
Breads and Muffins	Sprouted wheat bread (eg. Ezekiel brand), soy flour bread	Brown rice bread, corn muffin, gluten-free bread, millet, oat bran bread and muffin, rice cake, rye bread, spelt bread	Bagel, English muffin, matzo, multi-grain bread, pumpernickel, wheat bran muffin, whole wheat bread, white bread
Grains and Pasta	Buckwheat/kasha, oat flour, rye flour, soba noodles (100% buckwheat)	Couscous, barley flour, bulgur wheat flour, durum wheat flour, quinoa, rice (all kinds), semolina pasta, spelt flour, white flour	Whole wheat flour
Vegetables	Alfalfa sprouts, artichoke, broccoli, carrot, celery, collard greens, garlic, ginger, kale, leek, lettuce, mushroom (common), okra, onion, parsley, pumpkin, spinach, turnip	Arugula, asparagus, beet, bok choy, brussel sprouts, cauliflower, cilantro, corn, cucumber, green olives, radicchio, radish, scallion, seaweed, squash, taro, water chestnut, zucchini	Cabbage, capers, chili pepper, eggplant, shiitake mushrooms, black olives, peppers (green/jalapeno/red/yellow), potato (sweet/white/yellow), tomato, yam
Fruit	Apricot, blackberry, blueberry, cherry, cranberry, fig, grapefruit, lemon, lime, pineapple, plum, prune	Apple, avocado, cantaloupe, date, grape (all), kiwi, nectarine, peach, pear, pomegranate, raisin, raspberry, strawberry, watermelon	Bananas, coconut, mango, melon (honeydew), orange, papaya, plaintain, tangerine
Condiments	Mustard, soy sauce	Jam/jelly (from acceptable fruits), non-dairy salad dressing	Ketchup, mayonnaise, pickles, relish, vinegar
Beverages	Juice from F&V listed above, chamomile tea, green tea, coffee, red wine, lemon water	Juice from F&V listed above, mint tea, white wine	Beer, liquor, seltzer water, soda (all kinds), black tea

Meal Replacement Guide

Emphasize foods in their natural state and eat fresh as much as possible. Avoid overly processed and refined foods.

If you usually eat...   Try instead!

Breakfast

If you usually eat...	Try instead!
• Bagel with cream cheese	• Oatmeal with berries and walnuts
• Shredded wheat cereal with cow's milk	• Corn flakes with soy milk
• Egg McMuffin	• Yogurt parfait with cherries
• Half and half in coffee	• Soy milk
• Black tea	• Green tea
• Orange juice	• Grapefruit juice

Lunch

• Hamburger	• Veggie burger or chicken sandwich
• French fries	• Carrot sticks
• Beef burrito	• Pinto bean burrito (no cheese)
• Potato chips	• Apple slices
• Ham sandwich	• Tuna sandwich
• Cobb salad	• Greek salad
• Soda	• Cranberry juice

Dinner

• Spaghetti with tomato sauce	• Pasta with basil pesto
• Beef stew	• Lentil soup
• Pork chops	• Baked, seasoned tofu
• Mashed potatoes	• Black beans and rice
• Bread and butter	• Mixed salad with oil & squeezed lemon
• Pepperoni pizza	• Vegetarian lasagna with pesto sauce
• Bottle of beer	• Glass of red wine
• Ice cream	• Frozen yogurt
• Cheese cake	• Reese's peanut butter cups

Snacks

• Nachos	• Rice cake with peanut butter
• String cheese	• Raw almonds
• Beef jerky	• Soy beans (edamame)
• Hummus and crackers	• Dried apricots

Pain Relief

• Aspirin, Advil, or Aleve	• Tylenol

March 2012

SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY
				1	2	3
4	5	6	7	8	9	10
11	12	13	14	15	16	17
18	19	20	21	22	23	24
25	26	27	28	29	30	31

April 2012

SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY
1	2	3	4	5	6	7
8	9	10	11	12	13	14
15	16	17	18	19	20	21
22	23	24	25	26	27	28
29	30					

INSTRUCTIONS

For every day you have complied with the study diet, please place an 'X' in that date's box. For days on which you eat items that are not allowed, please make a note of the food (eg. "4 oz steak"). Please do your best to keep an accurate record and report all deviations. Thank you for your participation!

Contact the researchers if you have any questions at:

Dr. Carol Johnston: Carol.Johnston@asu.edu, 602-827-2265
Jennifer Brown: Jennifer.M.Brown@asu.edu, 623-332-3335

APPENDIX C

STUDY TIMELINE

Timeline

An Evaluation of the Blood Type Diet

Visit 1

In-person screening: collect consent, medical history, FFQ, blood type, weight, height, age, blood pressure

Visit 2

Go over diet with exp. group

Blood draw

Experimental group follows Type A diet, control group continues normal diet

Visit 3

Blood draw

| Recruitment | Screening | Washout 2 weeks | Experiment 4 weeks |

Email listserv and post flyers

Respond to Survey Monkey initial screening

Abstain from medications including aspirin and NSAIDs

Follow up with weekly emails to assure compliance

FFQ to confirm compliance

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APPENDIX D

ANALYTICAL PROCEDURES

Procedure for EldonCard™ 2511-1 ABO-Rh Home Blood Typing Test Kit

(*Adopted from instruction manual included with kit.*)

Test principle:

       The test is based on direct haemagglutination. The antibody reagents on the card will agglutinate red blood cells with corresponding antigens. No agglutination in a field indicates the absence of the corresponding antigen. The blood group of the individual is determined from the agglutination pattern on the EldonCard.

Reagents:

- The anti-A field contains murine IgM monoclonal anti-A from cell line Birma-1
- The anti-B field contains murine IgM monoclonal anti-B from cell line LB-2
- The anti-D field contains human monoclonal IgM anti-D from cell line MS-201
- The control field contains no antibodies but the same phosphate buffer as the other fields

Additional materials required:

- Either clean tap water, distilled water, isotonic saline or phosphate buffered saline
- Lancet
- EldonSticks, 4 per card

Procedure for capillary blood:

1. Fill in the data of the person being tested.
2. With a pipette, apply 20 µl of water onto each of the circular fields.
3. Disinfect the finger at the puncture site with a cleansing tissue. Let the finger air dry.
4. Puncture the skin by pushing the lancet firmly against the fingertip.
5. Keep the arm down for about 10 seconds to stimulate the blood flow. Gently press the blood towards the fingertip. Repeat pressing until a drop with a 3 to 4 mm (1/8 inch) diameter is seen. Apply the blood onto an EldonStick, approached from beneath the finger. Don't smear the blood over the skin. Place the stick onto the first circular field. The blood shall touch the water applied in step 2.
6. To develop all possible agglutinates the card must be tilted at least 40 seconds. Tilt the EldonCard to an almost upright position and wait 10 seconds. A wave of blood will move the red cells slowly to the bottom of

the fields. Tilt to the opposite vertical position and wait another 10 seconds while the blood flows down the fields. Tilt twice more on the remaining edges for 10+10 seconds. The results can now be read and recorded.

7. The EldonCard may be disposed of at this point, or it can be left on a horizontal surface to dry. When dry, it can be covered with a piece of ELDON FOIL 2511 and kept as a permanent record of the result.

Results:

Agglutination of the red cells in a field is considered a positive result and denoted "+" in the table below. No agglutination is considered a negative result and denoted "-" in the table. The possible reaction patterns on the card and corresponding blood groups:

Reaction in Field				Blood Group	
Anti-A	Anti-B	Anti-D	Control	ABO	RhD
-	-	+	-	O	Pos
-	-	-	-	O	Neg
+	-	+	-	A	Pos
+	-	-	-	A	Neg
-	+	+	-	B	Pos
-	+	-	-	B	Neg
+	+	+	-	AB	Pos
+	+	-	-	AB	Neg
+	+	+	+	Invalid result	

If a positive reaction is observed in the Control field, the test result is invalid and the test has to be repeated.

Procedure for IMUBIND® vWF ELISA Kit

(*Adopted from instruction manual included with kit.*)

Intended use:

The IMUBIND® vWF ELISA kit is an enzyme-linked immunoassay for the quantitation of vWF antigen in human plasma. This assay is for research use only. It is not intended for diagnostic or therapeutic procedures.

Principle of the procedure:

The IMUBIND vWF ELISA is a "sandwich" ELISA using a goat polyclonal antibody as the capture antibody. Samples incubate in precoated micro-test wells and the same polyclonal antibody, horseradish peroxidase (HRP) conjugated, is used to detect the bound vWF antigen. The addition of perborate/3,3',5,5' - tetramethylbenzidine (TMB) substrate, and its subsequent reaction with the HRP, creates a blue colored solution. Sensitivity is enhanced by the addition of a 0.5M sulfuric acid stop solution, yielding a yellow colored solution. vWF levels are determined by measuring and comparing the absorbance of sample solutions at 450 nm against those of a standard curve developed using calibrated antigen.

Reagents:

- 6 strips of 16 antibody coated microwells in holder and acetate cover sheet
- 6 vials vWF standards, 0 - 10 mU/mL (lyophilized)
- 1 vial Detection Antibody, HRP-conjugated anti-human vWF (135 µL)
- 1 vial Detection Antibody Diluent (lyophilized)
- 1 vial Substrate, TMB (11 mL)
- 1 packet Wash Buffer, PBS with 0.05% Tween 20, pH 7.4

There are sufficient reagents to assay 42 plasma samples and generate a 6-point standard curve (both tested in duplicate). Samples may be patient, control or reference plasmas.

Reagent preparation and storage:

1. Precoated Microwell Strips: Once removed from the foil pouch, the microwell strips should be used within 30 minutes. Unused strips may be stored at 2°-8°C for 4 weeks when sealed in the original pouch with the desiccant present, protected from any moisture.
2. Standards: Add 1.0 mL distilled H20 to the 0.5, 1.0, 2.0, 5.0 and 10.0 mU/mL standard vials. Add 2.0 mL distilled H20 to the 0 mU/mL standard vial. Agitate gently. Do not shake! Reconstituted vWF standards are stable for up to 1 month when stored at –20°C or colder.

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3. Detection Antibody: Supplied as a concentrated solution. Detection Antibody is diluted to working strength immediately before adding to the microwells. Concentrated Detection Antibody is stable until the expiration date stated on the vial when stored at 2°-8°C. Unused working strength Detection Antibody should be discarded.
4. Detection Antibody Diluent: Add 20 mL of distilled H2O to the Detection Antibody Diluent vial. Mix well. Reconstituted Detection Antibody Diluent may be used for up to 1 month when stored at 2°-8°C.
5. Substrate: Supplied ready to use. Once opened, the substrate may be used for up to 1 month when stored at 2°-°C.
6. Wash Buffer: Dissolve the contents of the Wash Buffer packet in 900 mL distilled H2O. QS. to a final volume of 1 Liter with distilled H2O. Mix well and confirm pH is 7.4 (adjust if necessary). Wash Buffer may be used for up to 1 month when stored at 2°-8°C.
7. Sample Buffer: Prepare an appropriate amount of Sample Buffer by adding BSA to Wash Buffer to a final concentration of 3% w/v (3 gm BSA/100 mL Wash Buffer). Sample Buffer may be used for up to 2 weeks when stored at 2°-8°C.

Specimen collection and preparation:

Either citrate or EDTA collected platelet poor plasma may be used for this assay. See "Collection, Transport and Preparation of Blood Specimens for Coagulation Testing and Performance of Coagulation Assays", NCCLS Document H21-A3, Vol. 18, No. 20, December 1998. Plasma collection should be performed as follows:

1. Collect 9 parts of blood into 1 part of 3.2% (0.109 M) trisodium citrate anticoagulant solution.
2. Centrifuge the blood sample at 1,500 x g for 15 minutes.
3. Plasma should be stored at 2°-8°C and assayed within 4 hours. Alternatively, plasma may be stored at –20°C for up to 6 months.
4. Frozen plasma should be thawed rapidly at 37°C. Thawed plasmas should be stored at 2°-8°C and assayed within 4 hours.
5. Dilute plasma sample 1:100 in Sample Buffer.

Procedure:

1. Open the foil pouch and remove the microwell/frame assembly. Remove those microwell strips that will not be used and replace them in the foil pouch with the desiccant inside. Reseal the pouch and store at 2°-8°C.
2. Add 100 ìL of vWF standard or diluted sample to a microwell, cover with the acetate sheet and incubate for 1 hour at room temperature. It is recommended to perform measurements in duplicate.
3. Wash the microwells 4 times with wash buffer (250 ìL per well).

4. Prepare working strength Detection Antibody by diluting the concentrate 1:100 as follows: add 20 ìL of Detection Antibody to 2 mL of Detection Antibody Diluent. Then add 100 ìL of working strength Detection Antibody to each microwell, cover with the acetate sheet and incubate for 1 hour at room temperature.
5. Wash the microwells 4 times with wash buffer (250 ìL per well).
6. Add 100 ìL of Substrate to each microwell, cover with acetate sheet and incubate for 20 minutes at room temperature. A blue color will develop.
7. Stop the enzymatic reaction by adding 50 ìL of 0.5M H2SO4. Tap the sides of the strip wells to ensure even distribution of the H2SO4. The solution color will turn yellow. Immediately read the absorbances of the solutions on a microwell plate reader set at a wavelength of 450 nm.

Results:

Construct a standard curve by plotting the mean absorbance value for each vWF Standard versus its corresponding concentration. A standard curve should be generated each time the assay is performed. The following standard curve is for demonstration purposes only.

Representative Standard Curve

IMUBIND® vWF ELISA

$y = -0.007x^2 + 0.2715x + 0.1041$
$R^2 = 0.9995$

Absorbance, 450 nm

vWF Concentration, mU/mL
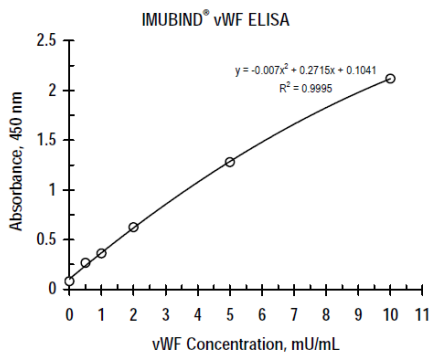
Calculation:

Use the mean absorbance value for each diluted sample to interpolate its vWF concentration from the standard curve. Multiply the concentration determined from the standard curve by 100 (the dilution factor) to obtain the vWF concentration in the original plasma sample.

**Procedure for Prothrombin time (PT) and
activated partial thromboplastin time (APTT)**

The following information was adopted from www.medical.siemens.com.

About the Sysmex® CA-7000 System:

      The Sysmex® CA-7000 System is a fully automated, high performance, cap-piercing analyzer that efficiently processes routine and specialty assays. Utilizing complete random-access capabilities and second generation cap-piercing, it is the fastest throughput coagulation analyzer available for routine testing.

PT reagents used:

- Thromborel® S
- Dade® Innovin

APTT reagents used:

- Dade Actin®
- Dade Actin FS
- Dade Actin FSL
- Pathromtin™ SL

The following information was adopted from www.sonoraquest.com.

Prothrombin time:

- Specimen Requirements: 1 refrigerated blue-top (sodium citrate) tube. Allow 2.7 mL blood to be drawn by vacuum. A completely filled blue-top tube is required. Do not centrifuge or freeze. Send unopened tube refrigerated. If testing is to be delayed beyond 48 hours, centrifuge tube and separate only top 2/3 of platelet-free plasma into a plastic vial and freeze immediately (1 mL minimum). Place in specimen bag with Frozen Specimen label applied and transport frozen.
- Stability: 48 hours refrigerated or room temperature if tube has not been opened. 4 hours if tube has been opened. 1 month if submitted as frozen plasma.
- Minimum Collection Volume: 1. 3.0 mL light blue top tube 2. 1.0 mL FROZEN citrated plasma
- Methodology: Photo Optical Clot Detection
- Rejection Reason: 1. Specimen clotted 2. Effective: 5/1/00, specimens submitted with incorrect blood anticoagulant ratio will not be tested. 3. Age of specimen. 4. Gross hemolysis 5. Clotted 6. Plasma not frozen. 7. High hematocrit.

- Transport Temperature: 1. Blue top tube - Refrigerated 2. Plasma - Frozen
- Container Type: Blue top tube, 3.2% sodium citrate tube Frozen plasma

Activated Partial Thromboplastin Time:

- Specimen Requirements: 1 refrigerated blue-top (sodium citrate) tube. Allow 2.7 mL blood to be drawn by vacuum. A completely filled blue-top is required. Do not centrifuge or freeze. Send unopened tube refrigerated. If testing is to be delayed beyond 24 hours, immediately centrifuge tube and separate only top 2/3 of platelet-free plasma into a plastic vial and freeze immediately. Place in specimen bag with Frozen Specimen label and transport frozen. NOTE: For heparinized patients, always submit frozen plasma (1 mL minimum).
- Stability: 1. 24 hrs - Blue top tube refrigerated or room temperature if tube has not been opened. 4 hours if tube has been opened. 2. 1 week frozen citrate plasma
- Minimum Collection Volume: 1. 3 mL Blue Top Tube 2. 1.0 mL FROZEN citrated plasma
- Methodology: Photo-Optical Clot Detection
- Rejection Reason: 1. Transported in a glass vial. 2. Specimen submitted with incorrect blood/anticoagulant ratio. 3. Age of specimen 4. Hemolysis 5. Plasma not recd frozen. 6. Clotted 7. High hematocrit
- Transport Temperature: Blue top tube - Refrigerated Plasma - frozen
- Container Type: 1. Blue top tube 2. Plastic aliquot tube- from 3.2% Sodium Citrate Tube.

Procedure for Total Cholesterol
COBAS® C 111

(*Adopted from instruction manual included with test.*)

Intended use:

In vitro test for the quantitative determination of cholesterol in human serum and plasma on the cobas c 111 system.

Specimen collection and preparation:

For specimen collection and preparation, only use suitable tubes or collection containers. Only the specimens listed below were tested and found acceptable.

- Serum.
- Plasma: Li-heparin, K3-EDTA plasma. (Use of EDTA plasma leads to slightly lower values).
- Do not use citrate, oxalate, or fluoride.
- Fasting and nonfasting samples can be used.

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer. Centrifuge samples containing precipitates before performing the assay.

Stability: 7 days at 15-25 °C, 7 days at 2-8 °C, 3 months at (-15)-(-25) °C

Assay:

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions. The performance of applications not validated by Roche is not warranted and must be defined by the user.

Application for serum and plasma:

cobas c 111 test definition:

- Measuring mode = Absorbance
- Abs. calculation mode = Endpoint
- Reaction direction = Increase
- Wavelength A/B = 512/659 nm

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- Calc. first/last = 6/37
- Unit = mmol/L
- Reaction mode = R-S

Pipetting parameters:

- Diluent (H2O)
- R 47 μL 70 μL
- Sample 2 μL 23 μL
- Total volume 142 μL

<u>Calculation:</u>

The cobas c 111 analyzer automatically calculates the analyte concentration of each sample.

Conversion factors:

- mmol/L x 38.66 = mg/dL
- mmol/L x 0.3866 = g/L
- mg/dL x 0.0259 = mmol/L

Procedure for LDL Cholesterol
COBAS® C 111

(Adopted from instruction manual included with test.)

Intended use:

In vitro test for the quantitative determination of LDL-cholesterol in human serum and plasma on the cobas c 111 system.

Specimen collection and preparation:

For specimen collection and preparation, only use suitable tubes or collection containers. Only the specimens listed below were tested and found acceptable:

- Serum.
- Plasma: Li-heparin plasma.
- EDTA plasma causes decreased values.

Fasting and non-fasting samples can be used. The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Centrifuge samples containing precipitate before performing the assay.

Stability:12 7 days at 2-8 °C, 30 days at -70 °C

Assay:

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions. The performance of applications not validated by Roche is not warranted and must be defined by the user.

Application for serum and plasma:

cobas c 111 test definition:

- Measuring mode = Absorbance
- Abs. calculation mode = Endpoint
- Reaction direction = Increase
- Wavelength A/B = 583/659 nm

- Calc. first/last = 16/37
- Unit = mmol/L
- Reaction mode = R1-S-SR

Pipetting parameters:

- Diluent (H2O)
- R1 150 μL
- Sample 2 μL 7 μL
- SR 50 μL
- Total volume 209 μL

Calculation:

The cobas c 111 analyzer automatically calculates the analyte concentration of each sample.

Conversion factors:

- mmol/L x 38.66 = mg/dL
- mmol/L x 0.3866 = g/L
- mg/dL x 0.0259 = mmol/L

Procedure for HDL Cholesterol
COBAS® C 111

(Adopted from instruction manual included with test.)

<u>Intended use:</u>

In vitro diagnostic test for the quantitative determination of the HDL-cholesterol concentration in human serum and plasma on the cobas c 111 system.

<u>Specimen collection and preparation:</u>

For specimen collection and preparation, only use suitable tubes or collection containers. Only the specimens listed below were tested and found acceptable.

Serum.

- Plasma: Li-Heparin, K3-EDTA plasma.
- EDTA plasma causes decreased results.

Fasting and non-fasting samples can be used. Collect blood by using an evacuated tube or syringe. Specimens should preferably be analyzed on the day of collection. The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer. Centrifuge samples containing precipitates before performing the assay.

Stability:19 7 days at 2-8 °C

30 days at -70 °C

<u>Assay:</u>

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions. The performance of applications not validated by Roche is not warranted and must be defined by the user.

Application for serum and plasma:

cobas c 111 test definition:

- Measuring mode = Absorbance
- Abs. calculation mode = Endpoint
- Reaction direction = Increase
- Wavelength A/B = 583/659 nm
- Calc. first/last = 16/37
- Unit mmol/L
- Reaction mode = R1-S-SR

Pipetting parameters:

- Diluent (H2O)
- R1 150 μL
- Sample 2.5 μL 7.0 μL
- SR 50 μL
- Total volume 209.5 μL

Calculation:

The cobas c 111 analyzer automatically calculates the analyte concentration of each sample.

Conversion factors:

- mmol/L x 38.66 = mg/dL
- mmol/L x 0.3866 = g/L
- mg/dL x 0.0259 = mmol/L

Procedure for Triglycerides
COBAS® C 111

(*Adopted from instruction manual included with test.*)

<u>Intended use:</u>

In vitro test for the quantitative determination of triglycerides in human serum and plasma on the cobas c 111 system.

<u>Specimen collection and preparation:</u>

For specimen collection and preparation, only use suitable tubes or collection containers. Only the specimens listed below were tested and found acceptable.

- Serum
- Plasma: Li-heparin, K3-EDTA plasma.
- EDTA tubes that are less than 1/2 full may cause a negative bias for triglycerides results.

Patients should refrain from eating for 10 to 14 hours before blood is drawn. Samples must be drawn in a soap and glycerol free collection device. The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer. Centrifuge samples containing precipitates before performing the assay.

Stability:7 5-7 days at 2-8 °C, 3 months at (-15)-(-25) °C, several years at (-60)-(-80) °C

<u>Assay:</u>

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions. The performance of applications not validated by Roche is not warranted and must be defined by the user.

<u>Application for serum and plasma:</u>

cobas c 111 test definition:

- Measuring mode = Absorbance
- Abs. calculation mode = Endpoint
- Reaction direction = Increase

- Wavelength A/B = 512/659 nm
- Calc. first/last = 6/21
- Unit = mmol/L
- Reaction mode = R-S

Pipetting parameters:

- Diluent (H2O)
- R 120 µL
- Sample 2 µL 28 µL
- Total volume 150 µL

Calculation:

The cobas c 111 analyzer automatically calculates the analyte concentration of each sample.

Conversion factors:

- mmol/L × 88.5 = mg/dL
- mg/dL × 0.0113 = mmol/L

Procedure for Triglycerides
COBAS® C 111

(Adopted from instruction manual included with test.)

<u>Intended use:</u>

In vitro test for the quantitative determination of C-reactive protein (CRP) in human serum and plasma on the cobas c 111 system. Measurement of CRP is of use for the detection and evaluation of inflammatory disorders and associated diseases, infection and tissue injury. Highly sensitive measurement of CRP may also be used as an aid in the assessment of the risk of future coronary heart disease. When used as an adjunct to other laboratory evaluation methods of acute coronary syndromes, it may also be an additional independent indicator of recurrent event prognosis in patients with stable coronary disease or acute coronary syndrome.

<u>Specimen collection and preparation:</u>

For specimen collection and preparation, only use suitable tubes or collection containers. Only the specimens listed below were tested and found acceptable.

- Serum
- Plasma: Li-heparin, K2-EDTA plasma

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer. Centrifuge samples containing precipitates before performing the assay.

Stability:24 11 days at 15-25 °C, 2 months at 2-8 °C, 3 years at (-15)-(-25) °C

<u>Assay:</u>

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions. The performance of applications not validated by Roche is not warranted and must be defined by the user.

Application for serum and plasma:

cobas c 111 test definition:

- Measuring mode = Absorbance
- Abs. calculation mode = Kinetic
- Reaction direction = Increase
- Wavelength A = 552 nm
- Calc. first/last = 17/34
- Unit = mg/L (nmol/L, mg/dL)
- Reaction mode = R1-S-SR

Pipetting parameters:

- Diluent (H2O)
- R1 82 μL
- Sample 6 μL 48 μL
- SR 28 μL 14 μL
- Total volume 178 μL

Calculation:

The cobas c 111 analyzer automatically calculates the analyte concentration of each sample.

Conversion factors:

- mg/L x 9.52 = nmol/L
- mg/L x 0.1 = mg/dL

APPENDIX E

DATA SUMMARY

Mean±SD in Four Study Groups at Week 0 and Week 4

	Control Type O (n=9)		Control Type A (n=9)		Experimental Type O (n=10)		Experimental Type A (n=11)		Normal Range[a]
	Week 0	Week 4	Week 0	Week 4	Week 0	Week 4	Week 0	Week 4	
Weight (lb)	162.2 ±42.5	162.7 ±41.9	138.2 ±14.7	139.0 ±15.4	145.7 ±20.8	**143.9 ±21.1[+]**	150.1 ±29.1	**142.9 ±20.9***	NA
BMI	25.0 ±5.3	25.1 ±5.4	23.3 ±2.7	23.4 ±2.7	23.3 ±3.3	**23.0 ±3.3***	24.3 ±3.3	**23.6 ±2.7***	18.5-25.0
PT (sec)	10.8 ±0.9	10.8 ±1.0	10.7 ±0.3	10.6 ±0.4	10.7 ±0.3	**10.9 ±0.4***	10.9 ±0.6	10.9 ±0.6	9.0-11.5
APTT (sec)	30.1 ±1.3	29.9 ±1.5	28.8 ±2.3	29.6 ±1.7	30.3 ±2.0	30.8 ±2.0	29.1 ±2.7	29.5 ±2.5	22.0-34.0
VWF (mU/ml)	627.7 ±152.6	888.5 ±186.8	991.3 ±374.1	1076.6 ±247.6	746.8 ±209.3	895.1 ±377.3	1029.1 ±237.9	1288.0 ±153.6	varies
Total Chol (mg/dl)	165.6 ±40.6	161.7 ±35.4	152.6 ±29.3	149.9 ±31.3	146.8 ±22.0	146.1 ±29.1	152.7 ±18.6	139.6 ±18.0	<200 mg/dl
HDL (mg/dl)	53.9 ±21.4	48.7 ±17.2	69.8 ±16.0	69.2 ±12.7	65.6 ±12.2	67.7 ±14.0	60.5 ±13.1	57.0 ±12.1	>60 mg/dl
LDL (mg/dl)	105.8 ±32.6	104.7 ±32.6	81.6 ±24.1	78.7 ±26.4	77.1 ±19.3	76.8 ±29.3	89.8 ±16.6	81.0 ±11.7	<130 mg/dl
TG (mg/dl)	104.5 ±35.2	102.7 ±36.4	69.7 ±27.0	78.6 ±41.7	78.9 ±35.4	76.0 ±30.4	77.1 ±24.1	74.3 ±26.6	<150 mg/dl
hsCRP (mg/L)	3.95 ±4.28	2.98 ±2.90	0.94 ±0.86	0.96 ±1.52	1.94 ±2.84	1.35 ±2.75	1.47 ±1.51	1.76 ±2.11	1.0-3.0
Kcal/day	1734 ±615	1591 ±435	1678 ±480	1574 ±651	1581 ±539	1360 ±583	1509 ±431	1156 ±504	2000

[+]p<0.10; *p<0.05. See Results section for further detail.

[a]See discussion section for references for normal ranges.